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The Methodology and Significance of Minimal Residual Disease Detection after Allogeneic Stem Cell Transplantation

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To my parents

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LIST OF ABBREVIATIONS

ALL	Acute Lymphoblastic Leukemia
ABL	Abelson
AML	Acute Myeloid Leukemia
BCR	Breakpoint Cluster Region
BM	Bone Marrow
CD	Cluster of Differentiation
CDR	Complementarity Determining Region
CLL	Chronic Lymphoblastic Leukemia
CML	Chronic Myeloid Leukemia
CP	Chronic Phase
CR	Complete Remission
CST	Conventional Stem Cell Transplantation
DC	Donor Chimerism
DLI	Donor Lymphocyte Infusion
DNA	Deoxyribonucleic Acid
cDNA	Complementary DNA
GVHD	Graft-Versus-Host Disease
GVL	Graft-Versus-Leukemia
HLA	Human Leukocyte Antigens
IgH	Immunoglobulin Heavy chain
MC	Mixed Chimerism
MHC	Major Histocompatibility Complex
MRD	Minimal Residual Disease
MUD	Matched Unrelated Donor
NST	Nonmyeloablative Stem Cell Transplantation
PB	Peripheral Blood
PBSC	Peripheral Blood Stem Cells
PBSCT	Peripheral Blood Stem Cell Transplantation
PCR	Polymerase Chain Reaction
Ph	Philadelphia chromosome, t(9;22)
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcript - Polymerase Chain Reaction
RQ-PCR	Realtime Quantitative - Polymerase Chain Reaction
SCT	Stem Cell Transplantation
STR	Short Tandem Repeats
TcR	T-cell Receptor
TRM	Transplantation-Related Mortality
VNTR	Variable Number of Tandem Repeats
WT1	Wilms' Tumor gene 1

1 SUMMARY

Allogeneic stem cell transplantation (SCT) is the choice of therapy for leukemia patients who respond poorly to conventional chemotherapy. Despite high remission rates after SCT, relapse of the underlying disease remains one of the most frequent causes of treatment failure. Graft-versus-host disease (GVHD), a major complication after SCT, is caused by the activation of alloreactive donor T-cells. Although being life threatening in its severe forms, GVHD has a protective effect called the graft-versus-leukemia effect (GVL). In order to use the GVL effect of donor T-cells, donor lymphocyte infusions (DLI) is now used as a treatment for relapse after SCT. Response to DLI is usually better when the tumor load is low. Therefore, sensitive methods to detect residual leukemic cells are needed in order to identify patients who are at the highest risk of relapse and to start immunotherapeutic interventions when the tumor load is still low. Minimal residual disease (MRD) refers to the presence of leukemic cells below the detection limit of standard morphological analysis. The most sensitive and widely used techniques for MRD detection are based on the PCR technology. The aim of this thesis is to evaluate the clinical significance of MRD detection in leukemia patients receiving SCT.

In patients with acute myeloid leukemia (AML), we evaluated the significance of mixed chimerism (MC) analysis, the detection of recipient-derived hematopoietic cells after SCT. MC analysis was performed in the leukemia-affected cell lineage to increase the specificity and sensitivity of the method. MC was detected in 14 of 30 patients. Ten of these 14 patients relapsed as compared to 2 of 16 with donor chimerism ($p<0.01$). MC was detected a median time of 66 (range 23-332) days before hematological relapse.

Using immunoglobulin and T-cell receptor gene rearrangements as clonal markers, we analyzed MRD levels before and after SCT in patients with acute lymphoblastic leukemia (ALL). MRD detection before SCT was associated with increased risk of relapse. However, GVHD was shown to protect against relapse in patients with high levels of MRD. MRD detection after SCT was also associated with a high risk of relapse. Relapse occurred in 8 of 9 MRD positive patients as compared to 6 of 23 MRD negative patients ($p<0.01$). MRD was detected a median of 5.5 (range 0.5-30) months before relapse.

In recent years, nonmyeloablative SCT (NST) has been studied as a safer approach for patients who are not eligible for the toxic conditioning regimens given before SCT. We studied the kinetics of MRD and MC in chronic myeloid leukemia (CML) patients receiving NST and compared the results to those obtained from CML patients receiving a conventional SCT (CST). A competitive PCR approach was performed for quantitative MRD analysis of BCR-ABL transcripts. In the early posttransplant period, higher incidence and levels of MC and MRD were found in NST patients as compared to CST patients. However, molecular remissions were subsequently achieved in most NST patients.

Wilms' tumor gene (WT1) has been reported as a "panleukemic" MRD marker in many studies. We wanted to evaluate WT1 as a MRD marker by comparing the kinetics of WT1 levels with that of BCR-ABL using realtime quantitative PCR. We found a background expression of WT1 healthy controls. In addition, WT1 analysis was not sensitive enough to predict relapse.

In conclusion, MRD analysis in leukemia patients provides the possibility to identify patients at high risk of relapse after SCT. Adoptive immunotherapy based on MRD results may prevent relapse and improve outcome for patients with poor prognosis.

2 INTRODUCTION

2.1 LEUKEMIA

The term leukemia is derived from the Greek and means “white blood”. Leukemia is a malignant disease (cancer) of the bone marrow and blood.[1] Leukemia accounts for approximately 2% of all cancer cases and affects all ages and sexes.[2] Although the cause of leukemia is unknown, some risk factors are exposure to radiation and chemicals such as benzene.[3] As leukemia progresses, function of the bone marrow becomes impaired and if the disease is unchecked, the abnormal cells become dominant and are carried throughout the body by the bloodstream. Uncontrolled, leukemia causes infections, due to the lack of normal white blood cells; severe anemia, due to lack of red blood cells; and bruising and hemorrhaging, due to lack of platelets. The aim of leukemia treatment is to bring about a complete remission (CR). CR means that there is no evidence of the disease and the patient returns to good health with normal blood and marrow cells. Relapse indicates a return of the cancer cells and return of other signs and symptoms of the disease. For leukemia, a CR that lasts five years after treatment often indicates cure.
Leukemia is divided into four categories, myeloid or lymphoblastic, each of which can be acute or chronic. Acute leukemia affects immature white blood cells, progresses rapidly, and is the type most often seen in children. Chronic leukemia occurs most often in adults and progresses slowly, often over a period of many years.

Acute Lymphoblastic Leukemia (ALL). ALL is the most common leukemia in children. It is usually diagnosed in children less than 10 years old of age but increases in frequency in older individuals (>50 years).[4] ALL is divided into T-ALL and B-ALL according to the cell type involved. About 85 % of the ALL cases involve the B-cell subtype, which is usually less aggressive than T-ALL. In addition, B-ALL can be further subdivided according to the French-American-British (FAB) classification (L1, L2 and L3).[5] Other aspects, such as surface markers and chromosomal aberrations are also used for identification of different subtypes. These biological features of the leukemic cells are important prognostic factors.
Using chemotherapy only, more than 70% of children with ALL are alive and disease-free at five years.[6] The corresponding number in adults is lower. Patients with initial poor prognostic factors and those who relapse after chemotherapy are considered for allogeneic stem cell transplantation (SCT).[7]

Acute Myeloid Leukemia (AML). AML can occur at any age but increases exponentially in incidence after 45 years old of age.[4] This leukemia can have many different genetic alterations and the appearance of the leukemic cells can be represented by many different subtypes. AML is subdivided into eight FAB subgroups according to the different patterns of blood cell involvement (AML M0 to M7). Although several genetic changes, especially translocations of chromosomes, are relatively common, a large proportion of patients has uncommon or rare genetic changes.
In childhood AML, chemotherapy has improved outcome but the results are not as dramatic as in ALL; 5-year disease-free survival rate is 40-50%. Therefore, a high proportion of AML patients is considered for SCT.[8]

Chronic Myeloid Leukemia (CML). CML is considered an "adult" leukemia because it usually occurs in individuals >30 years of age. It is uncommon in children (<2% of the CML cases). CML is distinguished from other types of leukemia by the presence of a genetic abnormality in leukemia cells, the Philadelphia chromosome (Ph).[9] Ph is detected in ~95% of the CML cases and is the result of a chromosome translocation involving chromosomes 9 and 22, t(9;22). This fusion leads to an abnormal fused gene called BCR-ABL. The protein produced by the BCR-ABL gene functions abnormally and leads to dysfunctional regulation of cell growth and survival. Most CML patients are diagnosed in the chronic phase (CP) of the disease. In time, the CP can evolve into a more rapidly progressive phase, referred to as "accelerated phase" and ultimately "blast crisis", resistant to current treatment.

Interferon-α has been an important drug in the treatment of CML. However, at the present time, SCT is the only curative form of treatment for CML.[10] Recently, a new drug has been introduced. Imatinib mesylate (Glivec, STI571) binds to the BCR-ABL protein and block its effects. Current studies indicate that patients undergoing treatment with imatinib have an increased likelihood of achieving a complete remission. Because this therapy is only a few years old, it is unknown at this time if the complete remissions achieved with imatinib therapy will be as long lasting as the case after successful SCT.

Chronic Lymphoblastic Leukemia (CLL). CLL is the most prevalent form of leukemia.[11] The disease is very uncommon in individuals under 45 years of age. At the time of diagnosis, 95 percent of patients are over age 50, and the incidence of the disease increases dramatically thereafter. As in the case of ALL, the B-cell type of CLL is more common than the T-cell type.

CLL is a type of leukemia that can be stable and not disturb the patient's well being for prolonged periods without treatment. Chemotherapy is usually used to treat progressive CLL. SCT is used in very few cases of CLL and therefore this disease will not be discussed further.

2.2 ALLOGENEIC STEM CELL TRANSPLANTATION (SCT)

The first studies of human SCT were pioneered by Thomas E. Donnall and colleagues in the late 1950s.[12,13] Although all the early clinical transplantation efforts failed, most probably due to poor human leukocyte antigen (HLA) matching, research continued and more successful transplantations were reported in the early 1970s.[14-16] For his pioneer work in this field, Thomas E. Donnall received the Nobel Prize in medicine in 1990.

