Aspects of Long-term Treatment with Tyrosine Kinase Inhibitors in Chronic Myeloid Leukemia

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Abstract

Chronic myeloid leukemia (CML) is caused by the tyrosine kinase activity of the oncoprotein BCR-ABL. The introduction of tyrosine kinase inhibitors (TKIs) targeting BCR-ABL has profoundly changed the prognosis of CML. Currently, there are three TKIs approved for treatment of CML, imatinib, nilotinib and dasatinib. The five-year-survival is about 90% in CML patients treated with TKI in first chronic phase and the side-effects are generally mild and manageable. However, some concerns remain in CML treatment. Some patients fail TKI therapy. They need to be identified by regular evaluations of hematologic, cytogenetic and molecular response. Moreover, TKI therapy is life-long and the long-term side effects are in part unknown.

The aims of this doctoral thesis were to investigate some aspects of long-term treatment with TKIs in CML, especially side-effects of TKIs on bone in vitro and in vivo, variations in molecular response and adherence to imatinib therapy.

We showed that CML patients treated with imatinib had stable bone mineral density (BMD) over time despite a high incidence of secondary hyperparathyroidism (Papers I and VI). Imatinib and dasatinib inhibited proliferation of mesenchymal stem cells, i.e. osteoblast progenitors, in vitro (Papers II and IV). Dasatinib significantly and dose-dependently inhibited osteoblast differentiation in vitro (Paper II), whereas the imatinib-mediated inhibition of osteoblast differentiation was most marked at low concentrations (Paper IV).

Molecular response to TKI therapy is determined by serial measurement of the BCR-ABL transcript level in leukocytes from peripheral blood. In CML the BCR-ABL fusion gene is predominantly expressed in myeloid leukocytes. We showed that changes in the relative proportion of myeloid and lymphoid leukocytes induced by exercise, significantly affected the BCR-ABL transcript level measured in peripheral blood (Paper III).

CML patients treated with imatinib at the Sahlgrenska University Hospital were interviewed in a structured way to assess adherence. Contrary to previous studies from United Kingdom and Belgium, adherence to imatinib was estimated as good in our cohort. The study also revealed factors known to predict adherence to therapy, namely the patients being well-informed and having sufficient access to the treating clinic (Paper V).

In conclusion, imatinib and dasatinib affect osteoblast differentiation in vitro and bone metabolism in vivo, but the bone quality measured as BMD remains unaffected in imatinib-treated patients. Moreover, variations in molecular response may simply be due to pre-analytic variations in blood counts rather than real changes in CML burden. Thus, small variations in the BCR-ABL transcript level should be interpreted cautiously. Finally, good adherence to imatinib can be obtained through simple measures.
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List of papers

   Increased cortical bone mineralization in imatinib treated patients with chronic
   myelogenous leukaemia.

    Second-generation TKI dasatinib inhibits proliferation of mesenchymal stem
    cells and osteoblast differentiation in vitro.

III. **Jönsson S**, Olsson B, Jacobsson S, Palmqvist L, Ricksten A, Ekeland-Sjöberg K,
     Wadenvik H.
     *BCR-ABL1* transcript levels increase in peripheral blood but not in granulocytes
     after physical exercise in patients with chronic myeloid leukemia.

    Imatinib inhibits proliferation of human mesenchymal stem cells and promotes
    early but not late osteoblastogenesis in vitro.
    *J Bone Miner Metab* 2011, in press.

   Good adherence to imatinib therapy among patients with chronic myeloid
   leukemia - a single-center observational study.
   *Ann Hematol* 2011, in press.

    Secondary hyperparathyroidism but stable bone mineral density in patients with
    chronic myeloid leukemia treated with imatinib.
    Manuscript.
Abbreviations

ABL  Abelson murine leukemia viral oncogene
ALP  Alkaline phosphatase
Ara-C  Cytarabine
AP  Accelerated phase
ARS  Alizarin Red S
ATP  Adenosine triphosphate
BC  Blast crisis
BCR  Breakpoint cluster region
BMD  Bone mineral density
b.i.d.  lat. *bis in die*, twice a day
C-KIT  Stem cell factor (CD117)
CCgR  Complete cytogenetic response
CHR  Complete hematologic response
CgR  Cytogenetic response
CML  Chronic myeloid leukemia
CMR  Complete molecular remission
CSF1R  Colony stimulating factor 1 receptor
Ct  Cycle threshold
DDR1  Discoidin domain receptor 1
DXA  Dual energy X-ray absorptiometry
EphB4  Ephrin type-B receptor 4
FISH  Fluorescence *in situ* hybridization
GUS  Beta-glucuronidase
HR  Hematologic response
INFα  Interferon alpha
IS  International scale
MMR  Major molecular response
MR  Molecular response
PDGFR  Platelet derived growth factor receptor
PFS  Progression free survival
Ph  Philadelphia (chromosome)
PTH  Parathyroid hormone
q.d.  lat. *quaque die*, once a day
pQCT  Peripheral quantitative computed tomography
qRT-PCR  Quantitative reverse-transcriptase polymerase chain reaction
SCT  Stem cell transplantation
STI571  Signal transduction inhibitor number 571 (imatinib)
TKI  Tyrosine kinase inhibitor
Introduction

Clinical features of CML

Chronic myeloid leukemia (CML) is an uncommon disease with an annual incidence of 1-2 per 100,000 individuals. At diagnosis, most patients are middle-aged or elderly (median age 62 years), but the disease may occur at all ages. Males are affected more frequently than females (m:f 1.3:1). The natural course of CML is divided into three phases: chronic phase, accelerated phase and blast crisis (Table 1). Most patients (90%) are in chronic phase at diagnosis, but occasionally patients present with accelerated phase or blast crisis.

CML patients in chronic phase have few symptoms, and the disease is sometimes detected accidentally at routine check-ups. Nearly all CML patients have leukocytosis at diagnosis and the patients may have bone pain due to a packed bone marrow.

The spleen is frequently enlarged and the patients may suffer from abdominal fullness. Some have lost weight and some have night sweats and fever. The chronic phase, if left untreated, usually lasts for 2 to 6 years.

The chronic phase is followed by the accelerated phase characterized by an increasing amount of immature leukocytes (blasts) in peripheral blood and bone marrow. During this phase, which seldom lasts more than 1 year, the patient shows more symptoms such as fatigue, weight loss and night sweats. Eventually the patient develops blast crisis that resembles acute leukemia with severe infections, bleedings and symptoms of anemia. Interestingly, 25% of the patients in blast crisis have blasts with a lymphoid phenotype, i.e. the disease has transformed from a myeloid leukemia into a lymphoid leukemia. Without treatment the median survival of patients in blast crisis is 3-6 months.

Table 1. The WHO criteria of the different phases of CML.

<table>
<thead>
<tr>
<th></th>
<th>Chronic phase</th>
<th>Accelerated phase</th>
<th>Blast crisis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blasts in blood or bone marrow</td>
<td>≤ 9%</td>
<td>10-19%</td>
<td>≥20%</td>
</tr>
<tr>
<td>Basophils in blood</td>
<td>&lt;20%</td>
<td>&gt;20%</td>
<td></td>
</tr>
<tr>
<td>Platelets in blood (x10⁹/L)¹</td>
<td>&gt;100</td>
<td>&lt;100</td>
<td></td>
</tr>
<tr>
<td>Extramedullary disease²</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>

¹ unrelated to therapy; ² excluding enlargement of the liver or the spleen
**Pathophysiology**

Blood cells develop from multipotent hematopoietic stem cells that reside in the bone marrow. In healthy individuals the hematopoietic stem cells proliferate and differentiate into blood cells in a tightly regulated manner (Figure 1). In CML, a reciprocal translocation between the long arms of chromosome 9 and 22 (t(9;22)(q34;q11)) has occurred in a hematopoietic stem cell.\(^5,6\) As a consequence of this translocation the *Abelson murine leukemia oncogene* (*c-ABL*) on chromosome 9 fuses with the *Breakpoint cluster region* (*BCR*) gene on chromosome 22. The shorter derivative chromosome 22 is named Philadelphia (Ph) chromosome and carries the *BCR-ABL* fusion gene (Figure 2).\(^6,7\)

Approximately 5-10 % of all CML patients have a variant translocation involving one or more partner chromosomes, most frequently chromosome 3, 4 or 5, in addition to chromosome 9 and 22.\(^8,9\)

Only one genetic aberration is sufficient to cause malignant transformation in CML, but if the disease is left untreated additional genetic aberrations will arise. This is called clonal evolution.\(^10\)

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**Figure 1. Normal hematopoiesis.** All blood cells originate from multipotent hematopoietic stem cells residing in the bone marrow. These stem cells can renew themselves and also differentiate to all cells seen in the peripheral blood. The different blood and bone marrow cells can be identified by morphology and cell surface antigens with a given cluster of designation (CD) number.
The most common secondary aberrations are duplication of the Ph chromosome, t(3;21), trisomy 8, 19 or 21, isochromosome 17, monosomy 7 and mutations in p16. Clonal evolution is associated with progression into accelerated phase and blast crisis.

