CHAPTER 1

General introduction and outline of the thesis

HISTORY OF CML

Chronic myeloid leukemia (CML) was recognized as a disease entity already more than a century ago. But it was not until 1960 that Peter Nowell and David Hungerford from the University of Philadelphia discovered that "chronic granulocytic leukemia", how CML was called these days, was consistently associated with an abnormal chromosome 22, which was later named after their city.[1] The story continues in 1973, when Janet Rowley from Chicago discovered that this Philadelphia chromosome was the result of a reciprocal translocation between chromosome 9 and 22.[2] In 1985, scientists from the Netherlands and USA demonstrated that this translocation leads to fusion of the *Abelson murine leukemia (c-ABL)* oncogene on the long arm of chromosome 9 with the truncated *breakpoint cluster region (BCR)* gene on the long arm of chromosome 22.[3] (Figure 1) This newly formed fusion gene encodes a constitutively activated tyrosine kinase, the chimeric oncoprotein BCR-ABL. BCR-ABL perturbs many molecular pathways and plays a pivotal role in the development of CML.[4]

Not long ago, prognosis of CML was poor with a median survival of 3-5 years.[5] Treatment options included allogeneic stem cell transplantation, interferon or chemotherapeutic agents like busulfan, hydroxyurea and cytarabine. The preferred first line therapy allogeneic stem cell transplantation, the only curative option, carried a high risk of morbidity and mortality and was restricted to those who had a donor available and who were fit and young of age.[6] The alternative for allo transplant, interferon, could induce durable major cytogenetic responses in around 10% of patients, however, its toxicity limited its use.[7] The addition of cytarabine to interferon increased major cytogenetic response rates, but induced even more side effects.[8] Busulfan and hydroxyurea were used for reduction of leucocyte count, but very rarely induced a reduction of Ph+ cells in the bone marrow and did not change the natural history of CML.[9,10] However, because of its low toxicity profile, hydroxyurea is still used for leukoreduction before diagnosis is established and as palliative treatment.

In the late nineties of the 20th century, Brian Druker from Oregon Health Sciences University Portland, Oregon, USA, together with Charles Sawyers at UCLA from Los Angeles and Nick Lydon from the pharmaceutical company Novartis developed a specific tyrosine kinase inhibitor (TKI), STI571 (imatinib), with *in-vitro* activity against BCR-ABL positive cells.[11] In 2001, the International Randomized Study on Interferon versus STI571 (IRIS study) showed impressive response rates and outcome and a new era of targeted therapy was born.[12] Whereas before imatinib 8-year survival was only between 5% and 53%, depending on risk score[13], in the IRIS study 85% of patients was alive at 8 years and CML related death rate was only 7%.[14] Following imatinib, other TKIs have been developed and introduced.

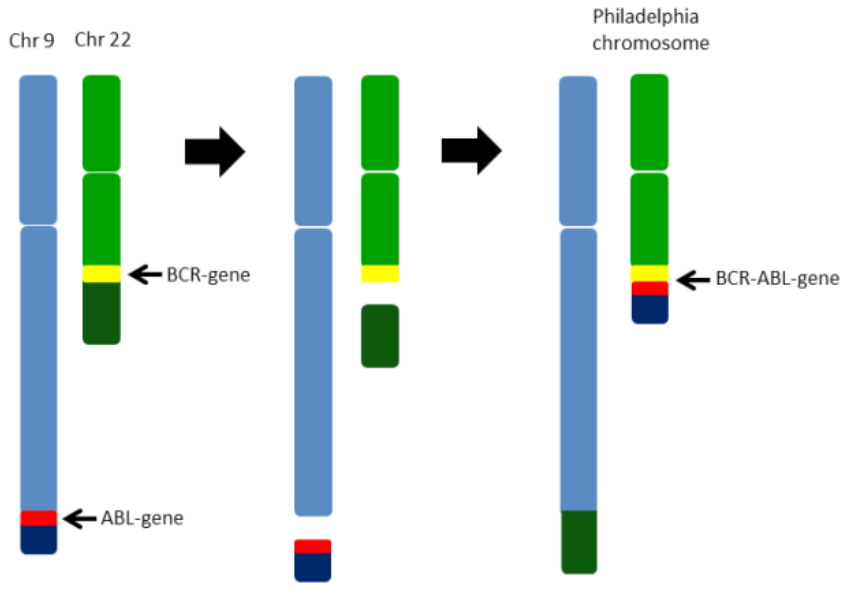

Figure 1. Forming of the Philadelphia chromosome. Part of the normal chromosomes 9 (including the ABL gene) and 22 break off and translocate: the part of chromosome 9 attaches to chromosome 22, forming an abnormally small chromosome 22, which is called the Philadelphia chromosome, carrying the BCR-ABL fusion gene.

