Clinical and Experimental Studies in Chronic Myeloid Leukemia

Studies of Treatment Outcome, In Vitro Cellular Drug Resistance and Gene Expression

ULLA OLSSON-STRÖMBERG
Dissertation presented at Uppsala University to be publicly examined in Enghoffsalen, Akademiska sjukhuset, Ingång 50, bv, Uppsala, Wednesday, May 16, 2007 at 09:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in Swedish.

**Abstract**


The aims of the studies described in the thesis were to investigate different treatment strategies in chronic myeloid leukemia (CML) patients. Furthermore, activity of imatinib was investigated by *in vitro* cytotoxicity assay, and the gene expression pattern in interferon treated patients.

In a randomized prospective national study, we examined the influence of busulphan (n=89) versus hydroxyurea (n=90) treatment on time to blast crisis, and survival. There was no significant difference in survival between hydroxyurea and busulphan treated patients; median survival was 3.5 and 3.2 years, respectively. The 26 patients who underwent allogeneic stem cell transplantation had a significantly longer median survival (4.7 years) than those who were not transplanted.

We investigated the feasibility of mobilizing Philadelphia chromosome negative blood stem cells with intensive chemotherapy and lenograstim in CML patients. Twenty-three patients (62%) were successfully mobilized. Twenty-one of these patients underwent autologous stem cell transplantation later on, with a 5-year overall survival at 68%.

Fluorometric Microculture Cytotoxicity Assay was used to analyze 32 tumor cell samples from CML patients, (26 chronic phase and 6 blast crisis). Imatinib showed a higher *in vitro* activity and more positive drug interactions in cells from blast crisis than from chronic phase. Interferon, daunorubicin and arsenic trioxide had the greatest benefit from a combination with imatinib.

Microarray-based gene expression analyses were performed on diagnostic CML samples prior to interferon treatment. We identified six genes that were differentially expressed in responders and non-responders to interferon. It might prove possible to use gene expression analysis to predict future response to interferon.

**Keywords:** chronic myeloid leukemia, adult, stem cell transplantation, mobilization of Ph-negative stem cells, in vitro assay, imatinib, gene expression

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Per, Clara och Maja-
 jag älskar er
 av hela mitt hjärta!
List of Papers

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


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<td>ACA</td>
<td>additional chromosome abnormality</td>
</tr>
<tr>
<td>ALL</td>
<td>acute lymphoblastic leukemia</td>
</tr>
<tr>
<td>AML</td>
<td>acute myeloid leukemia</td>
</tr>
<tr>
<td>AP</td>
<td>accelerated phase</td>
</tr>
<tr>
<td>BC</td>
<td>blast crisis</td>
</tr>
<tr>
<td>BCR</td>
<td>breakpoint cluster region</td>
</tr>
<tr>
<td>BCR-ABL</td>
<td>breakpoint cluster region-Abelson</td>
</tr>
<tr>
<td>BSC</td>
<td>blood stem cell</td>
</tr>
<tr>
<td>CCgR</td>
<td>complete cytogenetic response</td>
</tr>
<tr>
<td>CFU-GM</td>
<td>colony-forming-units-granulocyte-macrophage</td>
</tr>
<tr>
<td>CgR</td>
<td>cytogenetic response</td>
</tr>
<tr>
<td>CHR</td>
<td>complete hematologic response</td>
</tr>
<tr>
<td>CI</td>
<td>combination index</td>
</tr>
<tr>
<td>CLL</td>
<td>chronic lymphocytic leukemia</td>
</tr>
<tr>
<td>CML</td>
<td>chronic myeloid leukemia</td>
</tr>
<tr>
<td>CMolR</td>
<td>complete molecular response</td>
</tr>
<tr>
<td>CP</td>
<td>chronic phase</td>
</tr>
<tr>
<td>DA</td>
<td>daunorubicin and Ara-C</td>
</tr>
<tr>
<td>DiSC</td>
<td>differential staining cytotoxicity</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulphoxide</td>
</tr>
<tr>
<td>FDA</td>
<td>fluorescein diacetate</td>
</tr>
<tr>
<td>EFS</td>
<td>event free survival</td>
</tr>
<tr>
<td>FISH</td>
<td>fluorescence-in-situ-hybridization</td>
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<td>FMCA</td>
<td>fluorometric microculture cytotoxic assay</td>
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<tr>
<td>G-CSF</td>
<td>granulocyte-colony stimulating factor</td>
</tr>
<tr>
<td>GVHD</td>
<td>graft versus host disease</td>
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<td>HR</td>
<td>hematological response</td>
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<td>HU</td>
<td>hydroxyurea</td>
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<tr>
<td>IFNα</td>
<td>interferon-alpha</td>
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<td>LFS</td>
<td>leukemia free survival</td>
</tr>
<tr>
<td>LOOCV</td>
<td>leave-one-out cross validation</td>
</tr>
<tr>
<td>MCgR</td>
<td>major cytogenetic response</td>
</tr>
<tr>
<td>MEA</td>
<td>mitoxantrone, etoposide and Ara-C</td>
</tr>
<tr>
<td>MMolR</td>
<td>major molecular response</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>MNC</td>
<td>mononuclear cell</td>
</tr>
<tr>
<td>MRD</td>
<td>minimal residual disease</td>
</tr>
<tr>
<td>MTT</td>
<td>methyl-thiazol-tetrazolium</td>
</tr>
<tr>
<td>OS</td>
<td>overall survival</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PDGFR</td>
<td>platelet-derived growth factor receptor</td>
</tr>
<tr>
<td>PFS</td>
<td>progression free survival</td>
</tr>
<tr>
<td>Ph</td>
<td>Philadelphia</td>
</tr>
<tr>
<td>Ph-negative</td>
<td>Philadelphia chromosome negative</td>
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<tr>
<td>Ph-positive</td>
<td>Philadelphia chromosome positive</td>
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<tr>
<td>PMN</td>
<td>polymorphonuclear</td>
</tr>
<tr>
<td>RD</td>
<td>related donor</td>
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<tr>
<td>RT-PCR</td>
<td>real-time polymerase chain reaction</td>
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<td>SCT</td>
<td>stem cell transplantation</td>
</tr>
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<td>TCK</td>
<td>total cell kill assay</td>
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<tr>
<td>TRM</td>
<td>transplant related mortality</td>
</tr>
<tr>
<td>URD</td>
<td>unrelated donor</td>
</tr>
<tr>
<td>WBC</td>
<td>white blood cell</td>
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</table>
Introduction

Chronic myeloid leukemia

History
The word leukemia, derived from the Greek words *leukos*-white and *haima*-blood, means “white blood”. Chronic myeloid leukemia (CML) was first recognized as early as 1845 [1, 2] when several cases of splenomegaly, anemia and massive granulocytosis were described. Neumann deduced that the disease originated in the bone marrow and proposed the term “myeloid leukemia” [3]. However, it was not until 1960 that the discovery of the Philadelphia (Ph) chromosome by Nowell and Hungerford [4] led to a better understanding of the pathogenesis of the disease. In 1973, Janet Rowley [5] showed that the Ph chromosome results from a reciprocal translocation between the long arms of chromosomes 9 and 22. The therapies in the 1960s were early splenectomy and radiotherapy over the spleen. The first reports of the use of busulphan and hydroxyurea (HU) were published in the 1960s by Haut and Galton [6, 7]. These drugs are still in use today. In 1983, Talpaz published the first report on the efficacy of interferon-alpha (IFNα) which is also still in use today [8].

Clinical presentation
CML is a disease that originates in an abnormal pluripotent bone marrow stem cell [9]. The proliferating malignant clone expands and forces the normal hematopoietic cells aside. In blood and bone marrow there is a dominance of granulocytosis. The natural course on palliative treatment, with a median duration of approximately 3.5 years, goes from chronic phase (CP), followed by accelerated phase (AP), and finally, blast crisis (BC) where the leukemic process has acquired the characteristics of acute leukemia [10].

The great majority of patients are diagnosed in CP. A considerable percentage (20-40%) of these are asymptomatic, and their disease is diagnosed
after routine white blood cell counts (WBC) are obtained [11]. Some patients may have symptoms such as fatigue, weight loss, sweating and skeletal pain. At diagnosis, 50% of the patients have WBC counts >100 x 10^9/l with a dominance of granulocytopoiesis, and 50% have thrombocytosis. Atypical presentation in BC at diagnosis occurs in roughly 2% of the cases.

Epidemiology and etiology
CML is a rare disease. The incidence in western countries is around 10 per million persons/year. The disease represents about 15-20% of all cases of leukemia [12, 13]. The number of new adult cases in Sweden varies between 70 and 90 per year. A male predominance is observed in many studies [14, 15]. Median age at diagnosis is 60 years, and 20% of the patients are less than 40 years old (Swedish CML group, unpublished). CML is rare in children.

For the majority of patients, the etiology of CML is unknown. A recent report indicates a higher incidence of CML among clean-up workers after the Chernobyl catastrophe [16]. Individuals exposed to benzene or alkylating agents also have a higher incidence of CML, probably because of mutagenic effect [17, 18]. There does not appear to be an inherited disposition.

Diagnostic criteria
The CML diagnosis is based on morphological examination of blood and bone marrow, and detection of the Ph chromosome by cytogenetic or molecular analysis. The bone marrow in CML CP is hypercellular with a predominance of granulocytopoiesis. The morphologic features depend on the phase of the disease. The definitions of AP and BC are presented in Table 1 [19]. These have been used to distinguish CP from AP and BC in the most recent treatment reports and differ from those of the WHO, whose definition of AP also includes platelet count >1000 x 10^9/l, increasing spleen size, increasing WBC count, lack of response to therapy and cytogenetic evidence of clonal evolution. The WHO definition of BC states ≥20% blast cells in blood or and bone marrow compared to ≥30% in more recent publications [19].
Table 1. List of differences between CP, AP and BC that have been proposed by an international expert panel [19].

<table>
<thead>
<tr>
<th></th>
<th>CP</th>
<th>AP</th>
<th>BC</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Blast cells in blood or bone marrow &lt;15%</td>
<td>- Blast cells in blood or bone marrow 15-29%</td>
<td>- Blast cells in blood or bone marrow ≥30%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Blast cells plus promyelocytes in blood or bone marrow &gt;30%, with blast cells &lt;30%</td>
<td>- Extramedullary blast cell proliferation.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Persistent thrombocytopenia (&lt;100 x 10^9/l) unrelated to therapy.</td>
<td></td>
<td>- Large foci or clusters of blast cells in the bone marrow.</td>
</tr>
<tr>
<td></td>
<td>- Basophils in blood &gt;20%.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The Ph chromosome results from a reciprocal translocation between the long arms of chromosome 9 and 22 (Figure 1). This translocation results in the head-to-tail fusion of the breakpoint cluster region (BCR) gene on chromosome 22 at band q11 with the Abelson (ABL) proto-oncogene on chromosome 9 at band q34 [20]. In most cases of CML, the break occurs within the major breakpoint region of the BCR gene, that spans exons 12-16, and results in a fusion of either BCR exon 13 or 14 with ABL exon 2 (known as the b2a2 or b3a2 junctions, respectively). The fusion gene is translated into a fusion protein known as p210, because it has a molecular weight of 210 kDa. The BCR-ABL fusion can be detected by fluorescence-in-situ-hybridization (FISH) and the fusion mRNA by reverse-transcriptase polymerase chain reaction (RT-PCR) [21, 22]. The bcr-abl protein has an anti-apoptotic activity, and affects several signal transduction pathways that influence proliferation and adhesion of the leukemic cells. The protein is believed to play a central role in the development of CML [23, 24].