The BCR-ABL fusion gene is transcribed into messenger RNA (mRNA) which is translated into the BCR-ABL protein. Depending on the precise location of the breakpoints in the BCR and ABL genes, the molecular weight of the BCR-ABL protein can be of different sizes, i.e. 185 kDa, 210 kDa or 230 kDa.

The BCR-ABL protein is a tyrosine kinase that catalyzes the transfer of phosphate from adenosine triphosphate (ATP) to a tyrosine residue on a substrate protein. Both ATP and the substrate are bound to the BCR-ABL protein during the reaction. The phosphorylation modifies the activity of the substrate. BCR-ABL is overactive and the downstream phosphorylation is out of control. There is a long list of BCR-ABL substrates, e.g. CRKL, paxillin, CBL, RIN, and GAP, and phosphorylation of these leads to activation of several important signaling pathways involving RAS, RAF, JNK, MYC and STAT etc.

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**Figure 2. Formation of the Ph chromosome.** In 1960 in the city of Philadelphia (USA), Dr Nowell and Dr Hungerford discovered a small aberrant chromosome in a chromosome spread from a CML patient. It was named the Philadelphia (Ph) chromosome, but its relevance in CML pathogenesis was unclear and debated at this time. It was not until 1973 that Dr Janet Rowley could show that the Ph chromosome was formed by a balanced translocation between chromosome 9 and 22. A balanced translocation means that no genetic material is lost. The translocation results in two “new” chromosomes: derivative chromosome 9 (der(9)) and derivative chromosome 22 (der(22)). The Ph chromosome was found to be the der(22). Later in 1985 the BCR-ABL fusion gene on the long arm of the Ph chromosome was characterized. The alternative reciprocal translocation product on der(9), ABL–BCR, is thought to play no role in leukemogenesis.
The phenotype of the mutant cells involves an enhanced proliferation, a loss of attachment to the bone marrow stroma and an inhibition of apoptosis.\textsuperscript{6,11} This causes an expansion of the malignant clone at the expense of normal hematopoiesis. It is not fully understood why the malignant cells preferentially differentiate into granulocytes and platelets.

In untreated CML, the bone marrow is overcrowded with myeloid cells in different stages of maturation (Figure 3). The malignant cells adhere less well to the bone marrow stroma and have a tendency to enter the blood stream prematurely.\textsuperscript{8} The presence of immature myeloid cells in peripheral blood is a characteristic feature of CML and an important clue in diagnosing the disease (Table 2). Production of blood cells is sometimes seen in the spleen, liver and even lymph nodes. This phenomenon is called extramedullary hematopoiesis and leads to enlargement of the involved organs.

In chronic phase CML, the malignant cells are too many, but they are mature and have a reasonably well preserved function. This explains why chronic phase patients rarely suffer from infections. Most symptoms are related to high blood cell production, e.g. bone pain, abdominal fullness, night sweats and fever, rather than blood cell dysfunction or deficiency, e.g. anemia, infections, and bleedings.

**Diagnostic criteria**

The diagnosis of CML is based upon typical morphological findings in peripheral blood and bone marrow together with the presence of the \textit{BCR-ABL} fusion gene (Table 2). In 90-95\% of all CML patients the \textit{Ph} chromosome is detected in bone marrow cells by conventional cytogenetics.\textsuperscript{13} In this assay, the chromosomes are stained and identified in preferably 20 or more bone marrow cells in metaphase.
About 5-10% of all CML patients have a variant translocation and the Ph chromosome is not detected by conventional cytogenetics. In these cases the BCR-ABL fusion gene is detected by fluorescent in situ hybridization (FISH). In FISH, bone marrow cells are incubated with fluorescent probes that bind specifically to the BCR and ABL genes, respectively. Different signals will be obtained from the cells if the BCR and ABL probes co-localize as in CML or bind to different chromosomes as in normal cells. The FISH analysis can be performed on either metaphase or interphase cells.

Finally, the BCR-ABL fusion gene can also be demonstrated by quantitative reverse transcriptase PCR (qRT-PCR; see below). Some patients have the clinical features of CML together with typical morphological findings in peripheral blood and bone marrow, but the malignant cells do not carry the BCR-ABL fusion gene. These patients are referred to as Ph negative or atypical CML and should be considered a separate disease entity. Ph negative/atypical CML does not respond to treatment with tyrosine kinase inhibitors (TKI) and will not be further discussed in this thesis.

Quantification of BCR-ABL expression

BCR-ABL expression is quantified using qRT-PCR and the presence of BCR-ABL transcripts in peripheral blood confirms the diagnosis of CML. Moreover, the BCR-ABL transcript level is used for the evaluation of treatment response and stratifies the patients into different risk groups.

Briefly, mRNA is isolated from nucleated cells in peripheral blood. The mRNA is reversely transcribed into complementary DNA (cDNA). A qRT-PCR assay is then run using primers and probes that hybridize with the target (BCR-ABL cDNA). A certain number of PCR cycles are run. During each cycle the target doubles until the reaction reaches a plateau. At each cycle, probes that have hybridized with the target emit a fluorescent signal. Fluorescence is recorded after each cycle. As the target is amplified, more probes hybridize with target and the signal gets stronger. Finally, at one cycle the fluorescent signal reaches above a threshold level. This cycle is called cycle threshold (Ct). The Ct value is central in calculation of the quantity of the target. The Ct value depends on the amount of the target at start of the reaction (Figure 4).

Some patients have the clinical features of CML together with typical morphological findings in peripheral blood and bone marrow, but the malignant cells do not carry the BCR-ABL fusion gene. These patients are referred to as Ph negative or atypical CML and should be considered a separate disease entity. Ph negative/atypical CML does not respond to treatment with tyrosine kinase inhibitors (TKI) and will not be further discussed in this thesis.

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Table 2. Full blood count in a 48-year-old male with newly diagnosed CML in chronic phase. Reference ranges are shown within brackets.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Reference Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-Hb (g/L)</td>
<td>128</td>
<td>(134-170)</td>
</tr>
<tr>
<td>B-Platelets (x10^9/L)</td>
<td>892</td>
<td>(145-348)</td>
</tr>
<tr>
<td>B-Leukocytes (x10^9/L)</td>
<td>92.1</td>
<td>(3.5-8.8)</td>
</tr>
<tr>
<td>Immature myeloid cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B-Blasts</td>
<td>0.29</td>
<td>(0)</td>
</tr>
<tr>
<td>B-Promyelocytes</td>
<td>2.9</td>
<td>(0)</td>
</tr>
<tr>
<td>B-Myelocytes</td>
<td>11</td>
<td>(0)</td>
</tr>
<tr>
<td>B-Metamyelocytes</td>
<td>7.9</td>
<td>(0)</td>
</tr>
<tr>
<td>B-Band-formed granulocytes</td>
<td>14 (&lt;0.45)</td>
<td></td>
</tr>
<tr>
<td>B-Neutrophil granulocytes</td>
<td>48 (1.8-7.5)</td>
<td></td>
</tr>
<tr>
<td>B-Eosinophil granulocytes</td>
<td>1.2 (0.04-0.4)</td>
<td></td>
</tr>
<tr>
<td>B-Basophil granulocytes</td>
<td>3.5 (0.0-1.0)</td>
<td></td>
</tr>
<tr>
<td>B-Lymphocytes</td>
<td>2.9 (0.8-4.5)</td>
<td></td>
</tr>
<tr>
<td>B-Monocytes</td>
<td>0.58</td>
<td>(0.1-1.0)</td>
</tr>
<tr>
<td>B-Erythroblasts (x10^9/L)</td>
<td>1.5</td>
<td>(0)</td>
</tr>
</tbody>
</table>

About 5-10% of all CML patients have a variant translocation and the Ph chromosome is not detected by conventional cytogenetics. In these cases the BCR-ABL fusion gene is detected by fluorescent in situ hybridization (FISH). In FISH, bone marrow cells are incubated with fluorescent probes that bind specifically to the BCR and ABL genes, respectively. Different signals will be obtained from the cells if the BCR and ABL probes co-localize as in CML or bind to different chromosomes as in normal cells. The FISH analysis can be performed on either metaphase or interphase cells.

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To normalize for variations in the amount and quality of mRNA and cDNA, the expression of a housekeeping gene is determined in the same sample. A housekeeping gene is a gene that is expressed at a constant and stable level. At the Sahlgrenska University Hospital, beta-glucuronidase (GUS) is currently used as housekeeping gene.

Standard curves are used to calculate the amount of mRNA (Figure 4). These standard curves are based on results from qRT-PCR assays using known amounts of plasmids containing BCR-ABL and GUS, respectively. When the absolute amounts of BCR-ABL and GUS have been determined, the ratio between BCR-ABL and GUS is calculated.

The qRT-PCR methods differ substantially between different laboratories. Currently, there is on-going work to make results from different laboratories more comparable. Briefly, one laboratory exchanges patient material with a reference laboratory. Both laboratories perform qRT-PCR analyses on the same samples. The results are then compared and a conversion factor is calculated to adjust the first laboratory’s results with the results from the reference laboratory, thereby creating an international scale. Another way to harmonize the BCR-ABL quantification is the planned introduction and spreading of commercial kits of the BCR-ABL analysis.