Today, SCT is a well-established treatment method for hematological malignancies (e.g., leukemia, lymphoma and myeloma), nonmalignant bone marrow disorders (aplastic anemia) and genetic diseases associated with abnormal hematopoiesis and function (thalassemia, sickle cell anemia and severe combined immunodeficiency).[15-19] SCT allows the replacement of the patient's diseased hematopoietic system with a normal one. In autologous SCT, the patient's own bone marrow is cryopreserved prior to administration of chemotherapy and/or high-dose radiation therapy. The marrow cells are then thawed and infused into the patient to reestablish hematopoiesis. Because there is a risk that autologous stem cells may contain viable tumor cells, different methods have been developed in order to remove tumor cells from the stem cells.[20]

In allogeneic SCT, which is the main topic in this thesis, stem cells are mainly taken from an HLA identical sibling or an HLA matched unrelated donor (MUD). An HLA identical sibling, which is the ideal donor, can be found for only ~30% of all patients. Because HLA

molecules are highly polymorphic and important for the outcome of SCT, it has been necessary to develop large donor registries. Currently, more than 8 million individuals have volunteered to serve as donors and the chance to find a MUD is ~60-90%, depending on the ethnic origin of the patient.

2.2.1 Conditioning and nonmyeloablative SCT

Before the transplantation, patients receive a conditioning regimen in order to eradicate malignant cells and prevent graft rejection by immunosuppression of the patient. Total body irradiation (TBI) and chemotherapeutic agents like cyclophosphamide (Cy) and busulfan (Bu) are commonly used in different conditioning regimens.[15,16,21] These standard regimens are myeloablative and highly toxic for the patients, restricting its use to patients younger than 50-55 years of age who are in good medical condition. Therefore, less toxic and nonmyeloablative conditioning regimens have been developed for older patients and those with poor medical condition.[22-26] Also, the observation that the antitumor effect of transplantation derives not only from the conditioning regimen but also from the transplanted donor cells has led the investigators to ask whether nonmyeloablative SCT might be as effective as standard SCT.

Although, early results with nonmyeloablative SCT are encouraging, especially in older patients, the lack of comparative data between both transplant methods, the heterogeneity of the studies and the short follow-up have made it difficult to evaluate this new approach.[27,28] A direct comparison between different studies has also been complicated by the different nonmyeloablative regimens that have been used.

2.2.2 Stem cell source

While bone marrow (BM) traditionally has been the source of stem cells for transplantation, the use of peripheral blood (PB) has increased dramatically since the first reports in the mid-1990s and has now essentially replaced BM as the source of stem cells for allografting.[29-31] Therefore, the term “bone marrow transplantation” generally has been replaced by “hematopoietic stem cell transplantation”.

Hematopoietic stem cells usually circulate in the PB at very low concentrations, but following administration of hematopoietic growth factors such as granulocyte colony stimulating factor (G-CSF) or granulocyte/macrophage colony stimulating factor (GM-CSF), the concentration of stem cells in the PB increases substantially. Although the number of T-cells infused is ten times higher using PB stem cells (PBSC) as compared to BM, there is no increased risk for developing acute graft-versus-host disease (GVHD) after PBSCT.[32-34] However, the use of PBSC seems to be associated with more chronic GVHD.[35,36] Although the effect on relapse is still unclear, some of the benefits using PBSC are the ease of collection, acceleration of engraftment and immune reconstitution.[37]

Umbilical cord blood is a rich source of hematopoietic stem cells and has been successfully used to reconstitute hematopoiesis after SCT.[38,39] Banks of cryopreserved cord blood have been established as an alternative to unrelated SCT. Potential advantages include the rapid availability and because cord blood is relatively deficient in T-cells, some degree of HLA mismatching might be tolerated. However, the use cord blood has been associated with slower engraftment and an increased risk of graft failure.[40,41] The low cell content of cord blood collections has limited the use of this approach to children, although adult patients have been included in more recent years.

2.2.3 Graft-versus-host disease (GVHD)

Graft-versus-host disease (GVHD) is one of the major complications after SCT and is the main reason for transplant-related mortality (TRM). GVHD is an immunologically mediated disease where T-cells in the donor graft attack and destroy recipient cells.[42] GVHD occur in the majority of the patients (>80%) depending mainly on factors such as disparity in HLA, donor type and GVHD prophylaxis.[43-45]

Acute GVHD usually develops within the first three months following SCT. The main target organs include the immune system, skin, liver, and intestine. Depending on the involvement and severity of the damage on skin, liver and intestine, acute GVHD is clinically graded from grade I to grade IV.[46] In grade I (mild GVHD), only local skin rashes can be seen while in grade IV with severe organ damages, the mortality is almost 100%.

Chronic GVHD usually develops more than 100 days after SCT with an incidence of 40-60%. A prior acute GVHD increases the probability of chronic GVHD, which is graded as limited, or extensive.[47] The mechanism of chronic GVHD is less understood than acute GVHD. Interestingly, the usual symptoms of chronic GVHD resemble those of autoimmune disorders.

One of the main complications associated with GVHD, beside tissue damage, is severe immunological deficiency. Bacterial, viral and fungal infections are usually the causes of death in patients with more severe GVHD.[48-50]

In order to decrease the incidence of GVHD, immunosuppressive agents are given to patients for a prolonged period after SCT. A combination of cyclosporine A (CsA) and methotrexate (MTX) is usually used as GVHD prophylaxis.[44] T-cell depletion of the graft is an efficient way to decrease the incidence of GVHD.[51,52] However, T-cell depletion is associated with increased risk of graft failure and leukemia relapse[53,54], showing that T-cells are not only responsible for GVHD but also important in the engraftment process and the graft-versus-leukemia effect (discussed later).

2.3 RELAPSE

After SCT, high remission rates can be induced and in some cases, the remission status will continue without evidence of recurrent leukemia. However, in many patients, relapse of the underlying disease will be a major obstacle to successful SCT. The incidence of relapse depends on different factors, but patients can basically be divided into high-risk and low-risk groups, depending on the remission status at the time of SCT. Patients transplanted in second or later remission or in relapse are usually considered as high-risk patients. The relapse incidences in ALL, AML and CML patients transplanted between 1990-2003 at Huddinge University Hospital are shown in Table 1. The outcome for patients who relapse after SCT is usually poor and depends on the underlying disease and the time between SCT and relapse. Relapse early posttransplant is associated with high mortality and low rate of complete remissions.[55] Patients with post-SCT relapse may be treated with additional chemotherapy or with intensive conditioning followed by a second SCT. A second SCT, however, is quite toxic and the rate of long-term survivors is low, especially if relapse occurs within 6 months after SCT.[56] Current treatment methods against relapse after SCT are based on adoptive immunotherapy in order to increase the graft-versus-leukemia effect.

Table 1. Incidence of relapse at Huddinge University Hospital after SCT, 1990-2003

Disease	Stage at SCT	N=	Relapse incidence[1]	Mortality after relapse
CML	CP1	100	24% [2]	38%
	>CP1	22	31%	86%
ALL	CR1	38	40%	93%
	>CR1	55	56%	77%
AML	CR1	80	31%	88%
	>CR1	59	39%	91%

[1]Kaplan-Meier estimates at 5 year. [2]10/24 relapses were based on cytogenetics.

2.4 GRAFT-VERSUS-LEUKEMIA (GVL)

Evidence for a graft-versus-leukemia effect (GVL) effect in humans was first reported in 1979 with the observation that the risk of relapse was lower in patients who developed GVHD than in those who did not.[57] Later studies confirmed these results and showed that relapse rates are lowest in patients with both acute and chronic GVHD, higher in patients with no GVHD and highest in recipients of T-cell depleted allogeneic marrow or syngeneic - i.e., twin, marrow.[58] These findings led to the idea of using GVHD and T-cells in adoptive immunotherapy, to manipulate GVHD and T-cells in order to treat or decrease the risk of leukemic relapse.

Withdrawal of immunosuppression can be used to increase the reactivity of donor T-cell against recipient/leukemic cells. However, in most cases this is followed by the infusion of donor leukocytes.

Donor leukocyte infusion (DLI): Because T-cells were recognized to be important in the GVL effect, it seemed logical to use these cells to treat leukemia relapse after SCT. Kolb *et al* first reported that CML patients achieved complete cytogenetic remission when treated with infusions of “buffy-coat” cells from the original transplant donor.[59] The effectiveness of DLI to treat relapsed CML has since then been confirmed in many studies.[60] However, while remission rates of 70-80% have been reported for CML patients, the corresponding results for AML (<30%) and ALL (<20%) have not been encouraging.[61,62] This difference in GVL responsiveness is also evident in the case of T-cell depleted transplants, which increases relapse rates in CML patients more dramatically than in AML and ALL patients.[53,54,63] It is not clear yet why the GVL reaction is stronger in CML but some explanations may be the ability of leukemic cells to present antigens, the presence of costimulatory molecules and cell growth rate. In CML, differentiation toward antigen presenting cells (dendritic cells) can occur. These cells can stimulate and sustain a reaction against leukemia.

Major complications after DLI are pancytopenia and GVHD. Although GVHD and GVL are closely related, different strategies have been developed to separate GVL effects from GVHD in order to maintain (or increase) the antitumor activity of DLI but limit the damage to normal tissues.

1. Titration of the T-cell dose in DLI. The use of escalating doses may reduce the incidence and severity of GVHD, while preserving the GVL effect.[64,65]

2. Insertion of a "suicide gene" into donor T-cells prior to DLI and pharmacologically induce the death of the transduced cells when the antileukemic effect has been achieved or GVHD becomes more severe.[66,67]
3. Selective infusion or depletion of CD4+ or CD8+ T-cell subpopulations. Some clinical data suggest that depletion of CD8+ T-cells from the allograft or DLI can reduce GVHD without significantly decreasing the GVL effect.[68-70]
4. Co-administration of interleukin-2 (IL-2) to enhance the antileukemic effect of donor T-cells.[71]
5. Selective removal of alloreactive T-cells.[72,73]
6. Selection of donor cells with anti-leukemia activity or specific activity against antigens expressed only on cells of the hematopoietic lineage.[74,75]

One strategy to enhance the GVL effect is to reduce the leukemic burden before DLI or give DLI when the tumor burden is still low. The correlation between tumor burden and response to DLI has been described for CML, but is less clear for other hematological malignancies.[76-78] CML patients treated with DLI at the time of molecular or cytogenetic relapse have a higher rate of response to DLI than those treated at the time of hematological relapse.[77,79]
As mentioned earlier, patients with acute leukemia, AML and ALL, respond poorly to DLI given at the time of hematological relapse. However, the presence of a GVL effect in ALL is well established.[58,80] GVHD after SCT usually decrease the relapse rates in ALL. These findings suggest that GVHD and T-cells may protect against relapse in acute leukemias when the tumor burden is low. Although no major studies have started early interventions based on MRD results, some cases have been described with encouraging results.[78,81-84] Therefore, sensitive methods to detect residual disease are needed in order to identify those patients at the highest risk of relapse and to start immunotherapeutic interventions at the level of minimal residual disease.