The Ph chromosome is found in 95% of patients with CML; the remaining cases have no cytogenetically visible Ph chromosome, but are positive for the BCR-ABL fusion, which is then masked either as a cryptic translocation or within a complex karyotype [13]. The presence of extensive deletions in derivate chromosome 9 (der (9)) have been identified in 10-15% of CML patients in CP [25]. These deletions have variable breakpoints, and they often include sequences from both chromosome 9 and 22.
Figure 1. The Ph chromosome results when a piece of chromosome #9 switches places with a piece of chromosome #22. The translocation forms an extra-long chromosome 9 (called der 9) and an extra-short chromosome #22, which is the Ph chromosome that contains the abnormal, fused BCR-ABL gene.

Prognostic scores

Two sets of prognostic factors can be considered: one set identified prior to treatment (baseline factors) and one during treatment (response-related factors).

Baseline factors; Sokal score predicts survival for patients treated with busulphan or HU [26]. This score identifies three different risk groups: low, intermediate, and high. It is calculated from age, spleen size, blood platelet and blast cell counts. Twenty-three percent of the patients have high, 38% intermediate and 39% low Sokal risk score.

The Hasford score predicts survival for patients treated with IFNα and is calculated on 1303 patients from 14 different IFN clinical trials [27]. The Hasford score is based on age, spleen size and blood counts of platelets, eosinophils, basophils and blast cells. Three risk groups are identified. The low (40%), the intermediate (45%) and the high (15%) risk groups with median survivals of 98, 65 and 42 months, respectively. The Sokal and Hasford scores correlate with response, (but not survival) to imatinib (imatinib mesylate, Glivec®) treatment [21, 28, 29], which is considered as first line treatment in CML (page 21).

The Gratwohl score (Table 2) expresses the risk for transplant related mortality (TRM) and the chance of leukemia free survival (LFS) after allogeneic stem cell transplantation (SCT) [30]. This score was calculated on 3142 patients transplanted in 1989-1997. The patients are scored from 0 to 7.
The 5-year survival ranges from 72% in risk group 0-1, to 22% in risk group 5-7. The Gratwohl score is simple, based on five main factors, and provides adequate risk assessment for treatment decisions.

Table 2. Gratwohl transplantation risk score. The table lists the prognostic factors and the corresponding risk score.

<table>
<thead>
<tr>
<th>Prognostic factors</th>
<th>Risk score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0</td>
</tr>
<tr>
<td>&lt; 20 years</td>
<td>1</td>
</tr>
<tr>
<td>20-40 years</td>
<td>2</td>
</tr>
<tr>
<td>&gt; 40 years</td>
<td></td>
</tr>
<tr>
<td>Interval from diagnosis to transplantation</td>
<td>0</td>
</tr>
<tr>
<td>≤ 1 year</td>
<td>1</td>
</tr>
<tr>
<td>&gt; 1 year</td>
<td></td>
</tr>
<tr>
<td>Disease phase</td>
<td>0</td>
</tr>
<tr>
<td>CP</td>
<td>1</td>
</tr>
<tr>
<td>AP</td>
<td>2</td>
</tr>
<tr>
<td>BC</td>
<td></td>
</tr>
<tr>
<td>Donor-recipient sex match</td>
<td>1</td>
</tr>
<tr>
<td>Female donor and male recipient</td>
<td>0</td>
</tr>
<tr>
<td>Any other match</td>
<td></td>
</tr>
<tr>
<td>Donor type</td>
<td>0</td>
</tr>
<tr>
<td>HLA-identical sibling</td>
<td>1</td>
</tr>
<tr>
<td>Any other</td>
<td></td>
</tr>
</tbody>
</table>

Clonal cytogenetic evolution is defined as acquisition of cytogenetic abnormalities in addition to the Ph chromosome during the course of the disease. Only abnormalities that are demonstrable in at least two metaphases should be referred to as “clonal”. The tumor cells that carry these abnormalities could be Ph-positive or Ph-negative. Clonal cytogenetic evolution is often seen in AP and BC, and it is not clear whether the presence of this is an
adverse prognostic feature in the absence of other features of advanced disease.

CML patients who bear der (9) deletions and have been treated with IFNα have an adverse prognosis in terms of response to therapy and survival [25, 31]. However, there are few data regarding the impact of imatinib therapy on the outcome of CML patients with der (9) deletions [32, 33].

**Response-related factors:** Lately, factors related to therapy response seem to be of increased importance (page 22).

### Treatment

**Aim**

There are two treatment strategies in CML nowadays, remission inducing and palliative, respectively. Imatinib, other tyrosine kinase inhibitors, IFNα and allogeneic SCT are used for remission induction, busulphan and HU for palliation.

**Response**

The definition of response levels (i.e. remaining tumor burden) is summarized in Table 3.

Response to therapy is defined as hematological, cytogenetic or molecular. The majority of recent CML treatment reports define hematological (HR) and cytogenetic responses (CgR) in a uniform way, with only minor differences [29, 34, 35].

CML is a.o. caused by bcr-abl, an active tyrosine kinase. Imatinib is a small molecule that specifically inhibits the bcr-abl tyrosine kinase, and is recommended for up-front treatment of CP CML patients. With the introduction of imatinib, the cumulated rate of complete cytogenetic response (CCgR) in treated patients is high; therefore molecular methods for measuring minimal residual disease (MRD) (Figure 2) are required. RT-PCR has emerged as the method of choice for monitoring MRD in patients in CCgR. The status when no residual *BCR-ABL* transcripts can be detected with the most sensitive available methods is referred to as “complete molecular response” (CMolR). The use of the “log reduction” terminology has caused some confusion, as it implies the value is a relative. For this reason, it has been proposed to introduce a standardized numerical International Scale expressing the amount of *BCR-ABL* mRNA as a percentage of a control gene and anchored to two “absolute” values, based on validated reference materials of known value [22]. The degree of molecular response is an important prognostic factor. The goal for imatinib treatment is at least a 3-log reduction...
in BCR-ABL level. In this patient group the relapse rate at 54-months was only 3%, and no progression to AP or BC [29]. A major molecular response (MMolR) after 12 months of imatinib treatment is associated with even better event-free survival (EFS) and progression free survival (PFS) [21]. Patients with less than complete hematological response (CHR) at 6 months, less than partial cytogenetic response at 12 months, and less than CCgR at 18 months treatment with imatinib have a less good prognosis [21, 29]. A rise of BCR-ABL transcript level has been consistently associated with mutations [36].

In CP patients, there is a good correlation between BCR-ABL mRNA levels in blood and bone marrow. Therefore, RT-PCR analyses can be performed on blood samples, which is much more convenient for the patient [37].

Table 3. Definitions of treatment response.

<table>
<thead>
<tr>
<th>Level of response</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete hematologic response (CHR)</td>
<td>WBC &lt;10x10⁹/l, platelet &lt;450x10⁹/l and no palpable spleen</td>
</tr>
<tr>
<td>Minimal cytogenetic response (Minimal CgR)</td>
<td>66 - 95% Ph-positive metaphases *</td>
</tr>
<tr>
<td>Minor cytogenetic response (MinorCgR)</td>
<td>36 - 65% Ph-positive metaphases *</td>
</tr>
<tr>
<td>Partial cytogenetic response (PCgR)</td>
<td>1 - 35% Ph-positive metaphases *</td>
</tr>
<tr>
<td>Complete cytogenetic response (CCgR)</td>
<td>0% Ph-positive metaphases *</td>
</tr>
<tr>
<td>Major cytogenetic response (MCgR)</td>
<td>0 - 35% Ph-positive metaphases *</td>
</tr>
<tr>
<td>Major molecular response (MMolR)</td>
<td>BCR-ABL ratio &lt;0.1 International Scale</td>
</tr>
<tr>
<td>Complete molecular response (CMolR)</td>
<td>Negativity by RT-PCR</td>
</tr>
</tbody>
</table>

*Based on the analysis of at least 20 metaphases.
Figure 2. Approximate relationship between number of leukemic cells and level of \textit{BCR-ABL} transcripts.

Chemotherapy

Palliative chemotherapy is mainly based on HU and busulphan. These drugs are cytotoxic antiproliferative agents that are administrated orally, and they are well tolerated. HU was introduced for use in CML 1966 [38]. Patients treated with HU survive around 10 months longer than those on busulphan [39]. Currently, HU is used to quickly reduce the myeloid hyperplasia, or as long time palliative treatment. HU has very few side-effects, all of which are reversible on stopping treatment. Nausea and anorexia are usually transient. Atrophy and scaling of the skin and partial alopecia may occur.

Busulphan is an alkylating agent. It was one of the first drugs reported to be effective for hematological control in CML [40]. Side-effects of busulphan include severe prolonged myelosuppression. An uncommon side-effect is a severe idiosyncratic pulmonary reaction, a pronounced interstitial fibrosis, known as busulphan lung, which may be lethal [41]. Busulphan is not much in use as CML therapy nowadays.

Interferon-alpha

IFN\(\alpha\) was one of the first drugs that induced cytogenetic response in CML. The most important goal with IFN\(\alpha\) therapy is to reduce the tumor burden and thereby prolong time to BC [15, 42]. Not all patients respond to IFN\(\alpha\)
and the reason for this is largely unknown. The mechanism underlying the effect of IFN in CML is still poorly understood. In addition to the role in innate immunity, IFN modulates immune response and exerts antiproliferative effects in some cell types [43, 44] and it has been suggested that differences in immunological responses elicited by IFN underlie the individual differences in response [45, 46]. IFNα-based regimes were the treatment of choice for more than 10 years: several randomized prospective studies [15, 47-50] and a meta-analysis indicate that IFNα prolongs survival compared to palliative chemotherapy. The latest updates of the larger IFNα studies [51] report a 10-year overall survival (OS) ranging from 27 to 53% for all treated patients. IFNα induces CHR in about 80% of patients, CCR in about 50%, and major cytogenetic response (MCgR) in about 30%. A substantial survival prolongation is obtained only in cytogenetic responders. About 10% achieve a CCgR after 12 months treatment and here the 10-year survival is 70-80% for low and intermediate risk group patients.

The optimal IFNα dose is unknown. A multicentre trial comparing low dose (3 milj IE/m² x 1 sc, 5 days weekly) vs. high dose (5 milj IE/m² x 1 sc, seven days weekly) IFNα indicate that the low dose was as effective as, and better tolerated than the higher dose [52]. No significant differences in OS, PFS, CHR or MCgR were observed. As a palliative treatment, IFNα is used alone, or in combination with HU or Ara-C.