**Figure 4. Model of qRT-PCR.** A) At each cycle, the targets X and Y double until the reactions reach a plateau. The target-specific probes fluoresce when they hybridize with cDNA. As the target is amplified, more probes hybridize with cDNA and eventually the fluorescent signal reaches over the background level. The Ct value is the cycle number at which the curve intersects the threshold line. Above, the Ct value of target X is 21 and the Ct value of target Y is 26. A third target (Z) is tested, but the sample does not contain target Z and this target is therefore not amplified. B) Standard curves of target X and Y are shown to the right. The copy number of each target is easily calculated using the formula of the standard curve (Ct=a+b×copy number; a is the y-intercept and b is the slope of the line). Above, CtX 21 equals $10^{3.1} \approx 1250$ copies of target X and CtY 26 equals $10^{2.7} \approx 650$ copies of target Y. If target X would be BCR-ABL and target Y would be GUS, then the BCR-ABL transcript level is $(1250/650) \times 100=190\%$. That is a very high ratio that may be seen at diagnosis or at progression of the disease. In major molecular response (MMR) the ratio is less than 0.1%.
**Treatment of CML**

**Historic overview**

Chronic myeloid leukemia was recognized as a disease entity in the mid to late 19th century. At this time CML was treated with arsenic (Fowler’s solution) that reduced spleen size, fever and leukocytosis. When radiotherapy was developed at the turn of the century, splenic irradiation replaced arsenic in treatment of CML. In the 1950s the cytostatic drug busulfan was introduced to reduce leukocytosis. Busulfan was later replaced by hydroxyurea being less toxic. These treatments could at best relieve symptoms and the disease inevitably followed its natural course from chronic phase to blast crisis and death.20

In 1980 the first CML patients underwent allogenic stem cell transplantations (SCT) in Sweden. This treatment offered a chance of cure, but due to transplantation related mortality in the early days the median survival was not higher than 4.7 years in transplanted CML patients.2 Until 2001 allogenic SCT was the first line treatment in younger patients with a HLA-identical sibling donor and is still used in selected cases.2,21

In 1983 interferon alpha (IFNα) was introduced and remained the standard therapy until 2001.22 The treatment had considerable side-effects, such as fever, muscle pain, asthenia, and fatigue, but increased the median survival to 5-7.5 years.1 IFNα was sometimes combined with cytostatic drugs such as cytarabine (Ara-C).1

Finally, in 2000 the International Randomized IFN vs. STI571 (IRIS) trial started. This was a large-scale phase 3 study comparing treatment with the tyrosine kinase inhibitor (TKI) imatinib (STI571) vs. IFNα in CML patients in chronic phase.23 It soon became evident that imatinib was a major breakthrough in CML treatment. Today, a 5-year-survival of at least 90 % is expected in CML patients treated with imatinib or a second generation TKI.

**Treatment response**

There are factors identified at diagnosis (baseline) and during treatment that can aid in predicting treatment outcome. The most important baseline factor is the phase of disease (Table 1). Sokal and Hasford risk scores are calculated at diagnosis and are based on the following variables: age of the patient, blood counts and spleen size. These two risk score formulas differ slightly. The Sokal risk score was introduced in 1984 when standard therapy of CML was busulfan or hydroxyurea.24 The Hasford score was introduced in 1998 and was based on the outcome of patients treated with IFNα.25 Today, these risk scores have lost some of their importance and the timing and degree of treatment response to TKI therapy is the most important predictor of long-term outcome in chronic phase patients (Table 3).26

Hematologic response (HR) is a rough measure based on peripheral blood counts. In complete HR (CHR), the peripheral blood count is normalized and the spleen is not enlarged. Cytogenetic response (CgR) is determined by the proportion of Ph positive metaphase cells in bone marrow measured by conventional cytogenetics. At diagnosis, up to 100 % of the bone marrow cells examined carry the Ph chromosome. In complete CgR (CCgR) no Ph positive cells are detected (Table 3). Finally, molecular response (MR) is determined by serial measurements of BCR-ABL transcript level in peripheral blood by qRT-PCR.

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1 Sokal and Hasford risk scores are calculated at diagnosis and are based on the following variables: age of the patient, blood counts and spleen size.
2 Hasford score was introduced in 1998 and was based on the outcome of patients treated with IFNα.
3 Hematologic response (HR) is a rough measure based on peripheral blood counts.
4 Cytogenetic response (CgR) is determined by the proportion of Ph positive metaphase cells in bone marrow measured by conventional cytogenetics.
5 Molecular response (MR) is determined by serial measurements of BCR-ABL transcript level in peripheral blood by qRT-PCR.
Table 3. Definitions of treatment response.

<table>
<thead>
<tr>
<th>Definitions of treatment response</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hematologic response</strong></td>
</tr>
<tr>
<td>• Complete</td>
</tr>
<tr>
<td>B-Platelets &lt; 450x10^9/L</td>
</tr>
<tr>
<td>B-Leukocytes &lt; 10x10^9/L</td>
</tr>
<tr>
<td>B-Basophils &lt;5 %</td>
</tr>
<tr>
<td>No immature cells in peripheral blood</td>
</tr>
<tr>
<td>No palpable spleen</td>
</tr>
<tr>
<td>• Partial</td>
</tr>
<tr>
<td>1 – 35 %</td>
</tr>
<tr>
<td>• Minor</td>
</tr>
<tr>
<td>36 % – 65 %</td>
</tr>
<tr>
<td>• Minimal</td>
</tr>
<tr>
<td>66 – 95 %</td>
</tr>
<tr>
<td>• No</td>
</tr>
<tr>
<td>&gt;95 %</td>
</tr>
<tr>
<td><strong>Cytogenetic response</strong></td>
</tr>
<tr>
<td>% Ph positive cells</td>
</tr>
<tr>
<td>• Complete</td>
</tr>
<tr>
<td>0 %</td>
</tr>
<tr>
<td>• Partial</td>
</tr>
<tr>
<td>1 – 35 %</td>
</tr>
<tr>
<td>• Minor</td>
</tr>
<tr>
<td>36 % – 65 %</td>
</tr>
<tr>
<td>• Minimal</td>
</tr>
<tr>
<td>66 – 95 %</td>
</tr>
<tr>
<td>• No</td>
</tr>
<tr>
<td>&gt;95 %</td>
</tr>
<tr>
<td><strong>Molecular response</strong></td>
</tr>
<tr>
<td>BCR-ABL transcripts</td>
</tr>
<tr>
<td>• Complete</td>
</tr>
<tr>
<td>No detectable transcripts</td>
</tr>
<tr>
<td>• Major</td>
</tr>
<tr>
<td>3-log reduction</td>
</tr>
</tbody>
</table>

In the IRIS trial the BCR-ABL transcript level was compared with a standardized baseline that equaled the mean BCR-ABL transcript level in a group of untreated CML patients. Results were reported as “log reductions” compared with this standardized baseline. Major molecular response (MMR) was defined as a 3-log reduction compared with the standardized baseline, in other words a 1000 times (10^3) lower BCR-ABL transcript level compared with the standardized baseline.

There is on-going work to standardize the BCR-ABL quantification. The term MMR is used if the BCR-ABL transcript level is ≤0.1% according to the international scale, where the standardized baseline is set to 100%. However, in clinical practice a ratio of BCR-ABL/housekeeping gene below 0.1% is often considered as MMR.

**Tyrosine kinase inhibitors**

Imatinib was the first TKI introduced in treatment of CML. Imatinib is a small molecule that binds to the ATP-binding site in the kinase domain of BCR-ABL when the protein has adopted its inactive conformation (ATP not bound).

Nilotinib and dasatinib were introduced after imatinib and are referred to as second generation TKIs. Nilotinib is structurally related to imatinib and binds to inactive BCR-ABL. Dasatinib differs structurally from imatinib and nilotinib and binds to BCR-ABL in its active conformation (ATP bound). Active BCR-ABL has more structural similarities with other tyrosine kinases compared with inactive BCR-ABL. Hence, dasatinib is more promiscuous and inhibits more tyrosine kinases than imatinib and nilotinib (Table 4).