3 MINIMAL RESIDUAL DISEASE

A patient with leukemia is considered to be in complete remission (CR) when no blast cells are detected by light microscopic examination of the BM. The sensitivity of this method is 1-5%. At the time of diagnosis, the number of leukemic cells is approximately 10^{12}, which means that a patient in CR can still harbor as many as 10^{10} leukemic cells, cells which are responsible for relapse if they are not eradicated by chemotherapy or SCT. Minimal residual disease (MRD) refers to the presence of leukemic cells in the BM of patients in CR (Figure 1). A number of techniques have been developed that are substantially more sensitive than morphology for detecting MRD and assessing response to treatment. In the next sections, the most common used MRD methods after SCT are described and the specific advantages and disadvantages of each method are discussed (Table 2). The clinical significance of MRD detection, using different techniques, will also be discussed at the end of this chapter.

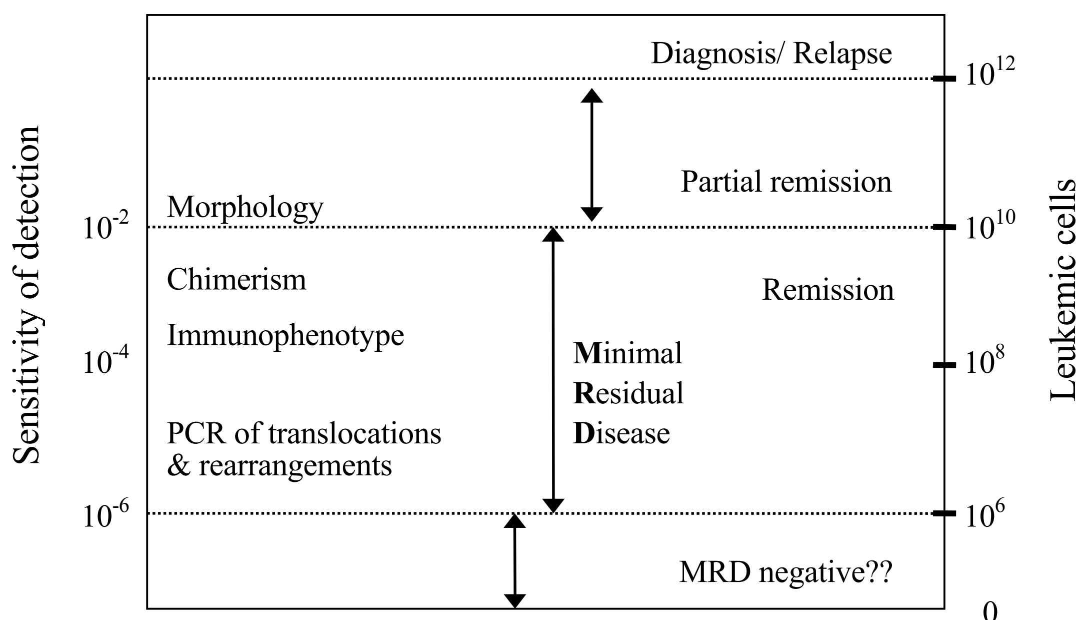

Figure 1. Minimal residual disease (MRD) refers to the presence of leukemic cells below the detection limit of standard morphological analysis (10^{-2}). Different methods have been developed for MRD analysis.

Table 2. Characteristics of the techniques currently used for MRD detection after SCT in patients with ALL, AML and CML.

	Flow Cytometric Immunophenotyping	PCR analysis of fusion gene transcripts	PCR analysis of Ig/TcR rearrangements	PCR analysis of VNTR/ STR Chimerism
Applicability:				
ALL	60-90% B-ALL 90-95% T-ALL	40-45% B-ALL 15-35% T-ALL	90-95%	>95%
AML	80%	20-40%	10%	>95%
CML		~95%		>95%
Sensitivity	10^{-4}	10^{-4}-10^{-6}	10^{-4}-10^{-5}	10^{-2}-(10^{-4} after cell separation)
Advantages	- applicable for most patients - quantification simple	- easy - sensitive and leukemia specific - stable target	- sensitive and patient specific - applicable in most ALL patients	- applicable for most patients - useful for engraftment analysis
Disadvantages	- limited sensitivity - immunophenotypic shifts can occur between diagnosis and relapse	- RNA degradation - limited applicability in AML and ALL - high risk of cross-contamination	- material from diagnosis or relapse needed - time-consuming at diagnosis - risk of false negative results due to clonal exchange	- limited sensitivity - not leukemia specific

3.1 METHODS

3.1.1 Immunophenotype analysis

Immunological methods were among the first used in MRD studies and rely on the use of specific monoclonal antibodies that bind to antigens expressed on the cell-surface membrane, in the cytoplasm or in the nucleus. Although, there are some abnormal proteins associated with specific leukemias (e.g., BCR-ABL in CML), the use of a single marker usually does not distinguish leukemic cells from normal ones. Therefore, immunological detection of MRD is based on identifying combinations of leukocyte antigens found on leukemic cells, but not on normal cells, in PB and BM. These phenotypes can be determined by double or triple color staining with antibodies conjugated to different fluorochromes and the labeled cells can be analyzed by a fluorescence activated cell sorter (FACS), flow cytometry. With the new cytometers, four and five color analysis is possible, increasing the specificity and informativity of the MRD analysis. Immunophenotype analysis is usually performed in ALL and AML patients only, because CML patients are monitored with molecular methods for the presence of BCR-ABL transcript.

Applicability. In T-lineage ALL, the combination of CD3 and TdT (Terminal deoxynucleotidyl transferase) is enough to monitor MRD in almost all patients.[85] Because the normal counterparts of T-ALL cells are immature T-cells in the thymus, detection of MRD in patients with this subtype simply consists of the identification of immature cells outside the thymus.
In B-lineage ALL and AML, however, the normal counterparts of leukemic cells are immature progenitors normally present in the BM. Therefore, MRD studies in B-ALL and AML are more complicated and a larger panel of antibodies is needed in order to distinguish leukemic cells from normal cells. Nevertheless, with the combination of 3-4 markers and the use of several combinations (phenotypes), MRD studies can be performed in 60-90% of B-ALL and AML patients.[86,87]

Sensitivity. The sensitivity of MRD detection with flow cytometry depends mainly on two variables: (1) the degree of morphological and phenotypic difference between the target cells and normal cells and (2) the number of cells that can be analyzed. Immunophenotypes that do not overlap between normal and leukemic cells will increase the sensitivity. The number of cells that can be analyzed in clinical samples is usually less than 10^6. Considering that 10-20 dots are needed to interpret a suspect flow cytometric event, the maximum sensitivity that can be achieved is 10^{-5}. However, most studies report a sensitivity of 10^{-4}.

Advantages and disadvantages. Because both leukemic and normal cells are counted directly in the flow cytometry, MRD quantification is more simple and accurate as compared with molecular methods.[88] Another advantage is that different parameters of the flow cytometry can be used to discriminate between viable and dying cells.
With a sensitivity of 10^{-4}, a fraction of the patients at risk of relapse will be missed. The method is difficult to perform and therefore restricted to highly specialized laboratories. Another limitation of the method is that the immunophenotype of leukemic cells may change during the course of treatment and disease progression, leading to false negative

results. This problem can be overcome with the use of several immunophenotypes per patient.[89]

3.1.2 Polymerase Chain Reaction (PCR)

Currently, the most sensitive and widely used technique for MRD detection is polymerase chain reaction (PCR). The PCR technique, first described in 1985, is a primer-mediated *in vitro* reaction for specific nucleic acid amplification.[90,91] The method is based on repetitive annealing and extension of two oligonucleotide primers that flank the region of interest in the template DNA. A DNA polymerase is required to catalyze the reaction in which the primer pair and four deoxynucleotide tri-phosphates (dNTPs) are used to create a complimentary DNA sequence. If RNA is the desired template for PCR amplification, a reverse transcription (RT) step is required to obtain a complementary DNA (cDNA) copy that can be used in the PCR reaction.
PCR is a very sensitive method. If you start with one copy of the original template, theoretically you will end up with 10^9 copies after 30 cycles of PCR amplification. This ability to produce large number of copies, however, is a problem when PCR products are carried over between samples. This cross-contamination might be difficult to recognize and will lead to false-positive results. Therefore, different precautions are needed to minimize the risk of cross-contamination when working with PCR.[92]
The first PCR based methods for MRD detection were reported in the late 1980s.[93-95] Most of these initial studies were performed using qualitative PCR. Although this approach may be useful in certain cases, it only gives limited information and does not allow analysis of tumor kinetics. Quantitative PCR methods were developed to monitor the change of tumor load during follow-up. Many of these semiquantitative MRD studies were based on end-point quantification. The PCR reaction generates copies of a DNA template in an exponential fashion. Due to accumulation of inhibitors during the PCR process, the PCR reaction will eventually reach a plateau phase where no further PCR product is generated. End-point PCR analysis is done when the plateau phase has been reached, and therefore there is usually a lack of correlation between the amount of PCR product and the initial amount of target molecules. More quantitative methods such as competitive PCR and limiting dilution are also based on post-PCR, end-point analysis.[96,97] These techniques require serial dilutions and the analysis of multiple replicates, both of which introduce variability and may be too difficult and time-consuming to be performed routinely.