Autologous stem cell transplantation

The role of autologous SCT for treatment of CML has been subject to a number of studies and reviews for more than 20 years [53, 54]. Several observations indicate that the procedure could be useful for achieving CCR and for prolonging survival [55]. However, a meta-analysis of six trials, in which patients were randomly allocated to autologous SCT or an IFNα-based regimen, did not prove advantageous for survival for autologous SCT [56].

Despite favorable PFS in imatinib-treated patients, there is still uncertainty about the durability of response. Maybe, in the future, cryopreserved autologous blood stem cells (BSC) collected at CCgR may provide an attractive autograft option either as salvage or consolidation. In this era, several different strategies are demonstrated to mobilize Ph-negative stem cells in 40-80% of the patients [57-60].

Allogeneic stem cell transplantation

The first report, 30 years ago, of a successful transplant of bone marrow from twin donors in patients with CML marked the beginning of a new era in the treatment of this disease [54, 61]. The concept was rapidly followed with transplants from siblings and was soon confirmed in a series of more than 100 patients by the International Bone Marrow Transplant Registry
The number of transplants increased rapidly up to 1999 and then began to decline. In the beginning of this transplant era, there was a clear consensus for early transplantation [63]. Early transplant is no longer the first choice management for CML [64]. This is also reflected by an increase in transplants performed at later stages of the disease [65].

The indication for using allogeneic SCT in CML has changed since the introduction of imatinib. Clearly, the general recommendation that patients with CML and an HLA-identical donor should be transplanted within the first 12 months after diagnosis can no longer be upheld. There is an ongoing discussion whether any subgroup of patients with newly diagnosed CP should be treated with allogeneic SCT as primary therapy or not. It has been proposed that patients classified as high risk Sokal and good risk Gratwohl should be transplanted upfront. In practice, almost all patients receive imatinib as first line therapy. Allogeneic SCT is the second line treatment for patients with a suitable donor.

The longest follow-up of patients who received transplants from a matched-related donor is that reported by the European Group for Blood and Marrow Transplantation (EBMT) between 1980-1990. OS at 20 years was 34% for all patients and 41% for patients in first CP [66]. In the cohort transplanted between 2000 and 2003, probability of survival at 2 years was 61% and for the subgroup transplanted in first CP with an HLA-identical sibling 74% [66]. TRM is considerable and ranges between 15 and 60% [67]. However, relapses were seen up to 21 years after treatment. The usefulness of the previously described main pre-transplanted risk factors for outcome [30] (Gratwohl score) were confirmed in this analysis. Survival was better for patients transplanted in CP than those transplanted in later stages or BC, due to increased transplant-related mortality and relapse rate among patients with more advanced disease. Long-term survival after allogeneic SCT in BC is <10%.

Progress in molecular DNA typing of HLA alleles, management of opportunistic infections and supportive care, as well as conditioning regimes and immunosuppressive therapy, have contributed to improved outcome after allogeneic SCT [68]. For CML patients receiving conventional transplantation, the use of blood stem cells has not proved better than the use of marrow stem cells [69].

Reduced intensity conditioning is currently being evaluated for CML [70-73] and may permit allogeneic SCT also in fragile patients. The long-term impact on OS, EFS and quality of life cannot yet be assessed.

Relapse after allogeneic SCT is still a problem. Donor lymphocyte infusion has in this situation been the standard therapy. It has been shown to restore full donor chimerism and produce long-term CMoIR [74]. However possible side effects, such as graft versus host disease (GVHD) and myelosuppression lead to a considerable amount of morbidity and mortality. Imatinib as treatment for relapse, has shown remarkable short term efficacy.
combined with low toxicity [75, 76]. A recently published study comparing imatinib and donor lymphocyte infusion treatment at relapse, [77] showed that imatinib treatment resulted in a higher incidence of relapse.

Imatinib

The **BCR-ABL** oncogene is thought to play a key role in the patogenesis of CML [20, 23, 24]. It encodes a chimeric bcr-abl protein having constitutively activated abl tyrosine kinase activity, and this is thought to be the underlying cause of CML. The first preclinical studies in 1996 showed apoptotic and antiproliferative effects on **BCR-ABL** positive cells by blocking bcr-abl-initiated signalling pathways [78, 79]. The imatinib (imatinib mesylate, Glivec®, Novartis) molecule binds to the ATP-binding pocket of the abl domain and stabilizes the inactive, non-ATP-binding conformation of the bcr-abl tyrosine kinase [80, 81] (Figure 3). This blocks tyrosine kinase activity and inhibits both bcr-abl mediated autophosphorylation and substrate phosphorylation, resulting in abrogated downstream cell signalling and reduced proliferation of the **BCR-ABL**-positive cells [78, 79, 82]. Although imatinib is highly specific for abl, the c-kit, platelet-derived growth factor receptor (pdgfr) and arg tyrosine kinase activities are also suppressed [83], which has been suggested to be part of the inhibitory function of imatinib on CML cells [84].

**Figure 3.** A schematic view of the mode of action for imatinib. a) **BCR-ABL** signalling without imatinib and b) with imatinib. Imatinib blocks the ATP-binding site and thereby inhibits tyrosine phosphorylation.
Early studies into bone marrow changes associated with imatinib therapy in CML patients suggested normalization of bone marrow, without any morphologic evidence of CML [85, 86]. In recent studies, long-term (mean 21 months) therapy with imatinib produced marked changes in bone marrow histopathology in most CML patients who respond to therapy [87]. Decreases in bone marrow cellularity, neutrophil granulopoiesis and the number of atypical micromegakaryocytes are accompanied by an increase in erythroid precursors and lymphoid nodules [87].

Imatinib is easy to administer orally, and has relatively few side effects. The drug is rapidly absorbed, reaching a maximum plasma concentration 2-4 hours after oral administration. It is metabolized by the liver, mainly via the cytochrome P450, and primarily eliminated via feces.

Imatinib induces a much higher rate of CCgR in CP than any other therapy (except allogeneic SCT) [29]. The superiority of imatinib 400 mg daily over IFNα and low dose Ara-C was established in a prospective randomized study of CP patients, the IRIS study [29] (International Randomized Study of Interferon and STI571). Patients were randomized to receive imatinib 400 mg/day or IFNα; plus low dose Ara-C. After 60 months treatment, PFS was 93%, OS was 90% and survival freedom from progression to AP/BC, as well as from hematologic or cytogenetic relapse, was 84% [29]. This ongoing study has been continuously updated and has shown thus far that response to imatinib is a most important response related prognostic factor (Table 4). The probability of PFS is strongly correlated with the level of response.

Cytogenetic response seems to be the most important response-related prognostic factor. If no CgR is achieved after 3 months, there is still a 50% chance of achieving a CCgR later. If after 12 months of treatment, the CgR is partial, the probability of achieving a CCgR at 2 years is still 50%, but if the response is less than partial, the chance is less than 20%.

The issue of the optimal imatinib dose is not yet settled, and the standard dose is 400 mg to CP patients. Circumstances permitting an increase in dosage from 400 to 600 mg/day, or from 600 to 800 mg/day, include disease progression and failure to achieve satisfactory HR and CgR. Results suggest that higher doses of imatinib, 800 mg/day, may be associated with faster onset and higher rate of cytogenetic response [89, 90]. The long-term outcome is not yet known.
Table 4. Relationship between the degree of early CgR, the CCgR rate at 2 years, and EFS at 42 months in IRIS study [88]

<table>
<thead>
<tr>
<th>Time of treatment and cytogenetic response</th>
<th>Probability of CCgR at 2 years, %</th>
<th>EFS at 42 months, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 months</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Partial</td>
<td>90</td>
<td>Not available</td>
</tr>
<tr>
<td>Minor</td>
<td>60</td>
<td>Not available</td>
</tr>
<tr>
<td>Minimal/none</td>
<td>50</td>
<td>Not available</td>
</tr>
<tr>
<td>6 months</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complete/partial</td>
<td>Not applicable/80</td>
<td>95</td>
</tr>
<tr>
<td>Minor or minimal/none</td>
<td>50/15</td>
<td>75</td>
</tr>
<tr>
<td>12 months</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complete/partial</td>
<td>Not applicable/50</td>
<td></td>
</tr>
<tr>
<td>Minor, minimal, or none</td>
<td>&lt; 20</td>
<td></td>
</tr>
</tbody>
</table>

An ongoing question is whether CML patients should be treated with imatinib for the rest of their life. An important unanswered question for patients who achieve a CMoIR is whether imatinib therapy may be discontinued safely. Only limited data are available regarding this, and the results are divergent [91-93]. In a recently published study 12 patients with undetectable residual disease for more than 2 years were included [91]. Six patients displayed a molecular relapse within 5 months, but when imatinib was reintroduced this led to a novel molecular response in most patients. Six patients still had an undetectable level of BCR-ABL transcript after a median follow-up of 18 months. In another report with three patients, all relapsed shortly after discontinuation of therapy [92].

A small fraction of CML patients show primary resistance to imatinib, and responding patients may later develop secondary resistance and relapse.
Combining imatinib with other antileukemic agents may be one way to further increase the remission rate and to prevent outgrowth of resistant clones. In *in vitro* studies, different drugs are shown to overcome imatinib resistance or to synergize with the effect of imatinib. These include leptomycin B, proteasome inhibitors, mTOR-inhibitors, arsenic trioxide, myophenolic acid, farnesyl-transferase inhibitors, bryostatin, decitabine, histone–deacetylase inhibitors, homoharringtonine and phosphoinositide-dependent kinase -1 inhibitors [94-100]. However, results are still preliminary and limited [101]. Intermittent exposure of CML cells to granulocyte-colony stimulating factor (G-CSF) enhances the effect of imatinib on CML cells [102].

Results of single agent imatinib in the management of AP and BC are less impressive than results achieved in CP [103, 104]. Some patients in AP appear to obtain a major benefit from treatment with imatinib. The responses for the majority of patients treated in BC are not maintained over a long time. Thus, for patients with advanced phases, imatinib should mainly be used only as a bridge to an allogeneic SCT. In patients with AP and BC, an initial dosage of 600 mg/day is recommended.

Treatment with imatinib prior to allogeneic SCT does not influence transplant related morbidity and mortality [105, 106].

**Side-effects**

Imatinib is generally well tolerated. Hematological adverse events are neutropenia, thrombocytopenia and anemia. This toxicity is managed by reducing the imatinib dosage or discontinuing treatment until the problem is resolved. Sometimes it is necessary to administer G-CSF.

The the most common non-hematological toxicities seen with imatinib treatment are edema (60%), nausea (50%), muscle cramps (49%), diarrhea (45%), skin rash (40%) and fatigue (39%). The most common manifestation of edema is the periorbital edema that is typically worse in the morning, and no specific therapy is required for most cases. Some patients have found that limiting salt intake helps to control edema, and in severe cases, diuretics may be indicated. Musculoskeletal complains are another common side effect of imatinib and are manifested as muscle cramps and bone pain. Calcium and magnesium supplementation often provides symptomatic relief, even in patients with normal serum levels of ionized calcium and magnesium [107]. Most cases with skin rash, is mild, self-limiting, and easily manageable with antihistamines or topical steroids, whereas a short course of oral steroids can be used to treat more severe cases [29].