Also, imatinib, nilotinib and dasatinib inhibit BCR-ABL with different half maximal inhibitory concentrations (IC_{50}): imatinib 260 nM/L, nilotinib 13 nM/L and dasatinib 0.8 nM/L.
Table 4. Targets of imatinib, nilotinib and dasatinib. The targets are tyrosine kinases, except NQO2 and CA.30,32

<table>
<thead>
<tr>
<th>TKI</th>
<th>Shared targets</th>
<th>Other targets</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imatinib</td>
<td>ABL, ARG, BCR-ABL, C-KIT, PDGFR α/β, DDR1</td>
<td>NQO2, CA</td>
</tr>
<tr>
<td>Nilotinib</td>
<td>ABL, ARG, BCR-ABL, C-KIT, PDGFR α/β, DDR1</td>
<td>NQO2, CA</td>
</tr>
<tr>
<td>Dasatinib</td>
<td>ABL, ARG, BCR-ABL, C-KIT, PDGFR α/β, DDR1</td>
<td>SRC, YES, FYN, LYN, HCK, LCK, FGR, BLK, FRK, CTK, BTK, TEC, BMX, TXK, DDR2, ACK, ATR2B, ACTR2B, ACVR2, BRAF, EGFR/ERBB1, EPHA2, EPHA3, EPHA4, EPHA5, EPHA8, EPHB1, EPHB2, EPHB4, EPHB6 ERBB2, ERBB4, FAK, GAK, GCK, HH498/TNNI3K, ILK, LIMK1, LIMK2, MAP2K5, MAP3K1, MAP3K2, MAP3K3, MAP3K4, MAP4K1, MAP4K5/KHS1, MAPK11/p38 beta, MAP14/p38 alpha, MYT1, NLK, PTK6/Brk, QIK, QSK, RAF1, RET, RIPK2, SLK, STK36/ULK, SYK, TAO3, TESK2, TYK2, ZAK</td>
</tr>
</tbody>
</table>

Response to TKI therapy

The first CML patients were recruited to the IRIS trial in June 2000. Within the first year, it became evident that imatinib was superior to IFNα and Ara-C in treatment of CML (Table 5). At 12 months, all patients randomized to treatment with IFNα and Ara-C were offered to switch to imatinib therapy.23 In 2006, a 5-year overall survival of 89% was recorded in patients treated with imatinib. An estimated 7% of the patients had progressed into accelerated phase or blast crisis during the 5-year period. The risk of progression was highest the first 2 years after start of therapy and then gradually decreased.33

Table 5. Results from the IRIS trial at 12 months of treatment.23,27

<table>
<thead>
<tr>
<th></th>
<th>Imatinib 400 mg q.d</th>
<th>INFα and AraC</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHR</td>
<td>95%</td>
<td>56%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CCgR</td>
<td>69%</td>
<td>7%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MMR</td>
<td>39%</td>
<td>2%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>AC or BC1</td>
<td>1.5%</td>
<td>6.9%</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

1Accelerated phase (AC), blast crisis (BC)

Imatinib became available in 2001 and was the only TKI approved for treatment of newly diagnosed CML patients until 2010. In a recent Swedish population-based study, the 5-year relative survival rate in CML patients aged ≤79 years was 0.44 in 1994-2000 prior to the introduction of imatinib and 0.84 in 2001-2008 after the introduction of imatinib.2

Nilotinib was approved in 2007 for treatment of CML patients with resistance or intolerance to imatinib. In 2010, nilotinib was also approved for treatment of newly diagnosed CML. This was based on results from a large, randomized, open-label study comparing upfront treatment with imatinib and nilotinib (Nilotinib Efficacy and Safety in Clinical trials – Newly Diagnosed Patients; ENESTnd). The ENESTnd study reported that treatment with nilotinib resulted in faster and deeper treatment responses and fewer cases of blast crisis during the first year of therapy compared with imatinib treatment (Table 6).34 These differences remained at follow-up after 24 months.35 However, the study has as yet not shown any significant difference in overall survival between the groups treated with imatinib and nilotinib, respectively.34,35
Dasatinib became available in 2006 and was approved for treatment of newly diagnosed CML in 2010. Dasatinib inhibits more tyrosine kinases compared with imatinib and nilotinib and is associated with an increased risk of pleural and pericardial effusions (Table 4).36 A recent study (Dasatinib versus Imatinib Study in Treatment-Naive CML patients; DASASION) showed that patients treated with dasatinib up-front had a better treatment response compared with patients treated with imatinib (Table 7).37

### Table 6. Results from the ENESTnd trial at 12 months of treatment.34

<table>
<thead>
<tr>
<th></th>
<th>Imatinib 400 mg q.d.</th>
<th>Nilotinib 300 mg b.i.d</th>
<th>Nilotinib 400 mg b.i.d</th>
<th>p—value2</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCgR</td>
<td>65%</td>
<td>78 %</td>
<td>80%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MMR</td>
<td>22 %</td>
<td>44 %</td>
<td>43 %</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>AC or BC1</td>
<td>4% (n=11)</td>
<td>&lt;1% (n=2)</td>
<td>&lt;1% (n=5)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Overall survival</td>
<td>100%</td>
<td>99%</td>
<td>100%</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

1Accelerated phase (AC), blast crisis (BC); 2Imatinib vs. nilotinib 300 mg b.i.d and imatinib vs. nilotinib 400 mg b.i.d.

### Treatment failure

Some patients fail TKI treatment due to different reasons (Table 8). There are BCR-ABL dependent and independent mechanisms. First, the drug may not reach the target. The patient may not follow the prescription, i.e. lacking adherence. The drug may not be absorbed in the gut or it is highly eliminated in the liver and kidneys. The malignant cells may not take up the drug (decreased drug influx) or they pump out the drug (increased drug efflux) with high efficacy. Second, the target may be resistant to the drug.

Several point mutations have been described in the kinase domain of the BCR-ABL gene. Such a mutation may lead to a conformational change in the BCR-ABL protein that partially or totally hinders the binding of TKI to the target. The most troublesome point mutation is the Threonine 315 Isoleucine mutation (T315I) that makes the cells completely resistant to imatinib, nilotinib and dasatinib.43,44

Finally, additional genetic aberrations may have rendered the malignant cells independent of BCR-ABL. This happens in progression to accelerated phase or blast crisis. In these cases TKI therapy only have modest effect and drug resistance will inevitably appear.

### Table 7. Results from the DASASION study at 12 months.37

<table>
<thead>
<tr>
<th></th>
<th>Imatinib 400 mg q.d.</th>
<th>Dasatinib 100 mg q.d.</th>
<th>p—value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCgR</td>
<td>65%</td>
<td>77 %</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MMR</td>
<td>22 %</td>
<td>46 %</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>AC or BC1</td>
<td>3.5% (n=9)</td>
<td>1.9% (n=5)</td>
<td>N.S.</td>
</tr>
<tr>
<td>Overall survival</td>
<td>99%</td>
<td>97%</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

1Accelerated phase (AC), blast crisis (BC)
When treatment fails the therapy needs to be changed or modified. Treatment failure may be successfully overcome. First, adherence to TKI therapy should be checked for. Second, in BCR-ABL independent resistance, dose escalation may help. Third, if there is a point mutation in BCR-ABL, a switch from one TKI to another may overcome resistance. For instance, nilotinib and dasatinib inhibit nearly all known imatinib resistant forms of BCR-ABL except for the T315I. Finally, there are several up-coming TKIs, e.g. ponatinib that inhibits T315I mutated BCR-ABL.

Optimal response to TKI therapy predicts an excellent outcome, whereas treatment failure implies a high risk of progression if the treatment is not changed. In fact, treatment failure may indicate that the disease has already started to evolve towards accelerated phase or blast crisis. A suboptimal response, however, is a grey area. As a group, patients with a suboptimal response have an increased risk of treatment failure and progression, but many patients will probably do very well despite a suboptimal response. There is no clear-cut action plan for patients with a suboptimal response. Instead, it is up to the individual hematologist to decide if extra monitoring or treatment changes are needed. It is even more unclear what to do when “warning signs” appear during treatment (Table 8).

**Treatment in advanced phases**

About 10% of all CML patients are in accelerated phase or blast crisis at diagnosis. Patients that present in accelerated phase comprise a heterogeneous group. They may have a disease only slightly more advanced than chronic phase or they may be on the verge of blast crisis. Patients in “early” accelerated phase may have an excellent response to TKI therapy with a long-term survival equal to patients presenting in chronic phase. Therefore, therapy with a single TKI may be started in a patient in accelerated phase, but the treating physician needs to be prepared to promptly change therapy if the patient has a poor or suboptimal response.

TKI may induce remissions in blast crisis but does not prolong survival. Patients in blast crisis need chemotherapy in addition to TKI. Allogenic SCT is the only treatment that offers long-term survival.

### Table 8. Definitions of treatment failure, suboptimal response and warning signs.

<table>
<thead>
<tr>
<th>Time</th>
<th>Treatment failure</th>
<th>Suboptimal response</th>
<th>Warning signs</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 months</td>
<td>No HR</td>
<td>&lt;CHR</td>
<td></td>
</tr>
<tr>
<td>6 months</td>
<td>No CHR</td>
<td>&lt;Partial CgR</td>
<td></td>
</tr>
<tr>
<td>12 months</td>
<td>&lt;Partial CgR</td>
<td>&lt;CCgR</td>
<td>&lt;MMR</td>
</tr>
<tr>
<td>18 months</td>
<td>&lt;CCgR</td>
<td>&lt;MMR</td>
<td></td>
</tr>
<tr>
<td>Anytime</td>
<td>Loss of CHR or CCgR</td>
<td>Loss of MMR</td>
<td>2-5 fold increase in BCR-ABL</td>
</tr>
</tbody>
</table>

Evaluation of treatment response at 3, 6, 12 and 18 months is the most important landmark analysis.
Bone and treatment of CML

Imatinib, nilotinib and dasatinib are promiscuous drugs and inhibit several other tyrosine kinases besides the main target BCR-ABL (Table 4). Previously, it has been reported that CML patients treated with imatinib have altered bone metabolism, probably due to inhibition of such other tyrosine kinases. A great part of my research has focused on this issue, and a short overview of bone physiology and metabolism is therefore given.