CLINICAL AND LABORATORY FEATURES

CML is a triphasic disease presenting with a prolonged indolent chronic phase with a duration of 3-5 years before progression occurs, via acceleration phase, to an inevitably and rapidly fatal lymphoid or myeloid blast crisis.[15] Around 90% of patients present in chronic phase.[16]

An estimate of 40% of patients is asymptomatic and is diagnosed by accident when blood is drawn for other reasons. Symptomatic patients often have mild symptoms, like anorexia, fatigue, night sweats, bone pain and abdominal discomfort due to splenomegaly.[15]

This clinical picture is accompanied by massive infiltration of tumor cells in blood, spleen and bone marrow. The peripheral blood smears shows hyperleukocytosis with extreme myeloid left shift with or without a mild increase in blast count, and in most cases, basophilia, eosinophilia and thrombocytosis.

For prognostication, 3 risk scores are available, the Sokal score, Hasford score (also called Euro-score) and EUTOS score. There is no consensus on superiority of one of the 3 scores, however, in most practices Sokal score is used.[13,17-19]

MOLECULAR PATHOPHYSIOLOGY

The hallmark of CML is the Philadelphia chromosome which can be detected by chromosome banding and/or by the BCR-ABL mRNA by polymerase-chain reaction (PCR). For future monitoring it is important to identify the *BCR-ABL* fusion subtype by PCR, which is dependent on the *BCR* breakpoint. The most common subtypes are b2a2 (fusion of BCR exon 13 with ABL a2) and b3a2 (e14-a2), which both produce the p210 oncoprotein. The smaller p190 BCR-ABL subtype, resulting from fusion of BCR exon 1 with ABL exon 2 is only seen in 1% of CML cases, but is on the other hand the most common subtype in Philadelphia positive acute lymphoblastic leukemia (ALL).[20]

The normal ABL protein is a tyrosine kinase. As a result of the translocation, its autoinhibitory domain, encoded by the first exon of ABL is lost and as a consequence tyrosine kinase activity is strongly enhanced.[21] This constitutively activated BCR-ABL protein activates numerous downstream signaling pathways. (Figure 1) Deregulation of these pathways leads to deregulation of cell-cycle, reduced apoptosis and adhesion, increased self-renewal and longevity, altogether ultimately resulting in leukemogenesis.[22,23]

TREATMENT WITH TKIs

The advent of imatinib in 2001 revolutionized the treatment of CML. This compound binds to the ATP-binding pocket of the ABL kinase domain, thereby blocking the phosphate transfer onto substrate proteins, ultimately inhibiting proliferation of Ph+ cells.[24] In the vast majority of patients, imatinib treatment induces cytogenetic and even molecular responses with very low or undetectable *BCR-ABL* transcripts levels.[14] In the 5-year update of the IRIS study, 87% of patients on imatinib had achieved a complete cytogenetic response (CCyR). From 124 patients in CCyR, BCR-ABL transcripts had been measured: in 80% of these patients, BCR-ABL transcripts were reduced more than 1000-fold ("3 log"; major molecular response, MMR).[25] The best achieved MMR rate was 86% in the 8-year follow up.[26] Achievement of a CCyR at 12 months and a MMR at 18 months was associated with 97% and 100% rate of survival without progression at 5 years, respectively.[25] Therefore, achieving a CCyR within 12 months and a MMR within 18 months after starting treatment became the target response levels in the European Leukemia Net (ELN) management recommendations. Patients failing to do so were designated as suboptimal or non-responders. For those patients, treatment strategies had to be reconsidered.[27] Recently, the ELN recommendations have been updated with new critera for optimal responses, which are more strict than before, and failure. A new category, "warning" was also introduced. [19]. In 2006 and 2007 the second generation TKIs dasatinib and nilotinib were introduced and even faster and deeper responses were achieved in first line.[28,29,30,31] Also less progression to accelerated phase and blast crisis was observed in patients treated with nilotinib (0.7% and 1.1%) or dasatinib (2.3%) compared with imatinib (4.2% and