A recently published study [108] indicates advanced heart failure could be a side effect in imatinib-treated patients. A follow-up examination of Novartis database found however that the incidence of advanced heart failure was only 0.2% per year [109]. Another small study reported the possible development of hypophosphataemia with associated changes in bone and mineral
metabolism in some patients receiving imatinib [110]. The long-term side effects are not yet known.

Resistance
Primary hematologic resistance, defined as a failure to obtain a CHR despite therapeutic doses of imatinib, occurs in approximately 5% of the patients. Secondary or "acquired" resistance refers to loss of previously established response. Although little is known about the molecular mechanisms responsible for the relatively rare cases of primary hematologic resistance, the mechanisms of secondary resistance are better understood. Secondary resistance may be multifactorial, including *BCR-ABL* mutations of the kinase domain interfering with imatinib binding, *BCR-ABL* amplification or over-expression, clonal evolution or decreased imatinib bioavailability.

The frequency of *BCR-ABL* mutations in resistant patients was reported to range from 42 [111] to 90% [112] depending on the methodology of detection, the definition of resistance, and the phase of the disease. Currently, more than 40 different mutations are associated with clinical resistance to imatinib [113-115]. The T315I mutation, and some mutations affecting the so-called "P-loop", are associated with poor prognosis [116]. Other mutations can be overcome by a dose increase, and some mutations are functionally irrelevant. Mutations are more frequent in AP/BC, and are in CP often identified in patients with more than 2-fold increase of the *BCR-ABL* transcript levels, rather than in those with stable or decreasing levels. Thus, screening for mutations in the kinase domain of bcr-abl can provide useful information for guiding therapeutic decisions [113-115], and need to be interpreted within the clinical context.

It has been postulated that cell survival mechanisms operating independently of *BCR-ABL* may be responsible for many cases of primary imatinib resistance, although the understanding of potential mechanisms of this *BCR-ABL*-independent resistance remains limited.

Dasatinib
The options besides allogeneic SCT to induce remissions are limited in patients who have failed imatinib treatment. Dasatinib (Sprycel®, Bristol-Myers Squibb) is a novel oral kinase inhibitor that binds to both the active and inactive conformations of the *ABL* kinase domain. It targets both the bcr-abl and the Src kinases. In preclinical studies, this compound is 300-fold more potent than imatinib, and harbors potent inhibitory activity against nearly all imatinib resistant mutants tested [117, 118], except for the T315I mutation. In 2003, the first cases to be treated with dasatinib were CML CP patients with hematologic resistance or intolerance to imatinib. A recently published study [119] showed CHR in 92% and MCGR in 45% of cases:
these responses were maintained in 95% of patients for a median follow-up time of more than 12 months. Myelosuppression was a common, but not dose-limiting adverse event. Non-malignant pleural effusions developed in 13% of the patients, and were usually successfully managed with diuretics, pleurodesis and glucocorticoids. The registered dosage is 140 mg/day administered orally in two divided doses [120].

New therapeutic options

Nilotinib
Nilotinib (AMN-107, Novartis) is another new oral kinase inhibitor with a 25-fold increased in vitro potency, relative to imatinib. As with imatinib, nilotinib binds to the inactive conformation of ABL. This drug harbors activity against most imatinib-resistant cell lines with mutant ABL kinases, but it has no effect on the T315I mutation [118, 121]. Unlike dasatinib, nilotinib does not inhibit Src family kinases. A recently published study [122] with nilotinib-treated imatinib-resistant CML CP patients showed CHR in 11/12 and MCgR in 6/12 patients. Common adverse events were myelosuppression, transient indirect hyperbilirubinemia and skin rashes.

Bosutinib
Bosutinib (SKI-606,Wyeth) is, like dasatinib, a dual Src-Abl inhibitor active against BCR-ABL-positive CML cell lines [123]. Resistance to imatinib is not entirely explained by bcr-abl mutations, and in vitro experiments have shown bosutinib activity in models where resistance was not caused by mutations [124]. In contrast to imatinib, no substantial inhibition of c-kit and pdgfr was noted with bosutinib, thus placing this molecule in a class of its own. The difference in selectivity could, compared with imatinib, result in fewer side effects since much toxicity associated with imatinib can be tracked to the inhibition of pdgfr and/or c-kit. Phase I-II clinical trials are ongoing.

MK-0457
MK-0457 (Merck) is a novel kinase inhibitor with preclinical antileukemia effect. The T315I bcr-abl mutation mediates clinical resistance to imatinib, nilotinib and dasatinib. MK-0457 has in vitro activity against cells expressing mutated BCR-ABL, including the T315I mutation [125]. The first patients with CML and Ph-positive ALL and T315I mutation have been treated, and they have achieved responses [126]. These cases are the first reported to respond to this kinase inhibitor.
**LBH589**

Treatment with the histone deacetylase inhibitor LBH589 (Novartis) depletes \textit{BCR-ABL} levels \textit{in vitro}. Co-treatment \textit{in vitro} with LBH589 and nilotinib is active against imatinib-resistant CML cells, including those with expression of \textit{BCR-ABL} mutation T315I [127]. A phase I study is ongoing.

**Vaccination**

Despite the fact that imatinib is the most effective debulking therapy for CP patients, the eradication of residual disease without allogeneic SCT still seems a difficult goal for the tyrosine kinase inhibitor approach alone [128]. An alternative attempt to target the \textit{BCR-ABL} gene and its derived p210 fusion protein is an active specific immunotherapy (eg, a vaccine). Sixteen CP CML patients with a stable residual disease were injected s.c 6 times with a peptide vaccine derived from the sequence p210-b3a2 (CMLVAX100). Vaccinations with CMLVAX100 were followed by an antitumor effect in most patients [129]. All patients’ cytogenetic responses improved after six vaccinations.

**Treatment of chronic phase**

Standard (non-investigational) treatment of CP includes HU, INF\textalpha ± low dose Ara-C, imatinib and allogeneic SCT. In countries where imatinib is available, and standard allogeneic SCT is feasible, we are now in the rather privileged situation of having two potent strategies that are both established. Imatinib is currently preferred as the initial treatment. In patients with Sokal high risk and low Gratwohl score, the choice between imatinib and allogeneic SCT should be discussed. The comparative 5-year survival for different treatments is presented in Table 5.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>5-year survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Busulphan-HU</td>
<td>30 %</td>
</tr>
<tr>
<td>IFN\textalpha</td>
<td>27-53%</td>
</tr>
<tr>
<td>Allogeneic SCT</td>
<td>50-60 %</td>
</tr>
<tr>
<td>Imatinib</td>
<td>90 %</td>
</tr>
</tbody>
</table>

Table 5. Approximate 5-year survival in CML patients treated with busulphan, HU, IFN\textalpha, allogeneic SCT and imatinib. The figures are based on references cited above.
The motives for treatments other than imatinib are intolerance or toxicity, failure, suboptimal response and “warnings” (definitions are presented in Table 6). In the case of imatinib intolerance or toxicity, the treatment choices are allogeneic SCT, dasatinib, INFα ± low dose Ara-C, or investigational trials with new agents.

In the case of failure, the first choice should be allogeneic SCT or dose-escalation of imatinib to 600 or 800 mg daily, provided that the patient tolerated 400 mg and that resistance to imatinib was not associated with the presence of a BCR-ABL mutation with a high level of insensitivity to imatinib. Dasatinib treatment is also an option, or investigational agents such as nilotinib [119, 122].

In the case of suboptimal response, the first choice could be dose-escalation of imatinib to 600 or 800 mg. In this case, allogeneic SCT could be offered to patients with a low or intermediate Gratwohl risk score.

In patients presenting “warning” features, standard treatment is still imatinib 400 mg, but any “warning” should alert the clinician that the patient may become eligible for imatinib dose escalation, for allogeneic SCT, or in selected cases for investigational agents [19].

There are of course several other possible scenarios. If the patient requiring imatinib reduction or frequent treatment discontinuations, due to myelosuppression or side effects, the recommendation is to continue the 400-mg dose insofar as possible. Appropriate supportive care should be provided, including G-CSF and erythropoietin.

Monitoring of blood imatinib concentration is not required, but is desirable in case of failure, especially in patients who need to take drugs interfering with cytochrome P 450, and in those experiencing a severe drug-related adverse event [130].
Table 6. Definition of failure, suboptimal response and warnings for upfront CML patients who are treated with imatinib 400 mg daily [19].

<table>
<thead>
<tr>
<th>Time</th>
<th>Failure</th>
<th>Suboptimal response</th>
<th>Warnings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diagnosis</td>
<td>NA</td>
<td>NA</td>
<td>Sokal high risk Del(9q) ACA in Ph+ cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 months</td>
<td>No HR (stable disease or disease progression)</td>
<td>Less than CHR</td>
<td></td>
</tr>
<tr>
<td>6 months</td>
<td>Less than CHR</td>
<td>Less than PCgR</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No CgR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 months</td>
<td>Less than PCgR</td>
<td>Less than CCgR</td>
<td>Less than MMolR</td>
</tr>
<tr>
<td>18 months</td>
<td>Less than CCgR</td>
<td>Less than MMolR</td>
<td></td>
</tr>
<tr>
<td>Any time</td>
<td>Loss of CHR</td>
<td>ACA in Ph+ cells</td>
<td>Any rise in transcript level</td>
</tr>
<tr>
<td></td>
<td>Loss of CCgR</td>
<td>Loss of MMolR</td>
<td>Other chromosome abnormalities in Ph-negative cells</td>
</tr>
<tr>
<td></td>
<td>Mutation</td>
<td>Mutation</td>
<td></td>
</tr>
</tbody>
</table>

Treatment of accelerated phase and blast crisis

As described before, the natural course on palliative treatment goes from CP followed by transformation into AP, and finally BC. BC may be myeloid or lymphoid, the former being twice as common. The pathophysiology of CML in transformation is incompletely understood. Many patients show additional cytogenetic abnormalities (ACA) and it is reasonable to hypothesize that clonal evolution plays a role. The most common ACA includes duplication of the Ph chromosome, trisomy 8 and iso-chromosome 17; however, these abnormalities are also observed in patients with earlier stages of CML [131, 132]. Since many patients now are treated with imatinib or other tyrosine...
kinase inhibitors, this change in therapeutic approach almost certainly affects the kinetics and molecular phenotype of CML in transformation.

Historically, therapy for patients with BC has been disappointing, with response rates far lower than achieved with standard induction regimens for de novo acute myeloid leukemia (AML) or acute lymphoblastic leukemia (ALL). The recently available bcr-abl tyrosine kinase inhibitors nilotinib and dasatinib, broaden the options for patients developing BC on imatinib. Ideally, the choice of therapy should be based on screening for imatinib-resistant BCR-ABL mutations, as both dasatinib and nilotinib are active against most of these, except for T315I [119, 122]. With dasatinib, HR is seen in 31 of 44 patients with AP, BC or Ph-positive ALL (imatinib resistant) [119], the rate of MCgR is 25%. At six months responses were 82% for AP patients, but nearly all patients with lymphoid BC and Ph-positive ALL have a relapse. Responses occurred among all BCR-ABL genotypes, with the exception of the T315I mutation. The major adverse effects of dasatinib are myelosuppression and non-malignant pleural effusions [119].