Bone composition

The skeleton has multiple functions. It is a container of hematopoiesis, protects our inner organs and is essential for locomotion and stature. The outer part of the bone is solid (cortex) and the inner part is woven (trabeculae). The bone matrix comprises a mixture of tough fibers (e.g. collagen type I fibrils) that resist pulling forces and solid particles (e.g. calcium hydroxyapatite \([\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]\)) that resist compression. The matrix is formed and continuously remodeled in a tightly regulated process mediated by osteoblasts, osteoclasts and osteocytes. In a 10-year-period all bone tissue has been replaced. Bone mass peaks in young adults between 20 and 40 years of age. Thereafter bone resorption exceeds bone formation and a gradual physiologic bone loss is seen.

Osteoblasts

Osteoblasts develop from mesenchymal stem cells that reside in the bone marrow. Key transcription factors in osteoblast differentiation are RunX2 and Osterix. Osteoblasts synthesize and secrete the proteins in the bone matrix. In addition, they regulate mineralization of the bone matrix by controlling the local level of calcium and phosphate, and secreting alkaline phosphatase (ALP) that promotes mineralization. The osteoblast progenitors line the inner and outer surface of the bone and differentiate into mature osteoblasts after stimulation with different factors, e.g. platelet derived growth factor (PDGF) among others. The receptor of PDGF is a TKI target which opens for side effects in bone with these compounds (Table 4).

Osteocytes

Osteocytes develop from osteoblasts that have been trapped inside the bone matrix. The osteocytes are star-shaped cells and reside in small matrix cavities that are connected by tiny fluid-filled channels. Increased work load on the bone compresses the bone that in turn raises the fluid pressure. A decrease in work load decompresses the bone and the fluid pressure drops. The osteocytes have long cytoplasmic extension that sprawl into the tiny channels and senses changes in fluid pressure. When an osteocyte senses increased fluid pressure it stimulates bone formation. When the fluid pressure drops the osteocytes stimulates bone degradation. This process is termed mechanotransduction.

Osteoclasts

Osteoclasts develop from monocytic precursors that fuse and form large, multi-nucleated cells with phagocytic capacity (Figure 1). Osteoclasts remodel newly formed bone and remove damaged bone. When activated the osteoclast is polarized. The active side of the osteoclast binds to bone matrix proteins and a tight seal is formed between the osteoclast and the underlying bone matrix. This allows localized bone degradation. The membrane on the active side of the osteoclast is rearranged into
a ruffled border and this increases the active surface facing the bone matrix. Signaling through the colony stimulating factor 1 receptor (CSF1R) is important in differentiation of osteoclasts. CSF1R is a tyrosine kinase receptor that is inhibited by the TKIs, and CSF1R inhibition is another mechanism by which bone can be affected by these drugs.

**Studying bones**

Bone can be studied indirectly by biochemical measurements of blood or urine, or directly by radiological measurements of the skeleton. Biochemical measurements can be further subdivided into: i) analyses of ions essential in the bone mineralization, e.g. calcium, phosphate, and magnesium, ii) analyses of regulators of bone metabolism, mainly parathyroid hormone (PTH) and vitamin D, and iii) analyses of bone markers, mainly peptides released from the bone matrix during bone formation or degradation.

The gold standard to measure bone mineral density (BMD) is dual energy x-ray absorptiometry (DXA) of the lumbar spine and the hip. DXA gives a two dimensional assessment of BMD (areal BMD; g/cm²). The DXA results are often compared with results from a study of BMD in a large American population (The Third National Health and Nutrition Examination Survey; NHANES III). For each DXA measurement, one can retrieve the patient’s BMD together with the mean BMD of an age, gender and weight matched population (reference BMD). Results are presented as Z- and T-scores. The Z-score denotes how many standard deviations (SDs) a person’s BMD is above or below the mean BMD in young adults (20-40 years old) of the same gender (Figure 5).

A new method to study BMD is peripheral quantitative computed tomography (pQCT) that gives a three dimensional assessment of BMD (volumetric BMD; g/cm³). With pQCT it is possible to make separate analyses of the cortical and trabecular part of the bone.

**Figure 5. DXA results.** The plots above are estimations made only to illustrate the relationship between BMD, T- and Z-scores. The black line marks the reference BMD (hip) of females aged 20 to 100 years. The upper dashed line marks Z-score +1, and the lower dashed line marks Z-score -1. A physiologic age-related decrease in BMD is seen. A 67-year female has performed DXA of the hip and the results are plotted in the graph: BMD 0.778 g/cm², T-score -1.7, Z-score +0.3.
Aims of the study

The objectives of the present thesis were to study the following aspects of TKI therapy in CML:

I. Long-term effects of imatinib therapy on bone metabolism and BMD in CML patients
II. Effects of imatinib and dasatinib on osteoblast differentiation *in vitro*
III. Variations in molecular response during imatinib therapy
IV. Adherence to imatinib therapy in CML patients
Results

Statement of official approval

The human studies (Papers I, III, V and VI) were approved by the regional ethics committee in Gothenburg. Studies using radiological techniques were approved by the radiation safety committee at Gothenburg University. Informed written consent was obtained from all study patients and controls.

Study patients and controls

All CML patients included in the studies were treated at the Sahlgrenska University Hospital according national and international guidelines.47,60 Treatment responses were defined using standard criteria.13,28 The controls included in the studies were healthy volunteers.

Papers I and VI

Case-control study of bone and imatinib

In 2006 it was first reported that CML patients treated with imatinib had decreased levels of phosphate in serum, increased excretion of phosphate in urine and elevated levels of parathyroid hormone (PTH).48 This was interpreted as signs of disturbed bone metabolism. We therefore decided to study whether these biochemical changes were accompanied by changes in BMD.

A case-control study was set up in 2007 and 17 CML patients (11 males, 6 females) and 17 healthy age and gender matched controls were recruited. All CML patients were in CCgR. The mean age of the patient group was 60 years (range 41-79) and the duration of imatinib therapy was 50 months (range 24-79).

The results (Paper I) showed that the patients had significantly lower serum levels of phosphate, calcium and magnesium compared with controls. The serum level of PTH tended to be higher in the patients, but the difference in PTH between the patients and controls only reached borderline significance (p=0.06). The patients had significantly higher areal BMD in hip and lumbar spine measured by DXA compared with controls. The patients had a mean T-score above 0 and a mean Z-score above 0.5 in both the hip and the lumbar spine. Volumetric BMD measured by pQCT was significantly higher in the cortex of radius and tibia in the patients compared with the controls (p<0.05). There were no differences in the trabecular compartments of radius and tibia between the cohorts.

Prospective study of bone and imatinib

The two aforementioned cohorts were 4 years later, in 2011, invited to renewed biochemical and BMD measurements (Paper VI). Three controls were excluded since they had started treatment against osteoporosis between 2007 and 2011. It was shown that serum levels of PTH increased significantly in the patients between 2007 and 2011, and 7 out of 17 patients had evidence of secondary hyperparathyroidism in 2011. However, the areal and volumetric BMDs were stable in the CML patients over the 4-year-observation period. The patients had a mean T-score above 0 and a mean Z-score above 0.5 in both the hip and the lumbar spine even when studied in 2011. The patients had significantly higher cortical volumetric BMD in tibia and radius compared with controls in both 2007 and 2011. Trabecular volumetric BMD did not differ between the groups at any location or time point.
Papers II and IV

The results of Paper I showed that CML patients treated with imatinib had signs of altered bone metabolism. This led us to study the effects of imatinib and dasatinib on osteoblast differentiation *in vitro*. Human mesenchymal stem cells (hMSCs) were purchased from Lonza Inc. (Walkersville, MD). Osteoblast differentiation was induced in the hMSCs by growing the cells in differentiating medium containing dexamethasone, glycerophosphate, L-ascorbic acid and bone morphogenetic protein 2 (BMP-2). The cells were treated with imatinib or dasatinib at concentrations corresponding to the therapeutic plasma concentrations of these drugs.\(^{28,61}\)

**Inhibition of MSC proliferation**

It was shown that imatinib and dasatinib inhibited proliferation of hMSCs in a dose-dependent manner as assessed by a \(^{3}\)H-methyl-thymidine incorporation assay (Figure 6). In this assay, radioactive \(^{3}\)H-methyl-thymidine is incorporated into DNA when the cells proliferate. At the end of the assay, the amount of incorporated \(^{3}\)H-methyl-thymidine is measured in a scintillation counter.

**Effects of dasatinib on differentiation**

Additionally, it was demonstrated that dasatinib dose-dependently inhibited early osteoblast differentiation measured by alkaline phosphatase (ALP) activity and late osteoblast differentiation (mineralization) as assessed by Alizarin Red Staining (ARS; Figures 7 and 8).