5%).[29,31] Up to now this has not resulted in improved overall survival for standard dose nilotinib or dasatinib, although CML related death rates decreased in the nilotinib arms of the ENESTnd study.[29,30] Recently, bosutinib and ponatinib were approved for second or third line therapy for patients who are resistant or intolerant for the other TKIs.[32-34] Table 1 lists the available TKIs in the Netherlands. Nowadays, imatinib, nilotinib and dasatinib are approved first line options. The choice of initial agent depends on different factors, like comorbidities of the patient and the expected drug adherence.[19,35] The majority of CML patients living today are treated with imatinib, since it was the first line option for a long time. Although nilotinib and dasatinib are more potent on different levels, most experience on efficacy, safety and side effects is obtained with imatinib. Interferon is no longer considered as suitable therapy, although it can be used during pregnancy.[19,36]

Table 1. Current TKIs approved for CML treatment in the Netherlands.

	approved	**dose**	**approved indication**	**possible side effects**	**estimated costs** *(€ per patient, yearly)*
imatinib	2001	400 mg once daily	all treatment lines	edema, muscle cramps, myalgia, rash, neutropenia	30.546
nilotinib	2006	300 mg twice daily	all treatment lines	rash, neutropenia, thrombocytopenia, PAOD, IHD, pancreatitis	40.322
dasatinib	2007	100 mg once daily	all treatment lines	edema, neutropenia, thrombocytopenia pleural effusion, pulmonary hypertension	49.539
ponatinib	2012	15-45 mg once daily	resistence or intolerance to nilotinib or dasatinib, T315I mutation	rash, neutropenia, thrombocytopenia, increased lipase, arterial occlusive disease	38.368 - 76.735
bosutinib	2012	400-500 mg once daily	resistence or intolerance to all other TKIs	diarrhea, thrombocytopenia, elevated ASAT and ALAT	41.054 - 51.318

PAOD = peripheral arterial occlusive disease, IHD = ischemic heart disease

TKI RESISTANCE

Unless the revolutionary change in response and survival for CML patients since the introduction of the TKIs, there are still patients resistant or intolerant for TKIs. In the 8-year follow up of the IRIS study, 16% of patients had discontinued imatinib because of unsatisfactory response and 6% discontinued because of intolerance.[14] Comparable results were observed at 5 years in a large single center study, in which 25% of patients discontinued imatinib after failure, loss of response or intolerance.[37] Refractoriness or acquired resistance to TKI treatment may have several causes, amongst them are altered pharmacokinetics (changes in gastrointestinal absorption, plasma-protein binding or CYP3A4/A5 activity or cytochrome polymorphisms), BCR-ABL protein overexpression, clonal evolution, alterations in drug transporter proteins (overexpression of the multidrug resistance P-glycoprotein or hampered expression of the cellular influx pump Oct-1), senescence of leukemic stem cells (LSCs), defects in repair mechanisms or apoptosis, development of alternative signaling pathways independent of BCR-ABL and mutations in the BCR-ABL kinase domain.[38-40] Mutations of the kinase domain account for 50% of resistant cases.[19] They hinder drug occupancy of the active site or may alter deformability of the phosphate-binding P-loop or the confirmation of the activation loop.[40] Mutations can already exist at baseline or can emerge during TKI therapy. It is postulated that mutations detected during therapy are pre-existing mutations that are only detected by highly sensitive methods and that the proportion of mutated cells rise to detectable levels due to clonal selection in the presence of drugs.[41,42,43,44] Although it is not recommended to perform mutation analysis at diagnosis routinely, the presence of baseline BCR-ABL mutations may be associated with imatinib resistance.[41-43] On the other hand, the detection of BCR-ABL mutations in patients with imatinib resistance is associated with higher risk of disease progression and shorter survival.[45,46] How the prognostic value of baseline mutations and mutations arising during treatment associates with second generation TKIs is not known. Moreover, patients already harboring a mutation have a higher likelihood of developing additional mutations.[43] Mutation analysis is preferably performed in resistant patients and the results will guide the selection of subsequent therapy, since the different mutations confer variable degrees of resistance to different TKIs. Most mutations are associated with imatinib resistance and may retain sensitivity to the other TKIs. Otherwise, nilotinib and dasatinib have much smaller spectra of mutations. The most troublesome mutation is the T315I mutation, conferring resistance to all TKIs except ponatinib.[47]

CML STEM CELLS

LSCs are quiescent self-renewing cells who derive from nHSCs after acquiring the chromosomal translocation (9;22), and are responsible for producing all malignant progeny. As outlined above, the bulk of CML cells are killed by TKIs with resulting deep molecular responses, however LSC are inherently unsusceptible to the different tyrosine kinase inhibitors and persist even after long term TKI treatment.[48-53] This relates to the fact that stem cells are, unlike more committed progenitor cells, not oncogene addicted. Shutting down BCR-ABL kinase activity does not result in apoptosis of CML stem cells as has repetitively been demonstrated *in-vitro*.[54-56,57]