In the nilotinib study [122], 39% achieved HR, and 27% CgR among the BC patients. In AP, the corresponding figures were 71% and 47%, respectively. Common adverse events included myelosuppression and transient indirect hyperbilirubinemia [122]. It is anticipated that these drugs will be useful in patients whose BC is driven by imatinib-resistant BCR-ABL mutants or increased levels of BCR-ABL, which may comprise 30-40% of cases [133].

For more traditional chemotherapeutic agents, high-dose Ara-C-based regimens are used for the treatment of myeloid BC, which in the pre-imatinib era had a worse prognosis than the lymphoid form [131]. CHR rates have ranged from 20% to 30%. Thus far, no high-intensity chemotherapy regimen has demonstrated clear superiority, and durability has been poor, with median survivals of only a few months. For CML in lymphoid BC, ALL-like induction regimens have been most commonly used, including the hyper-CVAD regimen [134]. As the response rates and durability have been disappointing, various new agents are being investigated for their capacity to induce remissions in CML BC. They include inhibitors of the mammalian target of rapamycin (MTOR) pathway; anti-vascular endothelial growth factor (VEGF)-based agents; histone deacetylase inhibitors; and other novel small molecule inhibitors [135].

As it is the only established curative modality, allogeneic SCT remains the ultimate salvage therapy for patients with imatinib-resistant CML. Patients in the appropriate age range should be HLA-typed at diagnosis so that the options for related donor (RD) or unrelated donor (URD) allogeneic SCT are known early in the disease course. Ideally, CML BC can be prevented with BCR-ABL inhibition. For those patients who progress despite this, the establishment of an early warning system for CML progression could allow initiation of more aggressive or alternative therapies at a stage where inter-
ventions have the optimal chance of being clinically meaningful. The fear is that patients will progress to BC too rapidly to allow sufficient time for allogeneic SCT or other investigative strategies to be implemented. Long-term disease-free survival /cure rate is about 10% for patients transplanted while still in BC [136]. Outcome is somewhat better for patients transplanted after achieving a second CP with salvage.

*In vitro* cytotoxicity assays

Cell culture assays for measurement of cytotoxic drug resistance in leukemia were used as early as in the beginning of the last century. In 1917 Pappenheimer added trypan blue to fresh thymic lymphocytes that had been exposed to toxic agents [137]. Trypan blue stained the dead cells blue, while the living cells remained clear. In 1954, Black and Spear compared the clinical outcome with the *in vitro* response of patient tumor cells [138].

Today, *in vitro* cytotoxicity assays are important tools for pre-clinical development of new anti-cancer agents. Basically, the leukemia cells are exposed *in vitro* to different cytotoxic drugs and the effect of the drugs on survival or proliferation of the cells is investigated.

Methodological aspects

There are two main groups of *in vitro* assays, total cell kill assays (TCK) and cell proliferation assays (clonogenic assays and short-term assays). The clonogenic assays are based on single cell culture in soft agar and subsequent counting of formed colonies, 2-3 weeks after exposure to cytotoxic drugs. The assay relies on the proliferative capacity of a small fraction of cells, presumed to be stem cells. The test is laborious and time-consuming.

Total cell kill assays are based on the concept of total tumor cell kill in the whole, mainly non-dividing, tumor cell population. These assays are short-term assays (2-4 days). The different TCK assays are in many aspects comparable, but the main differences are the various techniques used to estimate the proportion of viable cells after incubation with cytotoxic drugs, and the different drug exposures used. Examples of these forms of assay are methyl-thiazol-tetrazolium (MTT) assay, differential staining cytotoxicity (DiSC) assay and Fluorometric Microculture Cytotoxic Assay (FMCA).

The MTT assay and FMCA have many similarities. The FMCA was developed in Uppsala in the late 1980s, and is based on the uptake of fluorescein diacetate (FDA) by viable cells and its conversion to fluorescent fluorescein after exposure to cytotoxic drugs in primary cultures [139]. The end point for
recognizing cell death is loss of cell membrane integrity. The generated fluorescence is linearly related to the number of living cells. It is believed to measure cell death in the total population of tumor cells.

Use of primary cells in drug development

Tumor cell lines are the most commonly used model systems in cancer drug development, but the use of primary tumor cells from patients has gained increasing focus. The primary tumor cells from patients might predict drug activity patterns better than tumor cell lines, whereas the latter provide a practically unlimited source of samples facilitating multiple tests [140].

*In vitro* cytotoxicity assays can be used in several different ways. In preclinical development of new cytotoxic agents the assay can evaluate drug sensitivity patterns for groups of patients with different diagnoses [141-143] or subgroups, for example regarding genetic aberrations [144-146]. Combinations of cytotoxic agents can be evaluated regarding antagonistic or synergistic effects and can be the basis of future phase I and phase II trials. On primary tumor cells from patients, FMCA has been used to differentiate the *in vitro* drug effects between different diagnostic subgroups in chronic lymphocytic leukemia (CLL), AML and ALL [142, 144, 147].

Prediction of individual patient response

Human cancers are heterogeneous diseases with a diversity of pathological processes, cytotoxic drug sensitivity and resistance mechanisms, and there is a considerable variation in tumor cell sensitivity to cytotoxic drugs between patients with similar histological tumor types. Many groups have investigated the TCK assay as a method for improving disease treatment in individual patients, and the results have correlated well with the subsequent patient response and survival for a variety of diseases and drug regimes [148-152]. A meta-analysis, based on published papers from 1983-1997 and reporting data on 1596 correlations between drug sensitivity *in vitro* and clinical response, concluded that TCK performed well, with an overall sensitivity and specificity of about 0.9 and 0.7, respectively (www.weisenthal.org). For the individual patient, the assay can also be useful in giving prognostic information [153].

The American Society of Clinical Oncology (ASCO) has established a Working Group to evaluate the potential role of chemosensitivity assays in clinical practice. This evaluation suggests that no single assay is ready for routine integration into the clinical setting [151], which reflects problems in technical success of the assays, and a lack of adequate prospective evaluation in clinical trials. This conclusion has however gained criticism.
Assays in chronic myeloid leukemia

In most in vitro studies on primary tumor cells from CML patients, clonogenic assays are used, but this method is time-consuming [154, 155]. The drug effects are measured only in a small fraction of proliferating cells. The effects on resting cells, which might have proliferative potential in vivo, are not measured. Patients treated with imatinib to CCgR have detectable malignant primitive progenitors [128]. The primitive quiescent stem CML cells (CD34+, CD38-) have previously been shown to be insensitive to imatinib in vitro, and represent less than one percent of total CD34+ cells present at diagnosis, and may explain MRD in patients. Therefore, many laboratories use methods for enriching primitive CML stem cells (CD34+, CD38-) [156]. A critical issue when dealing with primitive CML populations is the coexistence of Ph-negative stem cells.

The FMCA, used in this paper, is a TCK assay and is believed to measure cell death in the whole tumor cell population. This method is easier to perform and less time-consuming than the clonogenic assay, but requires a fairly pure tumor cell population, since it cannot distinguish between normal and malignant cells. A tumor cell fraction exceeding 70% is a quality criterion normally used for the FMCA, and this is possible in newly diagnosed CML patients. The need for a high proportion of Ph-positive cells in the cell preparation currently makes testing of samples from drug-treated patients difficult, as the separation of malignant cells from normal granulocytes is complex.

This far most in vitro studies are performed on CML cell lines, which originate from patients in BC [157]. However, established cell lines may have lost some of their original tumor characteristics. Imatinib resistance is a clinical problem in the treatment of the patient, and mutations within the ABL kinase domain are a major cause. Therefore cell lines transfected with different mutations are used, for example Ba/F3, to develop drugs effective against specific mutations [117].

The use of in vitro assays based on primary tumor cells from patients might be an attractive alternative. Some studies also use a small number of patient samples [154, 155]. An important difference in using cell lines versus primary cells is that tumor cell lines are generally highly prolific, which is not the case for primary tumor cells. The primary patient cells generally share more properties with the tumor cells to be treated in the clinical situation. Primary tumor cells from patients more adequately reflect the disease-specific activity of new compounds, and may therefore be more relevant than cell lines when the effect of new drugs on different tumor types is studied [141].
Microarray expression analysis

The past decade has witnessed a revolution in the field of expression profiling in cancer. Microarray technology has changed both the view of cancer and how research in molecular oncology can be conducted [158]. The sequencing of the human genome [159, 160] and technological refinements allow for interrogation of the whole transcriptome (all expressed genes) in a single experiment. The challenge is to use the generated information for developing new diagnostic and prognostic indicators, and to identify new targets for therapeutic intervention.

Background

Microarray technology allows parallel analysis of the expression of thousands of genes at a fixed point [161], and relies on the inherent ability of single-stranded DNA to base pair with a complementary sequence [162]. Microarrays can be fabricated either by mechanical deposition of DNA or by de novo synthesis of oligonucleotides on a solid support [163, 164]. In spotted arrays, the probes are either pre-synthesized oligonucleotides or PCR products generated from cDNA clones. Spotted arrays are typically hybridized with cDNA from a sample and a reference (e.g. from patient and healthy control) labelled with two different fluorophores.

Briefly, two different fluorescent dyes are used to label the test and reference sample, which are then allowed to hybridize to known single stranded cDNA or oligonucleotide probes immobilized on a glass slide (Figure 4). As hybridization occurs competitively, the amount of bound target is proportional to the level of expression [161, 162]. Following hybridization and washing, the slides are scanned to measure the emission of the two dyes and the outcome is presented as a colour image of the spots, with red and green representing up- and down-regulation of the corresponding gene in the interrogated sample relative to the reference sample. The expression of each gene is then quantified as the ratio between the intensity values of the test and reference sample. Oligonucleotide expression arrays from commercial sources (e.g. Affymetrix, Agilent and GE Healthcare), are typically one-color-systems. In these systems, only one sample is interrogated at a time (i.e. without reference). Thus, these microarrays provide an estimate of the absolute values of gene expression, rather than ratios, as is the case with spotted arrays.
Image processing and subsequent data analysis are crucial for the extraction of useful information from microarray experiments. The raw data generated from scanning have to be filtered and normalized to remove noise and systematic variation. The goal of pre-processing is to exclude unreliable data and enable comparisons between individual experiments (recently reviewed in [165]).

![Figure 4. Brief overview of microarray technology. In this two-color-analysis, RNA samples obtained from patients and reference subjects are individually labelled and hybridized to a single DNA microarray consisting of individual gene-specific probes. Finally providing a color image with red and green representing up- and down regulation of the corresponding gene in the patient relative to the reference sample. Yellow spots designate genes with an equal expression level in the test and reference sample.](image)

Data analysis and applications

Supervised and unsupervised analyses are two commonly used but conceptually different approaches for acquiring information from microarray experiments [166]. In supervised analyses, information about tumor class is used to identify gene combinations specific for particular conditions e.g for cancer subtype differentiation. The term class refers to a characteristic shared by a group of samples, but not the other samples, for example responders to treatment and non-responders. Initially, a training dataset is used to identify a combination of genes (a classifier) that can separate the different classes. The classifier is subsequently evaluated on samples not used in the training procedure, i.e. the test set. This approach was pioneered by Golub et al [167] when they performed supervised classification on leukemias.