**Effects of imatinib on differentiation**

Imatinib, however, had a biphasic effect on osteoblast differentiation. Low concentrations of imatinib inhibited early and late osteoblast differentiation, whereas higher concentrations of imatinib had an enhancing effect on early osteoblast differentiation (Figure 7). Inhibition of mineralization was most profound at low concentrations and mineralization increased with increasing concentrations of imatinib (Figure 8).

![Figure 6. Dose-dependent inhibition of hMSC proliferation](image-url)
Figure 7. ALP activity as a measure of early osteoblast differentiation. These results are from experiments performed in Papers II and IV and are presented as mean values ± SEM. The mean ALP activity in untreated MSC cultures is set as baseline (100%).

Figure 8. Mineralization measured by Alizarin Red Staining (ARS). These results are from experiments performed in Papers II and IV and are presented as mean values ± SEM. The mean ARS concentration in untreated MSC cultures is set as baseline (100%).
Paper III

The molecular response is important in estimating the risk of disease progression in CML. Also, a 2-5 fold increase in the BCR-ABL transcript level is regarded as a warning sign for an evolving TKI resistance. However, variations are common and may be due to both pre-analytic and analytic variations.

The method to measure the BCR-ABL transcript level is a qRT-PCR technique applied on mRNA extracted from nucleated cells in peripheral blood. Nucleated cells comprise a mixture of different cells mainly granulocytes and lymphocytes. However, the BCR-ABL fusion gene is predominantly present and expressed in granulocytes.

Peripheral blood is an inconsistent compartment and the relative proportion of granulocytes and lymphocytes varies in response to different factors, e.g. exercise, stress and infections. It was hypothesized that variations in the nucleated cell fraction might affect the qRT-PCR results (Paper III). Changes in the nucleated cell fraction of peripheral blood were induced in a standardized way by letting CML patients (n=6) exercise on a cycle ergometer until maximal exhaustion (maximal exercise test). Venous blood samples were collected before and after exercise. All BCR-ABL quantifications were performed according to international guidelines by trained personnel in an accredited laboratory at the Sahlgrenska University Hospital.

Exercise induced an early increase in lymphocytes and a biphasic increase in granulocytes (Figure 9). The BCR-ABL transcript level increased significantly in the nucleated cell fraction after exercise ($p <0.05$). The mean BCR-ABL transcript level increased 3.3-fold (range 0.7 – 6.8) from start of exercise to three hours after exercise ($p <0.01$). However, the mean BCR-ABL transcript level was unchanged in isolated granulocytes after exercise (Figure 10).

**Figure 9. Leukocyte count during the exercise test.** Baseline samples were collected immediately before start of the exercise test (0 min). The exercise test was stopped when maximal exhaustion (15 min) was reached. Exercise induced significant changes in the amount of granulocytes and lymphocytes in peripheral blood. The results are presented as mean values ± SEM.

**Figure 10. The BCR-ABL transcript level.** The results are given as fold change in $BCR-ABL1/GUS$ ratio compared with baseline (0 min). The mean BCR-ABL transcript level increased significantly in the nucleated cell fraction but not in isolated granulocytes after exercise. The results are presented as mean values ± SEM.
Adherence is often referred to as compliance. According to WHO, adherence is “the extent to which a person’s behavior taking medication corresponds with agreed recommendations from a health care provider”.64 Previous studies from United Kingdom, Belgium and India have reported that poor adherence to imatinib is common.38-42 It was hypothesized that adherence to imatinib may be different in Sweden due to socioeconomic and demographic factors.

All CML patients (n=42) treated with imatinib at the Sahlgrenska University Hospital on 1 January 2010 were invited to participate in the current study. A high proportion (90%) accepted inclusion in the study. Their mean age was 60 years (range 26-88) and the mean duration of imatinib therapy was 63 months (range 1–120).

The patients were interviewed and adherence was evaluated in a standardized way using the 9-item Morisky Medication Assessment Scale (MMAS). The 9-item MMAS is based on four themes that involve forgetfulness, negligence, interruption of drug intake after clinical improvement, and restart of drug intake when symptoms worsen. The summary score ranges from 1 to 13 where higher scores reflect better adherence. In the current survey, good adherence was defined as a Morisky score of 11 or higher, a concept based on a previous study using the 9-item MMAS to assess adherence to antiretroviral therapy in HIV infected patients.65

In addition, pre-defined questions were asked to identify factors known to influence adherence to therapy, e.g. degree of social support, knowledge of treatment, and accessibility to treating clinic.

All but one patient was regarded as adherent to imatinib therapy according to the 9-item MMAS. The mean Morisky score was 12.3 out of 13. In the present study, no correlation was seen between the molecular response and Morisky score. The patient that was non-adherent to imatinib therapy had a Morisky score of 9. This patient was in CCgR with MMR. The in-depth interviews revealed factors that are known to positively influence adherence, i.e. the patients were well-informed and had sufficient access to the treating clinic.
Discussion

Methodological considerations

Study cohort

The present thesis deals with different issues related to treatment of CML. The topics originate from bedside and some of them have been brought back to the laboratory bench. The studies reported in Papers I, III, V and VI are single center observational studies. CML is a rare disease, and Sweden is a sparsely populated country (21 inhabitants per km²). In the Gothenburg area only 5-10 new cases of CML are expected every year. This creates obvious limitations when studying the disease. However, the limited number of patients managed by a few hematologists, allows a detailed overview of the total patient material in a defined area, and there is consistency as to how the disease is treated and monitored. The follow-up time in the bone studies was four years, which is a long time interval even in an international perspective (Papers I and VI). Moreover, four years are also regarded as sufficient follow-up time to capture a change in BMD.

Adherence measurement

There is no gold standard in the study of adherence. Previous research shows that a more accurate assessment of adherence is achieved if several different methods are combined. In the present work, only self-reported adherence was studied (Paper V). In such instances there is a risk of recall bias and the interview situation may elicit socially acceptable response, leading to an overestimation of adherence. However, the CML patients studied had an overall good treatment response and this supports our notion that clinically relevant non-adherence to imatinib is uncommon in this cohort. To further strengthen the results so-called objective tests could have been performed, e.g. pill counts, pharmacy refill rates and measurements of imatinib concentration in plasma.

Osteoblast differentiation in vitro

Osteoblast differentiation was induced in hMSCs harvested from bone marrow of healthy donors. In the present studies, hMSCs from two different donors were used in separate experiments. Multipotent hMSCs are rare cells that lack specific cell surface markers. The purity of the hMSC samples is therefore determined by both testing for different cell surface antigens by flow cytometry (CD105+, CD166+, CD29+, CD44+, CD14-, CD34-, CD45-) and functional testing for differentiation into adipocytes, chondrocytes and osteoblasts (Papers II and IV). However, there is a risk of contamination with committed hMSCs and hematopoietic cells. This may influence the results and lead to difficulties in reproducing the results with hMSCs from different donors. Another problem with hMSCs is their limited survival in long-term cultures. After a number of passages the proliferation rate of the hMSCs gradually decreases until the cells enter a state of growth arrest. The possibility for in vitro expansion of hMSC from one donor is therefore limited.

An alternative approach could have been to study the effects of TKI on osteoblast differentiation in animal or human osteoblast cell lines. Using cell lines, an unlimited number of experiments could have been performed on a homogenous population of cells (monoclonal cells). However, osteoblast cell lines comprise transformed cells and may behave and react...
differently compared with normal osteoblast progenitors. Thus differentiation of MSCs in vitro is considered superior in reflecting osteoblast differentiation in vivo. Yet, another approach could have been to use MSCs or osteoblast progenitors harvested from animals, e.g. rat calvariae (skulls). However, by using MSCs of human origin, species variability in sensitivity to TKIs or expression of tyrosine kinases was avoided.

The effects of imatinib on osteoblast differentiation may differ in vitro and in vivo. First, in vitro osteoblast differentiation is induced by adding a mixture of different factors. In vivo there are more factors stimulating or inhibiting osteoblast differentiation. Second, cell-to-cell interactions with osteoclasts and other cells of the osteoblastic lineage contribute to osteoblast differentiation in vivo.54

Bone formation by osteoblasts in vivo requires migration, proliferation and differentiation of osteoblast progenitors.54 In the present work migration was not studied and proliferation and differentiation was studied in separate experiments (Paper II, IV). Relevant animal models are needed to further study the effects of imatinib on osteoblast formation and activity.

General discussion

Effects of TKI on bone

Imatinib was approved for CML treatment in 2001. Ten years experience of imatinib tells us that the drug is well tolerated and efficient in treating CML. Second generation TKIs are even more efficient in treating CML and have similar side-effects. We believe that adherence to imatinib therapy is good among our patients (Paper V) and since the introduction of TKI therapy 10 years ago we have only seen a few cases of CML progression at our clinic.

However, there are remaining concerns regarding CML treatment and efforts have been made to deal with some of them in the present thesis. First of all, imatinib and the second generation TKIs evidently have long-term effects on bone metabolism in CML patients. The significance of this issue is still debated and there are two opposite views regarding the effects of TKIs on bone: i) patients on TKI therapy may be at risk of developing osteoporosis or osteomalacia;48,69 ii) TKIs may be potential agents in treatment of osteoporosis or osteolysis.70-78 This divergence is the result of different interpretations of complex and still unclear in vivo and in vitro effects of TKIs on bone cells.