On the other hand, and seemingly paradoxical to what is stated above, it has been demonstrated in clinical studies that approximately 40% of patients does not relapse after cessation of imatinib in case of undetectable bcr-abl transcripts.[58,59] Nonetheless, even in patiens with longstanding deep molecular responses with or without TKI treatment, BCR-ABL expressing cells are still detectable.[53,60] This implicates that although LSCs are detectable by PCR they fail to proliferate despite the absence of ongoing tyrosine kinase inhibition and do not cause molecular relapse.[61] A possible explanation for this phenomenon is suppression of the LSCs by the patient's immune system, or persistent BCR-ABL positive cells lacking stem cell capacity.

OUTLINE OF THE THESIS

As discussed above, the natural history of CML has changed a lot since the introduction of the various TKIs. The overall survival of CML patients has dramatically improved and most patients stay alive. However, a significant number of patients has to discontinue TKI therapy because of serious adverse events or TKI resistance, and are at risk for disease progression. In addition, many patient suffer from low grade adverse effects, like fatigue, which clearly reduces quality-of-life. Thus, there is still room for improvement and development of new therapeutic approaches is needed to achieve even better responses, to overcome TKI resistance and to eradicate persisting leukemic stem cells.

In the first part of this thesis we focus on CML epidemiology. Population-based studies are important as they avoid bias in incidence, treatment and survival and give more accurate real-life statistics of diseases. Furthermore, they can provide insight in cost efficacy and help healthcare planning. To assess the impact of TKI introduction on overall survival, and to assess to what extent CML patients are treated with TKIs in the Netherlands, we conducted a retrospective population-based study based on data from the National Cancer Registration (NCR) on all newly diagnosed CML patients diagnosed between 1989 and 2012. This study, one of the largest ever performed on this subject and is described in **Chapter 2**.

As the NCR only collected basic information on CML demographics and more detailed information was warranted to give better insight in CML treatment, monitoring, response and survival, the NCR database was extended. In 2008, the Population based Haematological Registry for Observational Studies (PHAROS) was founded and included amongst others all newly CML patients diagnosed from 2009 on. Part of the data on Dutch CML patients was transferred to the European Treatment and Outcome Study (EUTOS) as part of the European Leukemia Net (ELN), in order to investigate demography, treatment, response and survival of CML patients in twenty European countries. The first results on incidence and baseline characteristics of this study are outlined in **Chapter 3.**

In the second part of this thesis clinical studies are discussed. One of the new therapeutic approaches to improve molecular response rates and lower the incidence of disease progression in newly diagnosed CML patients is to combine TKI therapy with conventional chemotherapy, in order to try to achieve faster and deeper responses. In **chapter 4**, we discuss the results of a randomized phase III trial comparing high dose imatinib alone versus high dose imatinib combined with two cycles of intermediate-dose cytarabine.
Until recently, CML treatment was supposed to be continued indefinitely, since LSCs seemed not to be eradicated by TKI therapy and treatment discontinuation was expected to lead to loss of response. However, as stated above, a French study showed that some of the patients who were in long-term deep molecular remission and discontinued imatinib, did not lose their response.[58] Following the French, we conducted a randomized trial, wherein patients in deep molecular response on imatinib with or without previous cytarabine therapy, were randomized between imatinib continuation or discontinuation. The results of this study are described in **chapter 5**.

In the last two chapters, we focus on CML biology. As the BCR-ABL oncoprotein is held responsible for the transformation of normal hematopoietic cells into leukemic cells and these leukemic cells are characterized by increased cell proliferation, cell transformation, reduced apoptosis and alteration of cell-stroma adhesion, they must affect many molecular pathways in the cell. Identifying these pathways is important since they may function as potential therapeutic targets in future studies. In **chapter 6** we unravel the major pathways involved in BCR-ABL signaling.
Since we know that LSCs are responsible for disease persistence and CML relapse and that they might cause TKI resistance, we set out to investigate whether LSC burden at diagnosis affects response. We assessed the LSC burden at diagnosis by two different techniques, multiparameter flow cytometry (MPFC) and sorting plus FISH. Moreover, discriminating LSCs from normal hematopoietic stem cells by MPFC allows for developing targeted therapy. In **chapter 7** we present the results of this translational multinational study on the predictive value of LSC quantification in newly diagnosed CML patients.

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