Realtime quantitative PCR (RQ-PCR). The novel RQ-PCR technique circumvents many of the problems associated with semiquantitative PCR analysis and permits accurate quantification during the exponential phase of the PCR reaction. Accumulation of PCR products is continuously (realtime) monitored during the cycles allowing rapid quantification without post-PCR processing - e.g., gel analysis. The detection system in RQ-PCR is based on fluorescent signals generated during the PCR process. The increase of fluorescent signals after each cycle is detected by the realtime instrument. Depending on the method applied, fluorescent signals can be generated in different ways.
SYBR Green I. The simplest and cheapest RQ-PCR technique is based on the DNA binding dye SYBR Green I. This dye is included in the PCR reaction and generates fluorescent signals when it binds to double-stranded DNA. As the amount of PCR product increases after each cycle, more SYBR Green dye will bind and increase the fluorescent signal. The major disadvantage of using SYBR Green is that it binds to PCR products

nonspecifically. Therefore, the fluorescent signal will also include nonspecific PCR products and primer-dimers. A melting-curve analysis can be performed at the end of the PCR reaction to evaluate whether unspecific PCR products are present. This analysis is based on the fact that PCR products of different length and sequence will melt at different temperatures.
Hybridization probes. In this approach, two probes are included in the PCR reaction in addition to the amplification primers. The probes are designed to bind to closely juxtaposed sequences on the amplified DNA. One probe is labeled with a donor fluorochrome at the 3' end, and the other is labeled with an acceptor fluorochrome at its 5' end. A fluorescent signal is generated when the two probes are bound to the target and the fluorochromes are brought next to each other (within 1-5 bp nucleotides). Consequently, the fluorescent signal will be at maximum at the annealing phase of the PCR cycle, which is in contrast to SYBR Green I chemistry where the maximum signal is reached at the end of the PCR cycle. The RQ-PCR equipment usually used together with hybridization probes is the Lightcycler (Roche, Alameda, CA, USA).
TaqMan probes (hydrolysis probes). In this method, the single probe is conjugated with two fluorochromes, a 5' end reporter fluorochrome and a 3' end quencher fluorochrome. As long as the two fluorochromes are in close vicinity of each other on the intact probe, the fluorescence emitted by the reporter will be "silenced" by the quencher. However, during the polymerization phase of the PCR cycle, the TaqMan probe is initially displaced from the DNA strand by the *Taq* polymerase and subsequently hydrolyzed by the 5' to 3' exonuclease activity of this enzyme. This results in the separation of the two fluorochromes and the fluorescent signal from the reporter becomes detectable (Figure 2A). Currently, the most commonly used reporter and quencher fluorochromes are FAM (6-carboxy fluorescein) and TAMRA (6-carboxy-tetramethyl rhodamine), respectively. As an alternative to TAMRA, several "dark" fluorochromes have become available. These fluorochromes absorb the energy emitted from the reporter and release it as heat rather than fluorescence, reducing the background signal. The TaqMan based RQ-PCR approach is usually performed on the ABI sequence detection system (Applied Biosystems, Foster City, CA USA).
When the PCR reaction has been completed, the sequence detection software plots the measured fluorescence *vs.* the cycle number.(Figure 2B) This allows the calculation of a threshold cycle (Ct) defined as cycle number at which the fluorescence passes a fixed threshold. Samples with high copy numbers of target will reach the threshold value at earlier cycles than samples with less target copies - i.e., lower Ct value for a higher concentration. A standard curve can be generated from a serial dilution of a target with known starting copy numbers (Ct values *vs.* concentration, Figure 2C). Quantification is then performed by plotting the Ct value of an unknown sample on the standard curve.

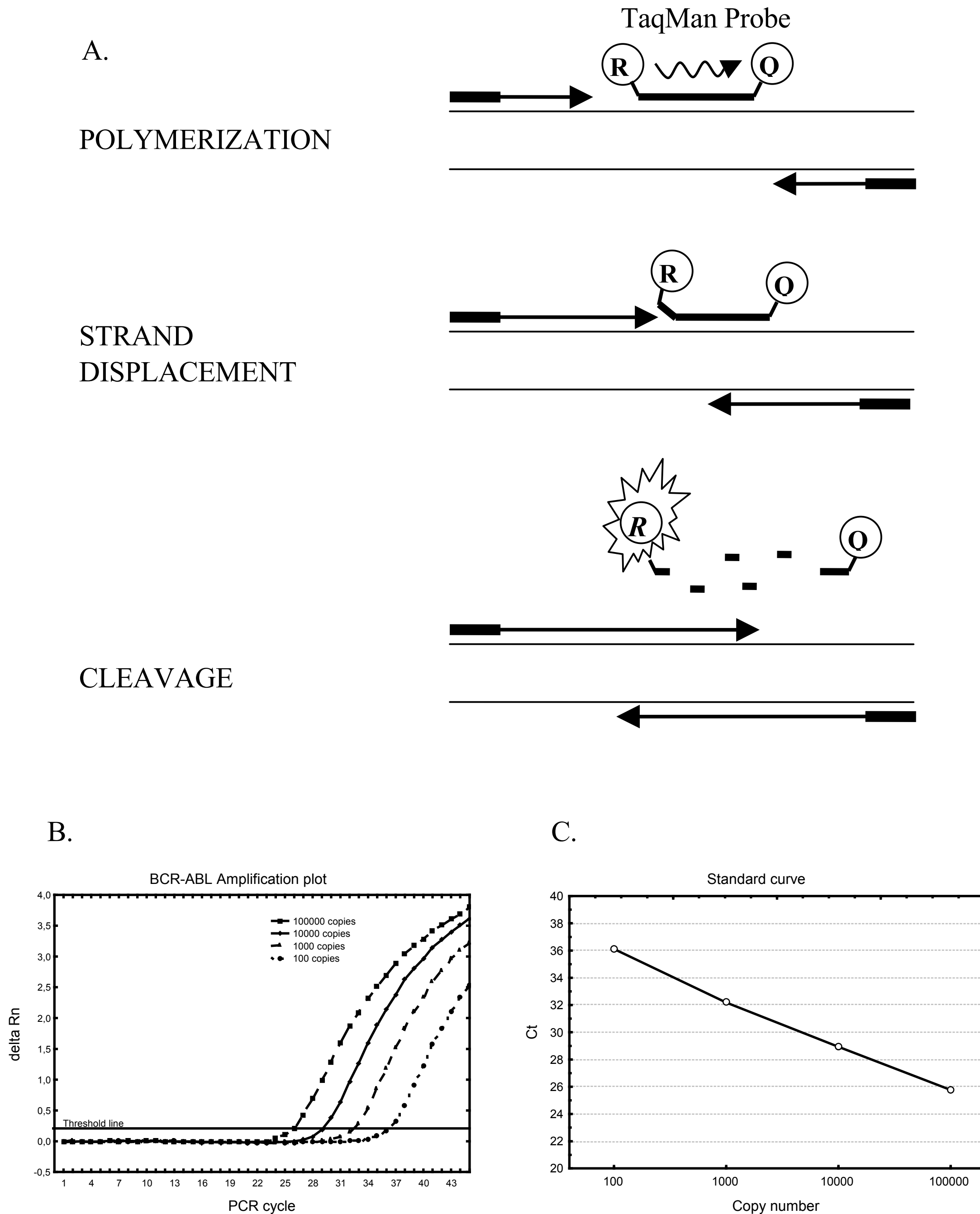

Figure 2. Realtime quantitative PCR. **A.** The Taqman probe is a oligonucleotide with a fluorescent 5'-Reporter dye and a 3'-Quencher dye. As long as the probe is intact, the reporter flurescence is quenched by the 3'-quencher dye. During polymerization, the probe is hydrolyzed by the 5'-nuclease activity of *Taq* polymerase and the 5'-reporter dye is released, yielding a signal. **B.** Amplification curves of serial dilution of a plasmid containing the BCR-ABL gene. The increase in fluorescence on the y-axis is indicated as delta Rn. Based on the background fluorescence, usually determined between cycles 3 and 15, a threshold line is determined. This threshold is used to calculate the threshold cycle (Ct) of each sample. **C.** A standard curve can be generated by the Ct values obtained from the amplification plot. The Ct values of unknown samples are plotted in the standard curve for quantification.

3.1.3 Fusion gene transcript analysis

Chromosomal abnormalities are present in 70-80% of the patients with AML and ALL and in >95% of the patients with CML.[98] These changes include gain or loss of chromosomes, gene deletions or insertions, chromosome inversions, and balanced chromosome translocations. Some of these abnormalities, especially the chromosome translocations, are recurrent and have been associated with leukemogenesis.[99] The fusion of two genes after a translocation may result in a novel chimeric protein.

Chromosome abnormalities can be used as leukemia specific targets for MRD analysis. Methods such as standard cytogenetics, southern blot and fluorescence in situ hybridization (FISH) have been used to detect chromosomal abnormalities but these techniques are associated with a low sensitivity (1-5%).[100]

With the PCR technique, it is possible to detect one leukemic cell among 10^5-10^6 normal cells. PCR can be performed directly on DNA if both breakpoints in a particular translocation cluster within a small region. However, in most cases, the translocation is more complex because the breakpoints occur within a large intronic region. The intervening segment of DNA between the primers will be too large to amplify. Therefore, RNA transcripts of the fusion genes are usually used as templates for PCR amplification.

Table 3. Some chromosome abnormalities used as MRD targets

Disease	Chromosomal abnormality	Molecular target	Frequency (%)
CML	t(9;22)	BCR-ABL	95
B-ALL	t(9;22)	BCR-ABL	5 (children)
			20-30 (adults)
	t(1;19)	E2A-PBX1	5-10
	t(4;11)	MLL-AF4	2-5 (50-60 in infants)
	t(12;21)	TEL/AML1	10-25 (children)
			1-3 (adults)
T-ALL	TAL1 deletion	Tal*	20-25
		SIL-TAL	
	t(11;14)	RBTN1/2-TCRδ*	5-10
AML	t(8;21)	AML-ETO	5-10 (20-40 in M2)
	t(15;17)	PML-RARα	5-10 (>95 in M3)
	inv(16)/t(16;16)	CBFβ-MYH11	5-10
	t(9;11)	MLL-AF9	1-10

*DNA as template, otherwise RNA (cDNA). References[100-102]

Applicability. The most common chromosomal abnormalities used for PCR analysis and the frequency of these abnormalities are shown in Table 3. In CML, the Ph-chromosome, t(9;22), is found in 95% of the cases and serves as an excellent MRD marker for this disease. The Ph-chromosome, which is also found in ALL patients, is discussed separately in the next section. In AML and ALL, there is no specific translocation associated with disease. There are several numbers of translocations, which occur in 1-30% of all cases, with larger frequencies in specific leukemia subtypes.

Sensitivity and quantification. Because chromosome abnormalities are highly disease specific, PCR amplification of the fusion gene transcripts can usually detect one leukemic cell among 10^4-10^6 normal cells. If necessary, higher sensitivity can be obtained by the use of "nested-PCR", a two-step PCR with a second PCR reaction performed on the products from the first PCR.