Osteoporosis is characterized by a generalized loss of bone mass and is defined as a T-score ≤ -2.5 determined by DXA.79 It is caused by an excessive osteoclast activity. Osteomalacia is characterized by a decreased mineralization of bone tissue and low BMD. It is most commonly caused by vitamin D deficiency (rickets). Osteolysis is no disease entity but a phenomenon seen in different diseases, e.g. multiple myeloma, and refers to focal pathologic bone resorption at one or multiple sites. It has been clearly shown that imatinib, nilotinib and dasatinib inhibit the formation and activity of osteoclasts in vitro and in animal models.73,80,81 This is partly why the TKIs are suggested as potential anti-osteolytic agents. We showed that imatinib does not increase BMD over a 4-year-observation period (Paper VI). This speaks against imatinib as a novel agent towards osteoporosis or osteolysis. Our in vitro data further supports this notion.
On the other hand, imatinib did not decrease BMD. Our results thereby contradict previous concerns about imatinib induced osteoporosis and osteomalacia.

The CML patients had been treated with imatinib for at least 2 years at the start of the study (Paper I). Bone status prior to start of CML therapy is therefore unknown. Untreated CML patients have a packed bone marrow and this may affect the surrounding bone tissue. Indeed, it has been reported that a hypercellular bone marrow as seen in untreated CML and chronic lymphatic leukemia may lead to osteoporosis and multiple focal osteolytic lesions of the axial skeleton. It is therefore uncertain if the patients’ bone status prior to TKI therapy would represent the patients’ bone status prior to CML when the patients were healthy. It is anticipated that there is a catch-up period during the first few months to a year after start of TKI therapy when the bone marrow microenvironment is restored. Indeed, O’Sullivan et al previously showed a biphasic change in bone turnover after start of imatinib therapy in 9 CML patients. In these patients an initial stimulation of bone formation was followed by a coupled suppression of bone resorption and formation. The authors speculated that the initial stimulation of bone formation represented recovery from illness. Alternatively, they reasoned, imatinib may stimulate the differentiation of a preexisting pool of osteoblast precursors leading to an initial increase in BMD. When the pool of osteoblast precursors is depleted, an imatinib mediated inhibition of MSC proliferation and migration would lead to a halt in bone formation. Such a mechanism might explain why our CML patients displayed high T- and Z-scores, even though BMD did not increase during the 4-year-observation period (Paper VI). Finally, the CML patients that we studied were middle-aged or elderly in a period of life when age-related physiologic bone loss is seen (Figure 5). Since BMD remained stable and T- and Z-scores tended to be high, we speculate that imatinib leads to a deceleration of physiologic bone loss.

We showed that CML patients on prolonged imatinib therapy displayed a high incidence of secondary hyperparathyroidism (Paper VI). The long-term consequences of a steady increase in serum PTH are unclear. We suggest that the increase in PTH is caused by a decreased release of calcium from bone, i.e. decreased bone resorption. The high incidence of hyperparathyroidism indicates that the osteoclasts are at least partly resistant to PTH stimulation. It is too early to say whether this TKI induced elevation of PTH has any long-term positive or negative effects on bone and other end-organs. For instance a persistent secondary hyperparathyroidism may eventually lead to an uncoupled autonomous production of PTH in the parathyroid glands, i.e. tertiary hyperparathyroidism.

The skeleton may appear as a solid and static organ, but in fact it is continuously remodeled throughout life. The bone cell population is constantly regenerated. Osteoclasts live for a few days to weeks while most osteoblasts live for a few months. In fetuses and children, coupled osteoclast and osteoblast formation and activity is essential for bone growth. A halt in osteoclast and osteoblast activity is deleterious in fetuses and children. An interesting recent paper showed that growth impairment was a major adverse effect of imatinib treatment in children with CML.
This supports our notion that bone remodeling is disturbed by imatinib. Moreover, skeletal deformities have been reported in off-spring of women treated with imatinib during pregnancy. Similar skeletal deformities have been described in rats exposed to imatinib during fetal life. In adults the effects of a halt in bone remodeling are less obvious. In adults, a concomitant TKI mediated decrease in bone formation and resorption may leave BMD unaffected. However, since bone remodeling is needed to heal microfractures in the bone tissue, the bone quality may be impaired in spite of normal BMD.

While repeated studies uniformly show that imatinib inhibits osteoclast formation and activity, there are studies reporting that imatinib inhibits or promotes osteoblast differentiation in vitro. The same is seen with the second generation TKIs. Several studies have shown that both nilotinib and dasatinib inhibit osteoclast differentiation, while studies on osteoblast differentiation are contradicting. As shown in Paper II, dasatinib inhibited osteoblast proliferation and differentiation in a dose-dependent manner. Other studies have confirmed the inhibitory effect of dasatinib on MSC proliferation, but showed a stimulatory effect of dasatinib on osteoblast differentiation. The results of Paper IV showed that imatinib had a biphasic effect on osteoblast differentiation. Low concentrations of imatinib inhibited early and late osteoblast differentiation whereas higher concentrations of imatinib promoted early osteoblast differentiation and had less inhibitory effect on late osteoblast differentiation. Thus, the effects of imatinib depended on both the concentration of imatinib and developmental stage of the osteoblasts. Interestingly, a recent paper showed that nilotinib had a similar biphasic effect on osteoblast differentiation. A biphasic effect of imatinib on osteoblast differentiation may explain why previous studies have been contradicting.

It is still unclear how TKIs affect osteoblast differentiation. There are several TKI targets that are thought to be important in normal osteoblast differentiation. c-ABL promotes osteoblast differentiation and mice deficient in c-ABL are osteoporotic. Inhibition of discoidin domain receptor 1 (DDR1) impairs osteoblast differentiation of MSCs in vitro. Deletion of c-KIT ligand results in delayed bone-growth, decreased bone mass and impaired osteoblast function in mice. The role of PDGFR in osteoblast differentiation is debated. It has been reported that depletion of PDGFR-β enhances osteoblast differentiation of murine MSCs. In contrast, a recent study showed that PDGF sustains osteoblast proliferation but does not affect differentiation. We suggest that the biphasic effect of imatinib is due to the effects on different tyrosine kinase signaling pathways. In addition, dasatinib, but not imatinib, inhibits ephrin type-B receptor 4 (EphB4) and Src that are thought to be important in osteoblast proliferation and differentiation. Different target spectra may explain why the imatinib and dasatinib seem to have different effects on osteoblast differentiation (Papers II and IV). Further research is needed to clarify the TKI effects on osteoblasts.
Molecular monitoring of CML

Much effort has been focused on standardizing the $BCR-ABL$ quantification. Nevertheless, small variations in the $BCR-ABL$ transcript level are common and do not necessarily reflect changes in tumor burden but may be due to pre-analytic and analytic variations in the assay. Patients in CCGR with MMR ($BCR-ABL$ ratio <0.1% according to the international scale) have an excellent prognosis. Currently, a 2 to 5 fold increase in the $BCR-ABL$ transcript level is regarded as a warning sign.\(^1\)\(^6\)\(^2\)\(^1\)\(^0\)\(^2\) We suggest that both the relative and absolute change in $BCR-ABL$ transcript level have to be considered in parallel. In the low range of the qRT-PCR assay, a small increase in $BCR-ABL$ transcript level generates a high fold change, e.g. when $BCR-ABL/GUS$ rises from 0.01 % to 0.05 % a 5-fold increase is recorded. Such a change is within the coefficient of variation (0.5 log) reported for the assay.\(^1\)\(^0\)\(^3\)

There are methodological differences between laboratories, e.g. in use of primers, probes, and technology employed. However, all laboratories perform the $BCR-ABL$ quantification on a mixture of lymphoid and myeloid leukocytes from peripheral blood, even though $BCR-ABL$ is mainly expressed in myeloid leukocytes. Peripheral blood is an inconsistent compartment and cells are mobilized and demobilized constantly from different pools, e.g. spleen, vessel walls, liver, bone marrow. We showed that exercise induced significant changes in the relative amount of myeloid and lymphoid cells in peripheral blood with a concomitant significant increase in the $BCR-ABL$ transcript level, above the 2 to 5-fold limit considered as a warning sign.