Unsupervised methods such as hierarchical clustering are exploratory methods useful for identification of previously unrecognized subgroups in tumor material, based solely on the global gene expression profiles.
Analysis in blood malignancies

A number of studies have shown that gene profiling analysis can correctly classify leukemia [167, 168] as well as predict response to therapy and clinical outcome in pediatric ALL [169], in adult T-cell ALL [170] and follicular lymphoma [171]. Gene profiling studies have also identified a previously unknown subgroup of large B-cell lymphoma [172].

Gene expression studies of primary CML cells have concentrated on the comparison of normal cells with those obtained in CP [173-177], and on the identification of a gene signature associated with disease progression, by comparing cells obtained in CP with AP/BC cells [178-180]. Gene expression profiling in CML has also been used to identify genes that can be used to predict the response and sensitivity to imatinib treatment [181-184]. These studies have reported gene expression signatures that can be used to distinguish between responders and non-responders to imatinib, responders defined as those patients that achieve a CCgR. However no consensus signature could be identified throughout the studies and Crossman et al [185] failed to identify a molecular signature associated with imatinib response. One major drawback is that several of these studies used a limited number of samples and the samples were heterogeneous with respect to the source of cells.

A recent study subtracted the normal CD34+ cells expression signature from the CD34+ CML expression signature, and identified a set of 103 differentially expressed genes [179]. At least four studies have used (CD34+ or AC133+) enriched cells from CML patients, with two of them focusing on identification of genes involved in disease progression, and one on prediction of an aggressive or indolent disease outcome from diagnostic CP CML samples [177, 178, 180, 186].

Recently, Radich et al [179] reported an extensive gene expression study in which 3,500 genes were identified as being differentially expressed across the different disease phases of CML. A very similar expression profile was observed between AP and BC, suggesting that, at least at the transcriptional level, CML may be considered as a two-step, rather than a three-step process. Of the genes involved in disease progression, SOCS2 has shown increased expression, whereas members of the CEBP family that regulate myeloid differentiation show reduced expression in advanced phases relative to CP CML [179, 180].
Aims for the doctoral project

- To evaluate a randomized study comparing the effect of busulphan versus HU influence on time to BC and on survival.

- To evaluate the mobilization of Ph-negative stem cells with intensive chemotherapy + G-CSF.

- To investigate the *in vitro* cytotoxic activity of imatinib alone, or in combination with other agents that could be useful in clinical trials.

- To identify a molecular signature predictive of response to IFNα.
Material and Methods

All studies were approved by the Ethics Committee in all regions.

Paper I

Study participants and data collection
Newly diagnosed CML patients in CP at regional and university hospitals in Sweden, from 1 January 1984 to 30 July 1989, were randomized to either busulphan or HU. Although cytogenetic analysis was not a requirement for inclusion, analysis for Ph chromosome was performed for a majority of the patients and therefore the outcome was separately analyzed for Ph-positive patients. Overall survival, time to BC, treatment side effects and outcome of allogeneic SCT were analyzed. The final follow-up of the study was year 2000.

Statistical analysis
Assuming 50% of the patients in one group experienced treatment failure, approximately 100 patients per treatment group were required to establish a difference of 20%, which was equivalent to 30% of the patients experiencing treatment failure in the other group, with a power of 80% and 5% significance level (two-sided). In each treatment group, 100 patients were planned, but the introduction of IFNα therapy decreased doctors’ compliance and forced a limit to the patient number. When comparing survival, patients were censored at the time of SCT.

Actuarial curves were estimated according to the Kaplan and Meier method [187]. The risk-group classification was performed according to Sokal [26].
Paper II

Study participants and data collection

Between 1994 and 1999, 37 patients were included in the study. The patients were aged 16-55 years, had not achieving CCgR after 6 months treatment with IFNα and HU, and were not eligible for allogeneic SCT. The patients were recruited from 12 regional and university hospitals in Sweden. All patients received daunorubicin and Ara-C (DA) for mobilizing BSC. Patients mobilizing poorly could receive a four-day cycle of chemotherapy with mitoxantrone, etoposide and Ara-C (MEA) or a second DA. At day +8, G-CSF s.c (lenograstim, Granocyte®, Aventis) was started at a dosage of 526 µg once daily and continued until leukaphereses were completed. Daily WBC count, with differential and number of blood progenitor cells (CD34+cells in blood: B-CD34+) [188], was checked from the time when WBC count was <0.1x10^9/l until the leukaphereses were completed. The leukapheresis products were analyzed daily for the number of mononuclear cells (MNC), B-CD34+, colony-forming units-granulocyte macrophage (CFU-GM) and for cytogenetics. Bone marrow cytogenetic analysis was performed on one of the leukapheresis days.

Twenty-one patients later underwent autologous SCT, aiming to induce long-standing CCgR. The final follow-up of the study was performed in 2001.

Treatment evaluation

BSC harvest was considered successful when MNC ≥3.5 x 10^9/kg, CFU-GM ≥1.0 x 10^7/kg, CD34+ cells ≥2.0 x 10^6/kg and the share of Ph-positive cells was 0% (complete success) or ≤1-34% (partial success). Malignant cell contamination of the BSC harvests was assessed by standard cytogenetic analysis, or when necessary, by FISH [189] or PCR [37]. The duration of hematopoietic recovery was calculated from the first to the last day of polymorphonuclear (PMN) count <0.5 x 10^9/l and of platelet count <20 x 10^9/l (without platelet transfusions).

Statistical analysis

The probability of OS was estimated according to the Kaplan and Meier method [187]. Surviving patients were censored on the last day of follow-up.
Paper III

Cell samples and cell preparation
This study contained three different parts:
For the methodological study, nine (6 CP, 3 BC) samples were used and the drug sensitivity in fresh tumor cells and cryopreserved cells from blood and bone marrow were compared.
In the imatinib single drug and imatinib combination study, the K562 cell line and cryopreserved tumor cells from newly diagnosed, untreated CML CP (n=37), CML BC (n=8), CLL (n=3) and AML (n=3) patients were used. None of the patients had received imatinib. Tumor cells from bone marrow or blood were isolated by 1.077 g/ml Ficoll-Isopaque density gradient centrifugation [148] within 24 h after collection. Viability was determined by the trypan blue exclusion test and the proportion of tumor cells calculated by inspection of May-Grunwall-Giemsa stained cytocentrifugate preparations on days 0 and 3. Culture medium RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin was used. The test was approved if viability was >70%. All CML samples were 100% Ph-positive.

Reagents and drugs
Cytotoxic agents were selected based on clinical usefulness in CML, diversity in mechanism of action and from literature data [94, 101, 155, 157, 190, 191]. The concentrations of the cytotoxic agents were chosen based on our own data from previous studies on CML cells (cytarabine 10 μM, daunorubicin 0.2 μM, arsenic trioxide 1 μM, IFNα 50000 IE/ml and homoharringtonine 0.1 μM) (unpublished data). Imatinib was supplied from Novartis and dissolved in dimethyl sulphoxide (DMSO) to a concentration of 10 mM. To examine the concentration-response relation, the drug was tested at concentrations ranging from 100 μM to 0.01 μM. For the CML CP samples, the concentration 1.0 μM of imatinib resulted in approximately 50% cell survival. This concentration was therefore chosen for the combination study. Experimental microtiter plates were prepared with 20μl/well of solution with the cytotoxic agents at 10 x the desired final concentration. All agents and concentrations were tested in triplicate.

Fluorometric Microculture Cytotoxic Assay
FMCA is a TCK assay based on the measurement of fluorescence generated from hydrolysis of FDA to fluorescent fluorescein in cells with intact cell
membranes, as previously described [148, 192]. After continuous 72-hour exposure to the cytotoxic agents in the 96-well microtitre plates at 37°C, the plates were centrifuged, and washed once with phosphate buffered saline (PBS). Hepes buffered saline containing FDA was added column-wise. The plates were incubated for 40 minutes before reading the fluorescence in a scanning fluorometer. The fluorometer was blanked against wells containing PBS including the dye, but without cells. The cell survival was expressed as a percentage and calculated as the ratio between the fluorescence in drug-treated wells and the fluorescence in control wells with blank values subtracted. Low numerical values thereby indicated a high cytotoxic effect.

Statistical analysis
Mean values for different groups were compared using the Mann-Whitney test for unpaired comparisons. Wilcoxon test was used to compare blood versus bone marrow and cryopreserved versus fresh samples. Two sided p-values with a significance limit of 0.05 were used throughout.

When the effects of drug combinations were analyzed, the cytotoxic effect of the combination was compared to the effect of the most active constituent alone (“best drug”). If the ratio between the survival index after combination treatment and the survival index after treatment with the most effective single agent was 0.8-1.2, the effect of the combination was considered as caused by the “best drug” only. Ratios below 0.8 were defined as a positive interaction and as antagonism when above 1.2. The “best drug model” for interaction between two drugs was compared with the median-effect method proposed by Chou and Talalay [193]. Drug interaction was evaluated using the combination index (CI) equation by the software CalcuSyn (Biosoft, Ferguson, MO) [194] and it was assumed that the substances acted mutually non-exclusively (conservative isobologram) [193].

Paper IV

Patient samples
Blood samples, or alternatively leukapheresis cells, were collected at diagnosis from 22 patients with CML CP. The majority of the patients received IFNα in combination with HU or Ara-C, while one patient received IFNα alone. Responders were defined as patients achieving a CCgR or MCgR after 12 months or earlier, after initiation of IFNα. Patients that had no cytogenetic response at six months were considered as non-responders. Mononuclear cells from two healthy blood donors were used as reference samples in all experiments.
Total RNA preparation

Total RNA was extracted by TRIzol® preparation (Gibco-BRL) for all 22 patients and reference samples. The RNA quality of all samples was analyzed with the RNA 6000 Nano Lab Chip kit in the bioanalyzer 400 (Agilent Technologies, Palo Alto, CA, USA).

Array fabrication and analysis

The cDNA microarray used was manufactured at the Microarray Core Facility at Rudbeck Laboratory, Uppsala University, Sweden, and contained 15,552 spots representing 7,458 CDNA clones and controls [195]. Three micrograms of patient RNA were labelled using the TSA Labelling and Hybridization Kit, mixed with the reference sample and hybridized to the array. Each patient sample was analyzed twice. The microarrays were scanned in a Gene Pix 4000B scanner. Raw data was normalized using the SMA package (Statistics for Microarray Analysis, http://www.stat.berkeley.edu/users/terry/zarray/Software/smacode.html). The gene lists for both groups were matched resulting in 2,916 genes, present on both lists, for further analysis. Hence, 2,916 genes, out of 7,458 genes, were used for supervised learning and hierarchical clustering analysis.

Supervised Learning

The discriminative power of selected groups of genes was evaluated through their performance in supervised classification. In a leave-one-out cross validation procedure (LOOCV), one sample at a time was withheld and not used for either gene selection, or training of the classifier [196]. In this series, the best discrimination between responders and non-responders was achieved by the top 20 genes. These genes (with the largest median differences between the groups) where then used in a selection procedure.