Quantification of the MRD target can be performed by comparing the PCR signal with serial dilutions of a standard with known amount of target DNA or RNA, by limiting dilution experiments,[103] and by competitive PCR.[97] However, with the introduction of RQ-PCR, quantification can be performed more easily and accurately than semiquantitative PCR analysis.[104]

Advantages and disadvantages. One of the major advantages of the PCR technique is the high sensitivity. In addition, these translocations are leukemia-specific and stable during the disease course.

The high sensitivity can be a problem if cross-contamination of RT-PCR products occurs, leading to false-positive results. RNA degradation and variations in efficiency of cDNA synthesis may also affect sensitivity of the method.

3.1.3.1 The Philadelphia chromosome, BCR-ABL

The Ph-chromosome was the first specific chromosome abnormality described in leukemia.[9] It is strongly associated with CML with an incidence of 95%. In ALL, the Ph is found in 2-5% of childhood cases and in 20-30% of adult cases, the incidence of which increases with age.[105-108] Ph is also found in approximately 1% of AML cases.[109]

The Ph arises from a reciprocal translocation, t(9;22), that joins 3' sequences of the ABL gene on chromosome 9 to the 5' sequences of the BCR gene on chromosome 22 (Figure 3).[110] The break on chromosome 9 regularly occurs 5' to the ABL exon 2 while the breakpoints on chromosome 22 can differ. In most cases of CML, the breakpoints within BCR occur in a region termed the major breakpoint cluster region (M-BCR) between either exons 13 and 14 (b2a2) or exons 14 and 15 (b3a2). In both cases, the hybrid BCR-ABL gene encodes a 210-kd chimeric protein (p210). In the majority of Ph-positive ALL cases, the breakpoint occurs in the first intron of the BCR gene, the minor breakpoint cluster region (m-BCR). This results in the expression of a p190 protein.

The vast majority of the CML cases possess the b2a2 and the b3a2 fusion types, with a higher prevalence for the b3a2 type. Co-expression of b3a2 and b2a2 is possible and detected in 5-10% of the CML cases. This is probably due to alternative RNA splicing and the reason has been proposed to be a polymorphism within the BCR gene.[111] In rare occasions (<1%), the e1a2 fusion type only can be found in CML, but it is detected in virtually all CML patients at the time of diagnosis and relapse, together with the other fusion types.[112-114] In Ph-positive ALL, the dominating fusion type is e1a2 with a frequency of 60-70%. Different locations of breakpoints or alternative splicing may also lead to other rare fusion types such as e19a2, b2a3, b3a3, e6a2.[115,116]

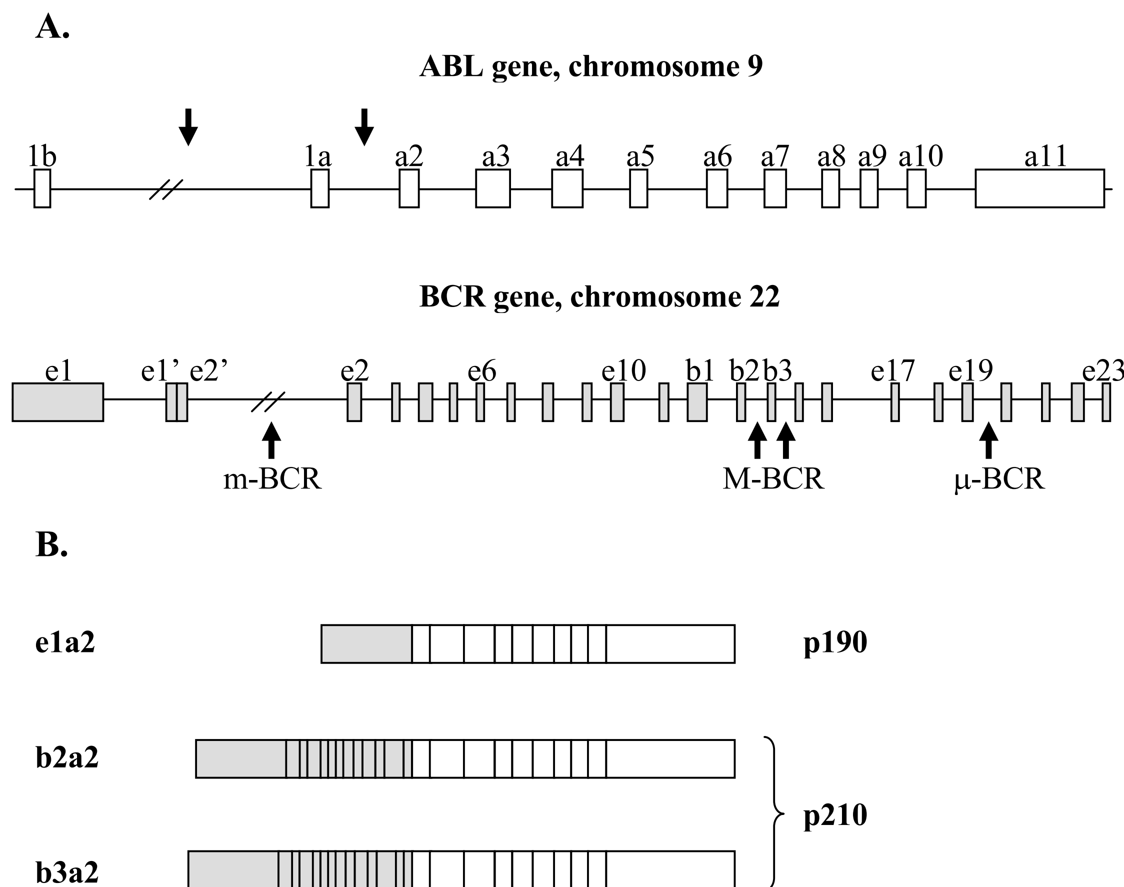

Figure 3. Schematic representation of the ABL and BCR genes and the BCR-ABL fusion transcripts involved in the Philadelphia chromosome translocation t(9;22),. **A.** Boxes and lines represent exons and introns, respectively. Breakpoint regions are indicated by arrows. The breakpoints in the ABL gene are located in a large region 5' of exon a2. In the BCR gene, three breakpoint cluster regions (BCR) have been identified. **B.** Breakpoints in the minor BCR (m-BCR) result in the e1a2 mRNA (p190 protein), which is usually found in Ph-positive ALL. The major BCR (M-BCR) is located between exons e13 and e15 (b2 and b4). The resulting b2a2 and b3a2 mRNAs and the encoded protein p210 are mainly found in Ph-positive CML. A rare product, e19a2 (p230), results from breakpoints occuring in μ-BCR.

Although the transforming potential of the BCR-ABL protein is well established, it is still unclear how this protein exerts its transforming effects. The BCR-ABL protein has a deregulated tyrosine kinase activity and is involved in the signal transduction pathways in the cell. Some of the cell mechanisms affected by the BCR-ABL protein is altered adhesion to stroma cells and extracellular matrix, constitutively active mitogenic signaling and reduced apoptosis.[110] Some data indicate that there are biological differences between the p190 and p210 fusion types. The *in vitro* tyrosine kinase activity of p190 protein is greater than that of p210.[117] In addition, in animal models, the p190 fusion type appears to induce leukemia that is more virulent than p210 leukemia.[118,119] Clinical data in ALL patients show that the p190 BCR-ABL transcript is associated with higher risk of relapse after SCT compared to the p210 BCR-ABL transcript.[120,121]

The presence of the BCR-ABL translocation in a hematopoietic cell seem not in itself sufficient to cause leukemia because BCR-ABL fusion transcripts are detectable in the PB

of many healthy individuals.[122,123] Using an optimized RT-PCR assay, with a sensitivity of at least 10^{-7}, the p210 and p190 type of transcripts were detected in 27% and 69% of the normal individuals, respectively.[123] It has been suggested that BCR-ABL in combination with a "correct" primitive hematopoietic progenitor cell may be sufficient to cause CML.
Because of its high sensitivity and easy and rapid performance, RT-PCR has been exclusively used for monitoring MRD in CML. BCR-ABL transcripts are stable over time in individual patients and there is no convincing data showing that clonal evolution may occur. Because qualitative PCR seems to have limited clinical value in CML patients after therapy, quantitative PCR methods have been developed to monitor the kinetics of MRD after SCT.

One important methodological aspect to the analysis of fusion gene transcripts is the use of an internal control gene.[124] Internal control genes (housekeeping genes, reference genes) are constitutively expressed genes, which are used for quality control of the patient samples. The yield and quality of RNA and cDNA can be highly variable. Usually, the number of fusion gene transcripts (BCR-ABL) is normalized to the number of transcripts of a control gene in order to compensate for variations that can occur between samples. Some of the genes, commonly used as controls in RT-PCR assays, include glucose 6-phosphate dehydrogenase (G6PD), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ABL, BCR, beta-2-microglobulin (β_2-MG), and porphobilinogen deaminase (PBGD). The selection of a control gene can be complicated by the presence of processed pseudogenes, nonfunctional and intronlacking genes with equal sequence length to endogenous mRNA, leading to false positive PCR results by genomic DNA. Identification of the appropriate control gene can be difficult and may need comparison analysis of different genes.[125]
The ABL gene is a commonly used control gene in BCR-ABL analysis and considered as a suitable control in different diseases.[126-131] Results are expressed as a ratio between BCR-ABL and ABL copy numbers (BCR-ABL/ABL). However, the use of the ABL gene as a control gene in Ph-positive diseases is complicated by the fact that the total number of ABL transcripts usually includes "normal ABL" + BCR-ABL. Thus, the BCR-ABL/ABL ratio will not be correct in cases, in which BCR-ABL levels are high (lower ratio). This, however, is a problem at relatively high BCR-ABL levels and will not have a major impact on the results at the level of MRD.