The molecular response is a useful tool in the monitoring of CML patients, but the results need to be interpreted cautiously and pre-analytic and analytic variations need to be accounted for. An early cytogenetic response is a major predictor of overall survival and progression free survival (PFS). In the IRIS trial, patients in CCGR with MMR at 18 months had a 100 % PFS at follow up after 5 years. However, patients in CCGR without MMR at 18 months had an almost equally high PFS (98%) and there was no statistical difference between the two groups.\(^3\)\(^3\) The majority of CML patients that present in chronic phase will obtain CCGR within the first 1-1.5 years. These patients have an excellent prognosis and small variations in molecular response commonly seen have limited clinical significance. Too much faith in molecular monitoring may lead to unnecessary investigations and treatments changes that may cause the patients harm and distress.\(^1\)\(^0\)\(^4\)

Recently, it has been shown that lymphocytosis is common in CML patients treated with dasatinib.\(^1\)\(^0\)\(^5\)-\(^1\)\(^0\)\(^9\) The lymphocytosis is correlated with higher rate of CCGR and MMR.\(^1\)\(^0\)\(^5\),\(^1\)\(^0\)\(^8\),\(^1\)\(^1\)\(^0\) An expansion of lymphocytes shifts the relative proportion of myeloid cells and lymphocytes in peripheral blood. Given that $BCR-ABL$ is expressed mainly in myeloid cells, dasatinib-induced lymphocytosis may result in a downshift of the $BCR-ABL$ transcript level without any change in the tumor burden. However, the higher incidence of CCGR in dasatinib-treated CML patients with lymphocytosis, supports the notion that the expanded clones of lymphocytes may have an anti-leukemic effect on the malignant CML cells.\(^1\)\(^0\)\(^5\),\(^1\)\(^0\)\(^8\)
Adherence to imatinib therapy

Most CML patients will have to ingest TKI daily for the rest of their lives. Thus, the patients need to remember to take the TKI regularly and tolerate the side-effects. For other drugs, it has been shown that the risk of poor adherence increases with duration of the therapy. In our study (Paper V), self-reported adherence was good among CML patients on prolonged treatment with imatinib (mean treatment duration 63 months). No relationship was seen between the degree of molecular response and adherence. Some known predictors of high adherence were identified. The patients were well-informed and took part in decisions concerning their disease and treatment. They had sufficient access to the treating clinic and the patients had scheduled follow-up appointments with “their own” hematologist.

Previous studies from United Kingdom, Belgium and India have suggested that poor adherence to imatinib is common among CML patients. The discrepancy between these studies and our may reflect differences in organization of health care, demographic and socioeconomic factors. When discussing adherence to imatinib and drugs in general, such factors need to be accounted for and it may be difficult to make general statements about adherence based on a national study.

A prerequisite of a successful imatinib treatment is of course that the patient takes the drug. Even though a lack of adherence seems to be a minor concern in our group of CML patients, adherence needs to be considered in all cases of treatment failure or suboptimal response. We suggest that relatively simple measures, e.g. patient information, easy access to the treating clinic and continuity of care, are sufficient to obtain good adherence to imatinib.

What is waiting around the corner

Imatinib is still first line therapy in CML. Recently, imatinib has been compared with the second generation TKIs, i.e. nilotinib and dasatinib. Nilotinib and dasatinib clearly show faster and deeper treatment responses, but no increased overall survival has as yet been shown with these drugs compared with imatinib. One reason may be that efficient rescue treatment exists in case of imatinib failure, e.g. switch to second generation TKIs and allogenic SCT. In a few years the patent on imatinib expires and that will probably sharpen the discussion about first line TKI therapy.

TKI therapy does not cure CML and cessation of therapy generally leads to recurrence of disease. There is on-going work to find ways to target the TKI resistant quiescent leukemic stem cells (CD34+ CD38-) which are thought to maintain the disease. If the leukemic stem cells could be eradicated, CML would be cured and TKI therapy could be discontinued. Until then we have to deal with different aspects of long-term treatment with TKI therapy.

CCgR is an established surrogate marker of survival. MMR (3 log reduction) is increasingly used as a primary endpoint in clinical trials, even though achievement of MMR has not yet been correlated with survival. Moreover, there is a lack of consistency as to when MMR should be recorded in clinical studies: i) MMR at any time point during the study; ii) MMR at pre-defined time-points
during the study, e.g. 12 months after start of therapy; or iii) sustained MMR, i.e. MMR at several time-points during the study. Further levels of molecular response are being introduced, i.e. 4- and 5-log reductions in \( BCR-ABL \) transcript level (CMR\(^4\), CMR\(^5\)), with the aim is to further stratify the response to TKI therapy.\(^{112,115}\)

**Conclusions**

The risk of imatinib-induced osteoporosis or osteomalacia is low in adult CML patients treated with imatinib. However, a high frequency of hyperparathyroidism is seen in CML patients on long-term imatinib treatment and the consequences of this are yet unknown. Hematologists treating CML need to be aware of potential off-target effects of TKI on bone and until this has been clarified a minimal intervention would be to check serum levels of PTH and calcium regularly, e.g. once a year.

We showed that imatinib and dasatinib displayed different effects on osteoblast differentiation that might be due to different target spectra of the drugs. It is therefore uncertain if dasatinib has the same effects on the bone as imatinib* in vivo*. Molecular monitoring is a useful tool in the management of CML patients, but small variations in \( BCR-ABL \) transcript level need to be interpreted cautiously due to pre-analytic and analytic variations. We showed that exercise induces significant changes in the \( BCR-ABL \) transcript level.

Adherence to imatinib is good among CML patients treated at the Sahlgrenska University Hospital. We suggest that adherence to imatinib may be obtained by simple measures such as easy access to the treating clinic, continuity of care and patient information.
Svensk sammanfattning (Summary in Swedish)


Trots stora framsteg i behandling av KML i kronisk fas, kvarstår några problem. En mindre grupp patienter svarar inte tillräckligt bra på behandlingen med imatinib. Detta kan bero på förändringar i BCR-ABL som gör att imatinib inte kan binda in till proteinet och hämma det. I sådana fall kan behandling med nilotinib (Tasigna®) eller dasatinib (Sprycel®) vara effektiv. Nilotinib och dasatinib är efterföljare till imatinib och kallas därför andra generationens TKI. En annan orsak till ett dåligt behandlingssvar kan vara bristande följksamhet till behandlingen (compliance).

Det är viktigt att tidigt identifiera de patienter som har ett otillfredsställande behandlingssvar. Detta sker genetiskt genom att regelbundet utföra olika analyser på blod- och benmärgsprover från patienterna. Ett sätt att indirekt mäta mängden kvarvarande cancerceller, är att kvantifiera uttrycket av genen BCR-ABL i vita blodkroppar i blodet. Ett 2-5-faldig stegring i mängden BCR-ABL mellan två provtagningar anses vara en varningssignal för ökad sjukdomsaktivitet. Variationer i mängden BCR-ABL är dock vanliga och beror troligen inte alltid på förändring i sjukdomsaktiviteten. Variationerna i
**BCR-ABL** skapar oro hos patienten och behandlande läkare och kan leda till mer eller mindre underbyggda förändringar i provtagning och behandling.

Imatinib, nilotinib och dasatinib hämmar utöver BCR-ABL ett antal normala tyrosinkinas. Detta skulle kunna ge önskade sidoeffekter. Eftersom behandlingen med TKI som är livslång är detta särskilt viktigt att undersöka.

Syftet med detta doktorandarbete har varit att undersöka:

I. Långtidseffekterna av imatinib på benmetabolism och bentsättning hos patienter med KML
II. Effekterna av imatinib och dasatinib på utmognaden av benceller *in vitro*
III. Variationer i **BCR-ABL**-nivån i blodet under behandling med imatinib
IV. Följsamhet till behandling med imatinib

**Långtidseffekter av imatinib på ben *in vivo***


**Effekter av tyrosinkinashämmare på benbildande celler *in vitro***

Imatinib och dasatinib hämmar flera tyrosinkinas som tros ha viktiga funktioner i benbildning. Efter den första benstudien 2007, valde vi att gå vidare och studera hur imatinib och dasatinib påverkar benbildande celler, osteoblast, *in vitro*. Vi kunde i dessa försök se att imatinib och dasatinib hämmade delningen av förstärtrade osteoblastceller. Vidare såg vi att dasatinib hämmade utmognaden av osteoblastceller på ett dosberoende sätt. Imatinib hade en mer komplett effekt på utmognaden av osteoblastceller. Låga doser av imatinib hämmade utmognaden av osteoblastceller, medan högre doser av imatinib stimulerade utmognaden av osteoblastceller i tidiga faser.

**Variationer i nivån av **BCR-ABL** i blodet***

Mängden **BCR-ABL** kvantifieras i blodet. Provmaterialen innehåller en blandning av olika vita blodkroppar, huvudsakligen lymfocyter och granulocyter, även om **BCR-ABL** huvudsakligen finns i granulocyter. En förändring av den relativa mängden granulocyter och lymfocyter skulle därför kunna påverka den uppmätta mängden **BCR-ABL**. Det finns flera faktorer som kan påverka antalet granulocyter och lymfocyter i blodet. Exempel är fysisk ansträngning, stress och infektioner. Vi lätt en grupp KML-patienter lämna blodprover före

Följsamhet till behandling med imatinib


Slutsatser


Sjukdomsaktiviteten följs vid KML genom att mäta nivån av BCR-ABL i blodet. Variationer i provtagningsmaterial och analysmetoden bör beaktas och små förändringar i nivån av BCR-ABL bör tolkas med varsamhet för att undvika onödiga förändringar i behandling och provtagnings.

Våra resultat tyder på att god följsamhet till behandling med imatinib kan uppnås genom enkla metoder, som ökad patientinformation, kontinuitet och tillgänglighet i vården.
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