In order to verify that the error rates obtained with the data set were unlikely to have occurred by chance, a permutation test was performed.

RT-PCR validation

The RT-PCR reactions were performed in duplicate. “Assay on demand” mixes for RNASE2, PRG2, NRGN, LTF, JARID1A and DEFA4 were purchased from (Applied Biosystem, Foster city, CA, USA). The expression in each gene was normalized to GAPDH expression in each sample.
Results and Discussion

Comparison of busulphan, hydroxyurea and allogeneic stem cell transplantation (Paper I)

The 179 patients registered in the study represented 35% of the estimated CML patients diagnosed over this five and a half year period. Ninety patients were randomized to HU, and 89 to busulphan treatment. Twenty-six patients underwent allogeneic SCT, 21 with RD, and 5 with URD. Analysis for Ph chromosome by conventional cytogenetics was obtained for 164 patients. The median age was 56 for the busulphan and 61 years for the HU group.

There was no significant difference in survival between busulphan and HU treated patients (Figure 5). Median survival was 3.2 years and 3.5 years, respectively. The treatment intensity did not differ between the two groups, nor did the time from diagnosis to therapy start (mean 24 for busulphan and 42 days for HU). One hundred and sixty-seven patients died, 85 due to BC (41 busulphan and 44 HU), and 81 had other complications (infections, bleedings, heart diseases and other malignant diseases). In one patient, the cause of death was unknown. Some of the latter 81 probably also had a BC, but no post-mortem bone marrow samples were taken to prove this. Two transplanted patients died a long time after diagnosis (10 and 14 years), one in BC and one in transplantation-related complications.

There was a significant survival benefit for patients who underwent allogeneic SCT (n=26) (Figure 6). In addition, when compared with non-transplanted patients in the same age group (up to 46 years) the outcome for the transplanted patients was significantly better. Thus, the age factor did not explain why allogeneic SCT was superior to conventional therapy. The median survival was 4.7 and 3.3 years, respectively. In all, 10 transplanted patients (38%) and 2 non-transplanted patients (1.3%) were still alive (both in the busulphan group). At the latest follow up, in year 2000, all the 10 surviving transplant patients were RT-PCR-negative for \textit{BCR-ABL} in blood, whereas, both non-transplanted survivors were 100% Ph-positive in bone marrow.

When this study began, it was not known whether HU prolonged survival compared with busulphan, although other groups were studying these two
drugs. The MCR CML III b [48] study did not find any difference in treatment outcome; however, the German CML I study [197] reported an advantage for HU treatment on OS. The MRC statistical group [39] published a meta-analysis of three randomized trials comparing HU and busulphan (ours, German CML I and MRC IIIb), showing that HU treatment is beneficial to survival. It might be speculated whether our study could not identify any differences. The reasons could be either that treatment intensity was the same for the two groups, or that the patient number was too small. In the German study, the proportions of patients with normal or subnormal WBC count were considerably higher in the HU group, which could explain the positive impact on survival.

An important finding in our study was the positive outcome of allogeneic SCT, the 10-year survival being 46% compared to 2% for the non-transplanted group. This study demonstrated the importance of a long follow-up time for revealing the positive effects of allogeneic SCT. It took between four and five years for the different regimen survival curves to separate, and it should also be noted that these patients were transplanted during the mid-1980s, when TRM was relatively high. Both tissue typing and supportive care have improved considerably since then. Thus in the Nordic countries, allogeneic SCT results improved during the 1990s reaching 65% OS [55].

OVERALL SURVIVAL FROM START OF THERAPY

![Graph showing Kaplan-Meier survival curves for CML patients treated with busulphan (n=88) versus HU (n=89).](image)

**Figure 5.** Overall survival from start of therapy. Kaplan-Meier survival curves for CML patients treated with busulphan (n=88) versus HU (n=89).
Successful mobilization of Ph-negative blood stem cells with intensive chemotherapy + G-CSF (Paper II)

In a national studying the period 1994-1999, 37 patients were reported: 27 were mobilized with one, and 10 patients received two chemotherapy regimens. Thus, there were 47 mobilization procedures. DA and MEA were used 41 and 6 times, respectively. G-CSF was administrated for a median of 20 (range 5-60) days/cycle. No treatment-related deaths occurred. In total, 114 leukaphereses were performed in 34 patients: three patients never underwent this procedure due to poor B-CD34+ mobilization (n=2) or fever (n=1). Leukaphereses were performed at a median of 27 days (range 15-59) from the first day of chemotherapy, with a median of 2 (range 1-7) aphereses per patient. Data on cells collected are presented in Table 7. Target cell count (B-CD34+ cells >2.0 x 10^6/kg) was reached in 30/34 patients (88%) in one or more leukapheresis procedures: no mortality was observed during the mobilization procedure.

Ninety BSC collections underwent cytogenetic analysis. In patients with sufficient collected BSC, complete success (0% Ph-positive cells) was achieved in 16 (43%), and partial success (1-34% Ph-positive cells) in 7 (19%). Thus, 23 of 37 patients (62%) were eligible for autografting.
Table 7. Results of harvest in 34 patients.

<table>
<thead>
<tr>
<th></th>
<th>Median</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day of first leukapheresis</td>
<td>27</td>
<td>15-59</td>
</tr>
<tr>
<td>Number of leukaphereses</td>
<td>2</td>
<td>1-7</td>
</tr>
<tr>
<td>MNC x 10^8/kg</td>
<td>3.9</td>
<td>0.9-17</td>
</tr>
<tr>
<td>CFU-GM x 10^4/kg</td>
<td>17</td>
<td>0-85</td>
</tr>
<tr>
<td>B-CD34+ x 10^6/kg</td>
<td>3.7</td>
<td>0.2-52</td>
</tr>
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</table>

The patients in the failure group did not have enough cells and/or had Ph-positive cells >34% in the harvest. There was a trend towards a lower percentage of Ph-positive cells in the first apheresis harvest compared with the last one (ns). Cytogenetic analysis of bone marrow and the same day’s apheresis product showed no statistical difference in Ph-positivity.

Twenty-three patients were eligible, but only 21 underwent autologous SCT: 15 with 100% Ph-negative and 6 patients with Ph-positive (1-34%) leukapheresis products. The remaining two patients underwent an URD allogeneic SCT, despite one having a successful, and the other a partially successful, mobilization. There was a rapid hematopoietic recovery after autologous SCT. The number of days with PMN count <0.5 x 10^9/l was 10 (range 7-49) and with platelets <20 x 10^9/l also 10 (range 2-173). The estimated 5-year OS was 68%, with a median follow-up time of 5.2 years. CCgR after autologous SCT was achieved in 18 of 21 patients (85%) and lasted 3-78+ months (median 7.5 months). There was no transplant related mortality. Four patients subsequently underwent URD allogeneic SCT due to cytogenetic relapse.

Twelve out of 21 patients were still alive 23 to 95 months post transplant (median 68 months). Eleven had a cytogenetic relapse, however: seven of these were due to treatment in CCgR at the latest follow-up. Four of these patients had received imatinib (one RT-PCR negative, two RT-PCR positive and one with unknown RT-PCR status), one IFNα (RT-PCR positive) and two had undergone URD allogeneic SCT (both RT-PCR-negative). One patient did not receive any CML treatment post autografting (RT-PCR-positive) and remarkably, was still Ph-negative 10 years after transplantation with Ph-negative BSCs. The remaining four surviving patients were Ph-positive and received imatinib (n=2), HU (n=1) and unknown treatment (n=1). Nine patients died (five in blast transformation, one from breast cancer, one in CNS relapse, one in GVHD and one for unknown reason).
survival for all 37 patients (intention-to treat) compared with the outcome for the 16 patients not autotransplanted was not significantly different.

In summary, this study showed that target cell count was reached for the majority of patients (88%). Twenty-three out of 37 (62%) were completely successfully, or partially successfully mobilized, as defined by the protocol (B-CD34⁺ cells ≥ 2 x 10⁶/kg and Ph-positive cells < 34% in the apheresis products). Out of these, 16 patients (43%) were Ph-negative in the apheresis products. These results compare favorably with other studies using cytostatic regimens for mobilization. Thus with ICE (idarubicine, Ara-C, etoposide) successful mobilization was achieved in 31% and partially successful mobilization in 17% of the patients [198]. Corresponding figures were 43% with idarubicine and 16% with Ara-C [199]. Pratt et al [200] used high-dose HU for mobilization, with a successful outcome in 4/14 (28%) and partially successful outcome in 4/14 patients (28%). Although other groups have reported treatment-related deaths, mainly due to prolonged cytopenia and infections [53, 201, 202], no mortality occurred in this study. Collecting BSC very early during hematopoietic recovery post chemotherapy may decrease the risk of tumor cell contamination. Previous publications have reported more Ph-negative products in the first collections than in later ones. In this study, a similar trend was revealed, but it was not statistically significant, probably due to too low a patient number.

Several groups have evaluated autologous SCT as an approach to treat patients with CML lacking a suitable donor, or too fragile for allogeneic SCT [53, 55]. In a review of the experience in eight centres, McGlave et al [203, 204] showed the 3-year survival after autologous SCT for selected CP patients to be approximately 60%. Bathia et al [205] reported cytogenetic responses after autologous transplantation in a range of 10-57%.

Since imatinib was introduced for CML treatment, the procedure of autologous SCT is rarely performed. However, given that imatinib often induces CCgR, it may in the future prove valuable to harvest BSC at the time of CCgR or MMolR with potential use at the time of progression in patients not eligible for allogeneic SCT. Many study groups have demonstrated the possibility of successfully collecting BSC in 40-80% of the patients achieving CCgR with imatinib [57-60].
In vitro activity of imatinib (Paper III)

In an initial investigation, the feasibility of using the FMCA method in drug sensitivity testing on tumor cells from CML patients was evaluated (Methodological study). Many other studies have been performed using the FMCA in other hematological malignancies as well as in solid tumors [148-150, 153, 157, 192], but not in primary CML tumor cells. No difference in drug sensitivity between tumor cells obtained from blood and bone marrow was found, indicating that blood can be used to make sampling easier for the patient. On the other hand, cryopreserved cells tended to be more drug sensitive than fresh cells, suggesting that it may not be optimal to use fresh and cryopreserved together in the same study. Considering this, only cryopreserved cells were used in the rest of this study.

Drug sensitivity analysis was performed with the FMCA method in samples from untreated CML CP (n=26), with CML BC (n=6), and the K 562 cell line (Imatinib single drug study). The CML samples from BC were significantly (p< 0.05, Mann-Whitney test) more sensitive to imatinib than the CP cells at the concentrations 1, 10 and 100 μM. The K 562 cell line (CML BC) had a similar pattern as the CML BC patient samples (Figure 7). Most published in vitro studies of imatinib use CML cell lines that originate from patients in BC [117, 157]. The use of such cell lines to draw conclusions concerning CML CP may be sub-optimal, risking an overestimation of drug efficacy and of drug combination effects. Imatinib is very efficient in patients with CML CP, [206, 207] whereas in AP and BC, the clinical effects are moderate and transient [103, 208]. In the present study, the BC samples were significantly more sensitive to imatinib than the samples taken in CP at diagnosis. The reason for this is unclear, although a possible explanation could be that BC cells are immature cells that generally have fewer mechanisms of protection against foreign stimuli. In the clinical settings, factors in addition to cellular drug sensitivity also come into play. The K 562 cell line had drug sensitivity comparable to the BC cells.