3.1.4 Antigen receptor rearrangement analysis

During early B-cell and T-cell differentiation the germline variable (V), diversity (D) and joining (J) gene segments of the immunoglobulin (Ig) and T-cell receptor (TcR) complexes rearrange, and each lymphocyte thereby obtains a particular combination of V-(D-)J segments (Figure 4). The huge diversity of antigen receptors is achieved by the random recombination of one individual member of each of theses gene segments. The addition and removal of junctional ("N") nucleotides increases this diversity even more. Therefore, the junctional regions of rearranged Ig and TcR genes, also called the third complementarity determining region (CDR3), are unique sequences that are assumed to be different in each lymphoid precursor. Because ALL cells are clonal proliferations of one precursor cell, analysis of Ig and TcR gene rearrangements can be used as "DNA-fingerprints" for each particular ALL.[94,132]

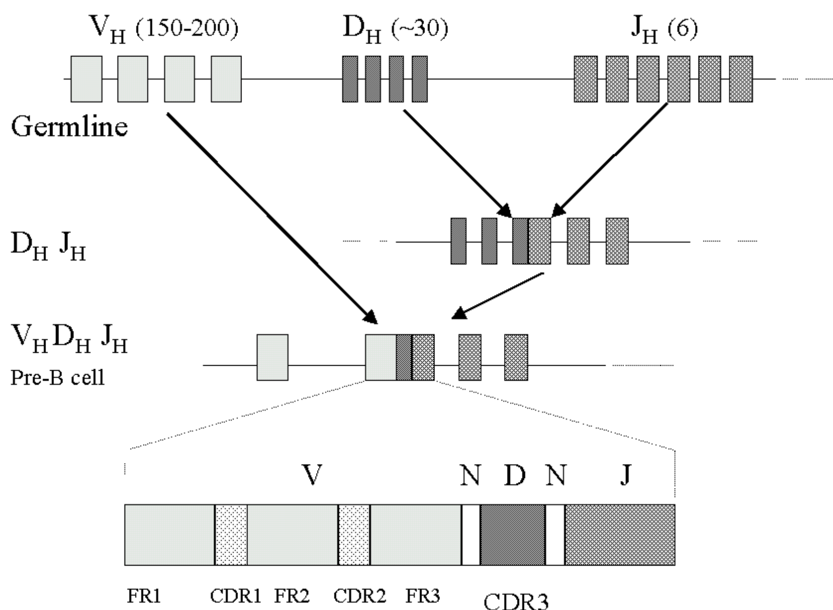

Figure 4. A schematic representation of the IgH gene rearrangement. Rearrangement is a two-step process: first D to J joining occurs, followed by V to D-J joining. Conserved regions are grouped into three framework regions (FRs) and the most variable part of the antigen binding site of immunoglobulins are grouped into complementarity determining regions (CDRs) Similar gene rearrangements occur in other Ig and TcR genes. The unique sequence of the CDR3 region is usually used to design patient-specific primers and probes for MRD analysis.

In principal, all antigen receptor genes can be used as MRD targets, - e.g., Ig heavy chain (IgH), Ig light chains kappa (IgL_κ) and lambda (IgL_λ), TcR alfa (TcRα), TcR beta (TcRβ), TcR gamma (TcRγ) and TcR delta (TcRδ). The first step in the methodology of rearrangement analysis is identification of junctional regions of Ig and TcR gene rearrangements. Usually, this is done by PCR analysis of BM samples taken at the time of diagnosis or relapse. Primer combinations, designed to conserved sequences flanking the rearrangement region, are used to amplify the leukemia (clone) specific sequences. The choice of primer combinations is complicated by the presence of large number of genes. The IgH gene complex consist of ~200 V_H-, 30 D_H- and six J_H-gene segments. The gene segments can be grouped into subfamilies based on sequence homology and therefore the number of primer combinations needed is reduced. The IgH rearrangement can be identified by using only five V_H family-specific primers in combination with one consensus J_H primer.[133,134] The V_H specific primers can be designed for all framework regions (FR1, FR2 and FR3).

TcRγ and TcRδ gene rearrangements are also relatively easily analyzed by limited number of primer combinations, but PCR analysis of IgL_κ, IgL_λ, TcRα and TcRβ requires more primers.[134] The detection of leukemia specific IgH/TcR rearrangements by PCR analysis is therefore limited by the choice of primers.

When the leukemia specific rearrangement is identified, different approaches can be applied to monitor MRD in remission samples.

In "gene fingerprinting" analysis, the same primer combination used for identification of the leukemia specific rearrangement is used to amplify remission samples.[135] This single step approach relies on the resolution of PCR products on a sequencing gel, which provides single base separation and permits differentiation of clonal products from the background produced by normal rearrangements. This technique is not sequence specific and obtains a sensitivity of 10^{-3}. Other methods like heteroduplex and single-strand conformation polymorphism (SSCP) analyses are also based on differentiation of monoclonal clones from polyclonal ones.[136,137]

In most strategies, the leukemia specific rearrangement is used to provide probes and primers for MRD detection in remission samples. Usually, the leukemia specific rearrangement is sequenced and when the different gene segments (V, D, and J) are identified, oligonucleotides specific for the junctional regions are designed. These oligonucleotides can be used as probes in hybridization experiments to detect PCR products derived from follow-up samples. The other possibility is to use the oligonucleotides as patient specific primers in PCR to amplify the leukemia specific clone.

Applicability. Due to the nature of the antigen receptor gene rearrangement analysis, this method is restricted to lymphoid malignancies although Ig and TcR rearrangements have been reported in ~10% of the AML cases.[138] IgH and TcR rearrangements can be detected in >95 of the ALL cases (Table 4). In ALL, IgH and TcR rearrangements are not lineage-restricted and this is referred to as lineage infidelity or cross lineage rearrangements. Thus, clonal rearrangements of TcR genes are seen in a large proportion of B-ALL and a smaller proportion of IgH rearrangements are found in T-ALL.[139] In addition to complete rearrangements (V-J), incomplete rearrangements (V-D, D-D or D-J) are usually detected in ALL cells, the occurrence of which seems to be age related.[140]

Table 4. Frequency and stability of Ig and TcR gene rearrangements.

Target		Frequency at diagnosis (%) B-ALL	Frequency at diagnosis (%) T-ALL	Monoclonality at diagnosis (%)	Stability at relapse (%)*
IgH		**>95**	**20**	60-70	65
	V_H-J_H	95	5		70
	D_H-J_H	20	20		40
Igκ		**50**	**0**	90	90
	Vκ-Kde	45	0		90
	Intron-Kde	25	0		85
TcRγ	(Vγ-Jγ)	**55**	**95**	60-65	75
TcRδ		**40**	**55**	60	60
	Vδ2-Dδ3/Dδ2-Dδ3	40	5		60
	Vδ-Jδ1/Dδ-Jδ1	1	50		

*Higher stability rates (>80%) in monoclonal leukemias as compared to oligoclonal leukemias (<50%). References[134,141,142]

Sensitivity and quantification. The detection limit of PCR analysis of junctional regions generally varies between 10^{-4} and 10^{-6}. The sensitivity is dependent on the type of rearrangement and on the background of normal lymphoid cells with comparable Ig or

TcR gene rearrangements.[143,144] Normal cells can contain the same rearranged gene segments as the leukemic cells with the only difference being the junctions with different "N" nucleotides. Therefore, with a longer junctional region it is possible to get higher primer specificity and thereby higher sensitivity. A "nested-PCR" approach can increase sensitivity if needed.
MRD quantification by PCR analysis of IG and TcR gene rearrangements is basically performed in the same way as quantification of fusion gene transcripts. Dilution series of diagnosis DNA is generally used to determine the tumor load in follow-up samples. Limiting dilution experiments and competitive PCR are other approaches that have been used for quantification.[96,145] However, the use of RQ-PCR has been increased and replaced many of the standard time-consuming PCR analyses.[104]

Advantages and disadvantages. The main advantages of this method are the high sensitivity and its applicable in virtually all ALL patients. The need to sequence junctional regions and to develop probes and primers for each ALL case is time-consuming and a limiting factor of the method.
The main disadvantage of using Ig and TcR rearrangements as MRD targets is that continuing rearrangements can occur during the disease course.[142,146-148] Such changes in rearrangement patterns will lead to false negative PCR results. Rearrangement changes between diagnosis and relapse are particularly observed in patients who show oligoclonality at diagnosis (Table 4). Oligoclonality is defined as the development of subclones from the primary leukemic cell and it is found in 30-40% of ALL cases.[149] The problem of oligoclonality is the uncertainty as to which clone is going to emerge at relapse and which should therefore be monitored as a MRD target.[150] Continuing IgH rearrangements might also occur between diagnosis and relapse and is usually due to V_H replacements without changes in the D_HNJ_H region.[147,151] Therefore, primers specific for the D_HNJ_H region can be designed in order to prevent false negative PCR results. It is now generally accepted that at least two Ig/TcR gene targets should be used for reliable and sensitive MRD detection in ALL patients.

Some studies have made methodological comparisons between flow cytometry and rearrangement analysis for MRD detection.[88,152] High concordance was found between both methods. Discrepant results were usually due to low sample cellularity or the presence of PCR inhibitors.[152]

3.1.5 Chimerism analysis

The term chimera originates from the ancient Greek mythology and describes a mixed biological creature with a lion's head, a goat's body and a serpent's tail. According to the medical terminology, a chimera state means a biological organism in which cell populations originating from another individual are living, differentiating and functioning. A chimera state can emerge spontaneously in twins during pregnancy when there is a communication between the blood circulation of the two placentas. An artificial chimera state can be developed by medical interventions, such as transplantation. After SCT, a state of chimerism develops when donor cells in the graft reconstitute the hematological and immunological system.[153] However, in some cases, host cells of hematopoietic origin survive the conditioning treatment and co-exist with donor cells. This state, which is

termed mixed chimerism, may be stable or transient. There are some terms describing the chimeric status after SCT.[154]

- Donor chimerism (full chimerism, complete chimerism) means that all the circulating hematopoietic cell populations are of donor origin.
- Mixed chimerism means that there is a mixture of donor and host cells in PB or BM.
- Split chimerism describes the situation when one cell lineage is of host origin and another cell lineage is of donor origin - e.g., B-cells are host and T-cells are donor. Mixed chimerism and split chimerism can be difficult to distinguish if chimerism analysis is performed using whole blood without prior cell separation.