Then, the combination of imatinib with conventional (IFNα, daunorubicin, cytarabine, vincristine) and experimental (arsenic trioxide, homoharringtonine) anti-CML agents was analyzed to determine if the activity of the single agent in vitro could be enhanced or reduced (Imatinib combination study). The highest mean difference in cell survival (%) of the combination imatinib and other cytotoxic agents to the most active single agent was compared. IFNα, daunorubicin and arsenic trioxide were the drugs with the highest frequency of positive interactions with imatinib in CML CP samples (Figure 8 a). Unfortunately, the high single agent activity of homoharringtonine, as well as of cytarabine, made any potential benefit of combinations with imatinib difficult to evaluate. Imatinib in combination with cytarabine, vincristine, arsenic trioxide and homoharringtonine showed a positive interaction in all BC samples (Figure 8 b). Remarkably, IFNα in combination
with imatinib had an antagonistic effect in two of the BC samples and this may explain the clinical situation, where IFNα is used only in patients in CP, and not in BC. Tumor cells from patients obtained in BC were more sensitive to combinations than samples from CP (<0.05, Mann–Whitney test: Figure 8 b).

![Graph of concentration-effect curve for imatinib in samples from CML patients in CP (n=26), BC (n=6) and from the K562 cell line. The error bars show ± 1 SEM.](image)

**Figure 7.** Mean concentration-effect curve for imatinib in samples from CML patients in CP (n=26), BC (n=6) and from the K562 cell line. The error bars show ± 1 SEM.

As primary and acquired resistance to imatinib therapy is problematic in CML therapy, trials combining imatinib with other anti-CML agents are of major interest. New oral tyrosine kinase inhibitors, such as nilotinib and dasatinib are promising drugs in case of imatinib intolerance/resistance [119, 122]. Previous combination studies performed in *BCR-ABL* positive cell lines, indicate synergistic effects of imatinib with cytarabine, vincristine and IFNα, and both additive and synergistic effects of imatinib combined with daunorubicin and homoharringtonine [94, 154, 155, 157, 190, 191]. Also cell lines transfected with different mutations are used, for example Ba/F3, to develop drugs effective against specific mutations [117]. There is evidence that CML stem cells are not eliminated by imatinib *in vivo*; therefore many laboratories investigate *in vitro* effects on enriched CML stem cells (CD34+, CD38) [156].

Our BC results were thus similar to previous results published on CML cell lines, as well as results on Ph-positive ALL cells [147]. In the current study, there were generally smaller combination effects in cells from CP,
indicating that cell line studies may overestimate the effects in CML CP. The chosen concentration of 1.0 μM of imatinib in the combination studies is relevant in treatment, as administration of 350 mg imatinib daily in the study of Peng et al [209] resulted in mean plasma concentration of about 1 μM.

Figure 8. The mean survival index (%) in samples from CML patients in CP (n=26) for imatinib and six drugs as single drugs, and in combination (a), and in samples from CML patients in BC (n=6) for imatinib and six drugs as single drugs, and in combination (b). The error bars show ± 1 SEM.
Gene expression analysis in interferon-α responders (Paper IV)

Twenty-two patient samples were included in the microarray-based gene expression study; however, seven samples not passing the quality requirements were excluded. Of the remaining samples, six were from patients responding, and nine from patients not responding, to IFNα.

In a first step unsupervised hierarchical clustering analysis was performed. The samples clustered into two distinct categories according to the sample processing procedure used before RNA extraction: ficoll density sedimentation or leukapheresis separation (Figure 9). This result indicates that the largest differences in gene expression between our samples arise from the different types of cells isolated by the two different methods. Unsupervised clustering was unable to separate responders from non-responders to IFNα. In a subsequent analysis we ranked the genes according to the differences in gene expression between the responders and non-responders. The top genes that showed the largest differential expression were used in a new hierarchical clustering. Again the analysis was unable to separate the two groups. Similarly, Crossman et al [185] could not identify a molecular signature associated with response to imatinib by hierarchical clustering when analyzing a mixed sample population consisting of both blood and bone marrow samples. These problems illustrate the importance of processing samples uniformly.

In order to identify genes that could potentially predict response to IFN, the LOOV procedure was performed, and the genes most frequently utilized in this selection procedure were RNASE2 (ribonuclease family 2), PRG2 (proteoglycan 2), and NRGN (neurogranin), LTF (lactotransferrin), JARID1A and DEFA4 (defensin alpha 4) (Figure 10). Classification of the withheld samples based on these genes resulted in an error rate of 13%. A permutation test showed that it was unlikely to obtain equal or more successful classification by chance (p=0.004).

Figure 9. Outcome of sample classification by hierarchical clustering of diagnostic CML samples. L=samples collected by leukapheresis. F=samples processed by ficoll density centrifugation.
The genes selected in the LOOV seem to be biologically relevant. Twelve out of 20 genes selected for the LOOCV procedure either had a cis element for the C/EBP transcription factor family in the promoter region (RNASE2, PRG2, LTF, MEFV, BPI, MT1F and CST), or was coupled indirectly to the transcription factors, e.g. MYC that antagonizes the transcription factor C/EBP. Previous studies have shown that C/EBPα is expressed early in the myeloid differentiation [210] and C/EBPc is essential for terminal differentiation of committed granulocyte progenitor cells. This finding suggests that the ability to respond to IFN might be associated with the capacity to differentiate in CML cells. C/EBP is negatively regulated by bcr-abl [211] and the introduction of C/EBPβ inhibits proliferation and promotes differentiation of BCR-ABL expressing cells [212].

RNASE2 and PRG2 are primarily expressed in eosinophils and have a higher expression in the non-responder group. These two genes have been identified to distinguish CD34+ cells derived from CP and BC CML samples [180]. The PRG2 is over-expressed in CD34+ cells from CML patients with a more indolent disease [186].

DEFA4 is highly expressed in neutrophils. Several of the genes reported in this study as differentially expressed between the two categories of patients, have been reported in other gene expression studies on CML cells. DEFA4, NRGN, CAMP, BPI, LTF, MYC and CD44 have been found to be deregulated in CML cells compared to normal controls [176]. Thus, a number of the genes identified in the present study appeared to be associated with CP or indolent CML, which suggests that they might have prognostic value not only with regard to IFN response but also perhaps in a general context for CML patients, irrespective of treatment.

The RT-PCR data confirmed that RNASE2, PRG2, JARID1A and DEFA4 had higher expression in non-responders, whereas, NRGN and LTF had a lower expression in this group. Analyzed individually by RT-PCR RNASE2, PRG2 and NRGN showed statistical difference among responders and non-responders (p<0.05). The results from the RT-PCR were used to perform a principal component analysis, which correctly clustered the samples into two distinct categories, with one exception. One responder patient clustered among the non-responders, and interestingly, this patient rapidly progressed to BC, despite the good initial response to IFNα.

We conclude that it might be possible to use gene expression analysis to predict future response to IFNα in CML diagnostic samples. Larger studies with uniformly processed samples are warranted in order to confirm our findings.
Figure 10. Expression level of RNASE2, PRG2, NRGN, JARID1A, DEFA4 and LTF transcripts relative to GAPDH expression determined by RT-PCR in CML samples (n=13). Filled circles represent non-responders and open circles represent responder patients. * Statistically significant.
Conclusions

Paper I
No difference between busulphan and HU therapy on OS was determined. Reports that allogeneic SCT improves survival in CML were confirmed.

Paper II
In the majority of patients with CML CP, intensive chemotherapy and G-CSF mobilize Ph-negative BSC sufficient for autologous SCT.

Paper III
FMCA may be a useful method for drug sensitivity testing in samples from CML patients, both in CP and BC. Patient tumor cells may have advantages over cell line for sensitivity testing. Combinations between imatinib and IFNα, daunorubicin and arsenic trioxide may be interesting for clinical trials in patients with CML CP.

Paper IV
With microarray-based gene expression analysis on diagnostic samples derived from CP CML patients, a set of genes that may possibly predict response to IFNα treatment were identified. In patients not responding to IFNα treatment RNASE2, PRG2, JARID1A and DEFA4 showed a higher expression, whereas NRGN and LTF showed a lower expression.
Future directions

Much effort has been invested in developing valuable prognostic scores to predict response and survival in patients with CML [26, 27]. Common for both scores are the possibility of distinguishing patients with a more indolent from patients having a more aggressive disease, and that the patients are subgrouped into low-, intermediate-, and high-risk patients. In the imatinib era, factors related to therapy response seem to be more important. Early cytogenetic response to therapy being the most important.

We are currently performing microarray analysis to explore differences in gene expression pattern among CP CML patients at diagnosis, in particular differences among the three different risk groups according to Sokal. The identification of genes and pathways involved in the malignant transformation could provide biomarkers that are useful for identifying “risk” patients already at diagnosis. Thirty-three patients (eleven from each Sokal risk group) were included in the study. We have started to analyze the data. Later, we would like to verify our results in an independent patient cohort.

Resistance to imatinib represents an important scientific and clinical issue in CML. Mutations in the ABL domain is a major cause, but we do not yet know all the mechanisms of resistance. To overcome resistance to imatinib, different strategies were developed, including dose escalation of imatinib or combination with conventional cytotoxic drugs, or new drugs such as dasatinib, nilotinib, bosutinib and MK-0457. It may be possible to combine these drugs. The FMCA system could be useful to study this, but it may be better to use primitive CML stem cells (CD34+, CD38-) in the future, or mutated cell lines.

Despite the fact that imatinib is the most effective debulking therapy for CP patients, the eradication of residual disease without allogeneic SCT still seems a difficult goal for the tyrosine kinase inhibitor approach alone. Peptide vaccines in eliminating and/or controlling residual cells by inducing a leukemia-specific immune reaction is an attractive solution. However, the peripheral blood of cancer patients frequently contains an increased number of T-regulatory cells, which appear to inhibit immune reactivity. We would like to investigate these T-regulatory cells in CML patients both at diagnosis and after treatment with imatinib.

I also hope to continue working with academical studies concerning CML therapy, in the Swedish and Nordic CML collaboration groups.
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References

1. Bennett JH.: Case of hypertrophy of the spleen and liver, in which death took place from suppuration of the blood. Edingburgh Medical and Surgical Journal 1845. 64: p. 413-424.


83. Buchdunger, E., et al., Abl protein-tyrosine kinase inhibitor STI571 inhibits in vitro signal transduction mediated by c-kit and platelet-


97. Nimmanapalli, R., et al., Histone deacetylase inhibitor LAQ824 both lowers expression and promotes proteasomal degradation of Bcr-Abl and induces apoptosis of imatinib mesylate-sensitive or -refractory


170. Chiaretti, S., et al., Gene expression profile of adult T-cell acute lymphocytic leukemia identifies distinct subsets of patients with dif-
200. Pratt, G., et al., Autologous stem cell transplantation in chronic myeloid leukaemia using Philadelphia chromosome negative blood pro-


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