In chimerism analysis, the relationship between recipient and donor cells is investigated in order to determine whether donor engraftment has occurred and if there are residual recipient cells, which may be responsible for relapse.(Santos 72 Transpl Proc 559-) Per definition, the chimerism analysis is not a MRD method because it does not specifically detect leukemic cells. Residual recipient cells that are detected can either be normal or malignant (or both). One way to overcome this problem is to perform leukemia lineage-specific chimerism analysis.[155-157] In this approach, follow-up sample cells are separated according to the leukemia phenotype found at diagnosis. Thus, in a patient with B-ALL, chimerism analysis is performed in B cells (CD19+ cells) which increases the specificity and sensitivity of the method by reducing the irrelevant background.[158,159]
Different methods have been developed to monitor chimerism. Most of these methods make use of polymorphic markers to differentiate between donor and recipient cells. Early studies relied on techniques such as red blood cell phenotyping, cytogenetics, fluorescence in situ hybridization (FISH) and restriction fragment length polymorphism (RFLP).[154,160] Limitations of these techniques include limited degrees of polymorphism, low sensitivity, and a requirement of a large number of cells.
The most widely used method for chimerism analysis is PCR amplification of short tandem repeats (STR, microsatellites) and variable number of tandem repeats (VNTR, minisatellites). VNTR and STR are repetitive DNA sequences dispersed throughout the genome. The main difference between VNTRs and STRs is the length of the repetitive sequence, 10-70 bp for VNTRs and 2-5 bp for STRs. These DNA sequences show a high degree of polymorphism because the number of repeats can differ from one individual to another. Therefore, PCR amplification of VNTRs or STRs will result in PCR products of different lengths depending on the number of tandem repeats.(Figure 5). Before SCT, the patient and donor pairs are “screened” with a panel of STRs or/and VNTRs to find markers that can differentiate between patient and donor DNA. One or two suitable markers are then used in the follow-up samples to monitor the chimeric status. PCR products are separated and analyzed after gel electrophoresis. While PCR products from VNTRs can be separated using low resolution agarose- and polyacrylamide gels (PAGE), STRs with smaller allele differences are analyzed using high resolution capillary electrophoresis and fluorescence detection.[155,161-163]
New approaches based on RQ-PCR have been developed for chimerism analysis. Because VNTRs and STRs are not suitable markers for RQ-PCR analysis, single nucleotide polymorphisms (SNPs) are used as polymorphic targets. This new approach seems to be more sensitive than VNTR and STR analysis and appears promising for chimerism analysis.[164,165]

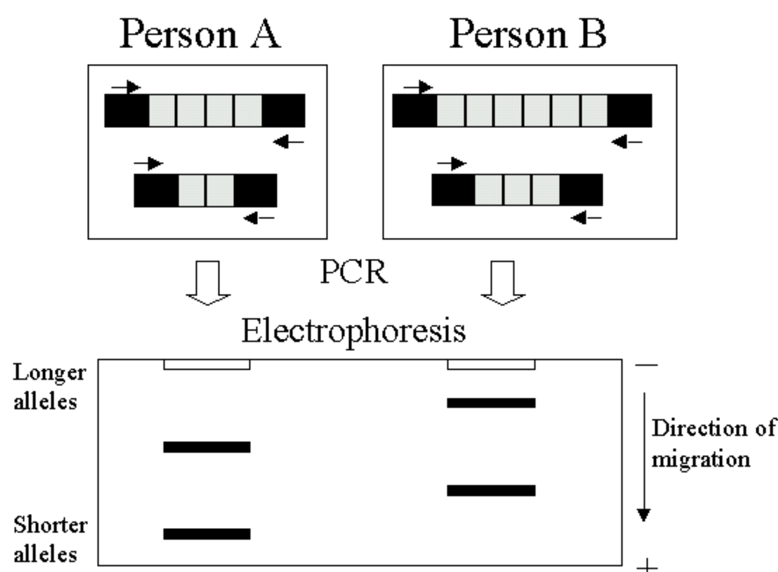

Figure 5. Chimerism analysis using PCR ampliciation of VNTRs. The number of repeats in a given VNTR can differ between one individual to another This will result in PCR products of different length. After SCT, the allelic pattern will reveal the relationship between recipient and donor cells.

Applicability. Using a panel of 5-10 VNTRs/STRs, an informative marker can be detected in >95% of the SCT cases.[166] With five different VNTRs, we were able to find an informative marker in all cases of MUD transplants and ~90% of the sibling transplants. Due to differences in primer sensitivity and other methodological considerations, the frequency of patients analyzed under optimal conditions will be decreased. For instance, when using PCR to amplify VNTR, preferential amplification of small allelic products relative to large allelic products has been reported.[167] Therefore, when choosing an informative VNTR, we preferred the marker that yielded a shorter PCR product in the patient as compared to the donor. Also when using STRs, there are ideal allelic constellations giving more specific and sensitive chimerism quantification.[162]

Sensitivity and quantification. PCR analysis of VNTRs/STRs amplifies both patients and donor DNA, which means that there is a competition for primers and nucleotides in the PCR reaction. Therefore, sensitivity is decreased as compared to PCR analyses where patient/leukemic specific targets are amplified. The sensitivity for detecting the minor population ranges from 1% to 10% but can be increased if cell separation is performed before the PCR analysis. Using this approach, we have increased the sensitivity by more than one log ($4x10^{-4}$).[155] High sensitivity (10^{-4}-10^{-5}) can also be achieved if Y-chromosome specific sequences are used as PCR targets.[168] However, this is only applicable in sex-mismatched transplants, male patients with female donor. Initial studies with RQ-PCR of SNPs report a sensitivity of 10^{-3}-10^{-4} (unseparated cells).[165]
Quantification using VNTRs/STRs is based on the ratio of donor and recipient signals after gel electrophoresis. In STR analysis with capillary electrophoresis and fluorescence detection (ABI Prism 310 genetic analyzer), quantification is performed by calculating different peak areas.[156] Serial dilutions of pretransplant DNA into donor DNA can be performed to construct standard curves. The ratio of donor and patient signals from follow-up samples are then compared to the standard curve for quantification.[169]

Advantages and disadvantages. The main advantage of the chimerism analysis is the high applicability, regardless of the underlying disease. Analysis in different cell population allows the investigation of the engraftment process, which is especially important after nonmyeloablative transplants. Limitations of the method are the low sensitivity and that it is not leukemia specific. As mentioned, these problems can be overcome partly by cell separation. Higher sensitivity may be obtained with RQ-PCR analysis of SNPs.[165]

3.1.6 Other markers

In addition to the most common methods described above, other genetic aberrations in hematological malignancies can be used as MRD markers.
FLT3 (Fms-like tyrosine kinase 3) is a receptor tyrosine kinase important for the normal development of stem cells and the immune system.[170] Increased expression of this gene has been reported in most of the AML and B-ALL cases.[171] Analysis of FLT3 mRNA levels can be used for MRD assessments, although this approach has not been widely used. This may be due to background expression of FLT3 in normal cells.[172] However, a mutation in the FLT3 gene, an internal tandem duplication (ITD), has gained more interest as a MRD target. This mutation involves duplication of an internal sequence, but additional nucleotides are often randomly inserted, resulting in a patient specific target. FLT-ITD is found in 20-30% of the AML cases and is associated with worse outcome.[170] Recent studies suggest that FLT3-ITD may not be stable between diagnosis and relapse and should therefore be used cautiously for MRD detection.[173,174]
Recently, a new translocation, t(5;14), has been identified in T-ALL with an incidence of 20-30%.[104,175,176] As a result of the translocation, the HOX11L2 gene is transcriptionally activated. HOX11L2 is not expressed in normal PB and BM and transcript analysis of this gene with RT-PCR may be used for sensitive MRD detection.
High expression of the PRAME gene (Preferentially expressed antigen of melanoma) has been detected in 40-50% of the leukemia patients.[177]

One of the new targets, which has been most widely used for MRD analysis is Wilms' tumor gene (WT1). WT1 is a tumor suppressor gene coding for a transcription factor and was originally identified for its involvement in the pathogenesis of Wilms' tumor, a childhood kidney neoplasm. High expression of WT1 has been shown at diagnosis in ALL, AML and CML.[178-181] Long-term monitoring of WT1 levels has been used to detect an early relapse and predict the prognosis after chemotherapy or SCT.[182,183] Based on these results, WT1 has been reported as a "panleukemic" MRD marker. However an association between WT1 expression and relapse, has not been found in some studies.[184-186] In a recent study, we showed that there is a high level of background expression of WT1 in the PB of normal individuals.[187] In addition, by comparing the kinetics WT1 with that of BCR-ABL after SCT, we could conclude that an up-regulation of WT1 occurs at the time of relapse, but the level and the time of increase are not sufficient to predict a threatening relapse.

3.2 PERIPHERAL BLOOD OR BONE MARROW?

The use of PB to detect and quantify MRD in leukemia is more practical as compared to the use of BM. BM aspirations are associated with pain and can not be carried out on

regular basis, especially in children. Therefore, it would be beneficial if BM sampling could be replaced by PB sampling. Studies comparing the incidence and level of MRD between PB and BM have been performed for different types of leukemia.
In CML, most studies have reported a high degree of concordance in BCR-ABL levels between PB and BM, suggesting that either PB or BM can be used for MRD analysis.[188,189]
Recently, two large studies analyzed MRD levels in PB and BM in patients with ALL.[190,191] In one study, MRD levels were investigated in B-ALL and T-ALL patients using PCR analysis of Ig and TcR rearrangements.[190] In T-ALL, MRD levels in the paired PB-BM samples were comparable and strongly correlated. However, in B-ALL, the incidence and level of MRD was higher in BM as compared to PB. In 107 PB-BM pairs with detectable MRD, MRD was detected in BM but not PB in 47 pairs. In 48 double-positive pairs, the level of MRD was usually much higher in BM than in the corresponding PB samples (up to 1000 times higher). In the second study, immunophenotype analysis was performed for MRD detection.[191] The difference in MRD distribution between B-ALL and T-ALL was also shown in this study. In addition, it was shown that MRD detection in PB of B-ALL patients was associated with a high risk of relapse. In T-ALL but not in B-ALL, BM sampling might be replaced by PB sampling.
In Ph-positive ALL, BCR-ABL transcript detection in PB and BM seem to be comparable.[120,192] When discordance occurs, MRD levels in BM are usually higher than in PB.[193]
In AML, no large studies have yet, to our knowledge, tried to compare MRD levels in BM with those in PB. AML is a heterogeneous group of diseases and it is possible that MRD distribution in PB and BM will be different in different AML subtypes. In patients with inv(16) and t(8;21), MRD analysis in BM samples seems to be more sensitive than in PB.[129,194,195] However, in patients with t(15;17), PML-RARα transcript levels in PB and BM have shown to be comparable.[196]
After SCT, analysis of BM samples have shown that stromal cells are of host origin while the macrophage component of the adherent layer originate from the donor.[197] Therefore, chimerism analysis of BM samples may lead to false positive results by contamination of recipient derived stromal cells.
Another problem related to BM sampling is nonhomogeneous distribution of leukemia in BM.[198,199] This means that the site of sampling may play a role in the incidence of false negative results and incorrect MRD quantification.

3.3 CLINICAL SIGNIFICANCE OF MRD DETECTION

3.3.1 Acute Lymphoblastic Leukemia

Conventional chemotherapy. Several retrospective and prospective studies indicate that analysis of MRD in ALL has prognostic value, both using immunophenotype and Ig/TcR rearrangement methods. [101,102,141] Low levels or absence of MRD in BM after induction therapy is associated with good outcome. In an extensive review of MRD analysis performed in 856 children with ALL, published between 1994 and 1998, Foroni *et al* showed that approximately 50% of childhood cases were MRD positive at the end of induction therapy and 45% of these patients relapsed.[102] Of the MRD negative cases, the relapse incidence was 7.5%. MRD detection at later time points is also associated with high risk of relapse.[200-204] By combining MRD results from two time-points, different risk-groups of patients can be identified.[204] This approach appears to be superior, in terms of

sensitivity and specificity, for predicting relapse, as compared to single time-point analysis. A MRD threshold level of 10^{-4} seem to differentiate between patients at high risk of relapse and those at lower risk of relapse.[200,201,204]

In adult ALL, the frequency and the level of MRD are significantly higher as compared to childhood ALL.[205,206] Adults respond to treatment more slowly and therefore the MRD status at later time-points have shown to be more predictive for relapse.[203,207] Similar differences are found when comparing T-ALL and B-ALL.[208] The frequency and the level of MRD was found to be higher in T-ALL than in B-ALL, reflecting the greater aggressiveness of T-ALL. However, in contrast to adult ALL, the prognostic value of MRD in T-ALL was high even at early time-points.

SCT.

MRD analysis before SCT. Many ALL patients transplanted in CR still relapse, which indicates the presence of leukemic cells, not detected by standard morphological analysis. Therefore, MRD studies before SCT have been performed in order to identify patients with persistent disease at levels below the remission threshold. Most of these studies showed that patients with persistent MRD before SCT were at higher risk of relapse as compared to MRD negative patients.[209-212] Furthermore, a GVL effect was usually observed in MRD positive patients who remained in CR. GVHD was shown to protect against relapse,[210,211] while T-cell depletion was associated with high risk of relapse in patients with high MRD levels.[209] Based on these results, a multicenter study has been initiated to evaluate the role of pre-SCT MRD in prospective studies by adopting a common protocol for MRD assessment.[213]

MRD analysis after SCT. All studies of MRD after SCT clearly show that MRD negativity is a good predictor of remission in patients with ALL.[82,83,214-216] However, the clinical significance of MRD positive samples is less clear. While most studies have found a strong correlation between MRD positivity and relapse,[83,214-216] regardless of the MRD quantity, some studies report a high frequency of MRD positive patients who do not relapse.[82,217] The median time interval between a positive MRD signal and relapse has varied between 1 month and 5.5 months in different studies.[83,214,216]

MRD analysis in Ph-positive ALL. RT-PCR analysis for BCR-ABL transcripts after SCT has been reported in Ph-positive ALL patients.[120,121,218-220] A strong association between a positive PCR assay and relapse was found in most of the studies although a high proportion of MRD positive patients without relapse has been reported. In a recent study by Stirewalt *et al*, 33 patients showed MRD positivity of which 15 (45%) relapsed.[121] Among 31 patients without MRD, the relapse incidence was 23%. Interestingly, patients with p190 BCR-ABL had an increased risk of relapse compared to those with p210 BCR-ABL.

Induction of GVHD by either DLI or rapid reduction of immunosuppression has been shown to induce molecular remission in ALL patients with residual BCR-ABL transcripts.[81]

3.3.2 Acute Myeloid Leukemia

The lack of widely expressed molecular markers in AML limits the systematic study of MRD by PCR. Therefore, correlative studies between MRD and treatment outcome have been performed only in selected groups of patients.

Conventional chemotherapy. In AML patients with t(15;17) and inv(16), high levels of MRD after consolidation or completion of therapy is associated with an increased risk of relapse.[196,221,222] The significance of detecting MRD in AML t(8;21) patients is less clear because AML1-ETO transcripts can be detected by qualitative RT-PCR in many patients in long-term remission.[223] This may be due to expression of AML1-ETO in normal hematopoietic cells.[224] However, quantitative monitoring of MRD have been shown to identify patients with a high risk of relapse.[129,225] MRD thresholds of predictive value have been identified for all three chromosome aberrations.[226] MRD studies using flow cytometry have also shown to be of clinical value.[227-229]

SCT. After SCT for AML, very few MRD studies have been reported. In addition, most of these studies have usually included a small number of patients. PCR analysis of fusion gene transcripts have been used in some studies, but the clinical significance of MRD detection is still not clear.[194,230,231]
Recently, Ogawa *et al* reported that quantitative analysis of the WT1 gene transcript could be useful for predicting relapse in ALL and AML patient after SCT.[183] However, this has not been confirmed by others.[185]

3.3.3 Chronic Myeloid Leukemia

Interferon-α (IFNα). INFα treatment is an effective cytoreductive therapy in early chronic phase CML and may induce complete hematological remission in 70-80% of the cases.[232] IFNα is the preferred therapy for patients without an available SCT donor. Qualitative RT-PCR is of very limited value in determining response to INFα, because almost all patients remain repeatedly positive.[126] However, by using quantitative PCR analysis, it is possible to identify patients at higher risk of relapse.[233]

SCT. The initial MRD studies after SCT were performed using qualitative “nested” RT-PCR. It was found that BCR-ABL transcripts could be detected in most patients for some months after SCT. Patients who were persistently MRD negative, especially more than 6 months after SCT had a very low risk of relapse.[234] Long persistent MRD could be detected in some patients with increased the risk of relapse.[235,236] A GVL effect in CML was evident by the fact that MRD detection was more common in patients with less severe GVHD and that T-cell depletion was associated with higher incidence of MRD and relapse.[237,238] Using qualitative MRD analysis it was also shown that MRD could be detected several months before relapse although this approach could not predict relapse for individual patients.[239]
With the introduction of quantitative PCR methods, the kinetics of BCR-ABL transcripts could be followed in more detail.[97,127,240] Serial quantitative RT-PCR analysis can distinguish patients who will most probably relapse (high or increasing BCR-ABL levels) from those who will remain in clinical remission (low or decreasing BCR-ABL levels).[241,242] Using ABL as the internal control gene, molecular relapse has been defined as a BCR-ABL/ABL ratio of >0.02% in 3 consecutive samples.[128] DLI treatment at the time of molecular relapse is associated with higher response rates as compared to DLI given at the time of hematological relapse.[77,79]

Imatinib. The tyrosine kinase inhibitor imatinib has been used in clinical trials for only a few years and long term results are still rare.[243] Preliminary results suggest that the use of

imatinib is a considerable improvement over INFα. However, as in the case of INFα treatment, BCR-ABL transcripts are detected in almost all patients after imatinib treatment.[244-246] Whether MRD negativity will be obtained with a longer follow-up remains to be seen.
A high incidence of MRD and relapse is found after imatinib treatment in patients with Ph-positive ALL.[247]

3.3.4 Chimerism results

Chimerism testing is used for routine analysis of engraftment after SCT and has been of great value for this purpose. Successful engraftment is associated with stable complete donor chimerism (DC). Whether chimerism analysis can be a useful tool for predicting relapse has been a matter of debate.[160,248-252] Although some studies have shown an association between detection of mixed chimerism (MC) and relapse,[155,157,161,169,253,254] others have failed to find such a correlation.[255-259] These conflicting results in the literature may be explained by a number of factors.

The time and frequency of sampling are important factors that influence the detection of MC. During the early posttransplant period, most patients will show some degree of MC. Investigating the kinetics of engraftment, Dubovsky *et al* showed that DC was usually achieved by day 28 after SCT.[260] Although, frequent sampling during this early time period may lead to a high incidence of MC without an association with relapse, it may be more valuable at later time points. Serial and quantitative chimerism analysis of samples taken at short intervals after SCT has been useful for prediction of relapse.[169,253]
T-cell MC is usually detected after SCT and can persist for some months after SCT.[261] While no clear association between T-cell MC and relapse have been found in acute leukemia patients, higher incidence of MRD positivity and relapse has been found in CML patients with T-cell MC.[238,255,262] This is probably due to the GVL effect of T-cells, which is stronger in CML than in acute leukemia. Because GVHD and GVL are closely related, we investigated whether T-cell MC was associated with GVHD and relapse.[261] We found that T-cell MC was significantly correlated to a decreased risk of acute GVHD. However, no association between T-cell MC and relapse was detected. A high incidence of MC, especially in T-cells, is found after T-cell depleted transplants and correlates with a higher risk of relapse in CML patients.[238,263]
PCR analysis of VNTRs and STRs yield similar sensitivities, 1-5%. In some studies, PCR analysis of Y-chromosome specific sequences has been performed in sex-mismatched transplants (female to male). This approach increases the sensitivity of the chimerism method by at least two logs, to 10^{-4}-10^{-5}.[168,264,265] Using this methodology, MC can be detected at low levels (10^{-4}), several years after SCT.[266] Whether these recipient cells are long-lived normal hematopoietic cells, malignant cells or contaminating non-hematopoietic cells in the samples is not known. Fehse *et al* showed that the level of MC was higher in BM compared to PB, which may indicate the presence of host-derived cells - e.g., stroma cells, collected during BM sampling.[267] They also showed that complete DC could be achieved after cell sorting.

Most chimerism studies have been performed using DNA samples obtained from whole PB or BM without prior cell separation. This approach has the disadvantage that sensitivity is limited to 1% if VNTRs/STRs are used. In addition, if MC is detected, the

identity of the recipient cells will not be known - i.e., whether detected recipient cells are potential malignant cells or not. Therefore, in recent years, the immunophenotype of the original leukemic clone have been used for FACS or immunomagnetic separation of specific cell populations expected to harbor tumor cells. After cell separation, the sensitivity and specificity of the chimerism analysis for detecting MRD are increased by reducing the irrelevant background of other cell types. This approach has been successfully applied in ALL, AML, and CML patients.[112,155,157,159] In addition, lineage-specific chimerism analysis may be useful in predicting response to DLI, as shown in some studies.[268-270]

Studies reporting the use of adoptive immunotherapy based on chimerism results are rare.[84,271] In one study, Bader *et al* reported 12 patients with increasing MC who received further immunotherapy consisting of either withdrawal of immunosuppression or DLI.[84] Seven of these 12 patients responded to the treatment and remained in continuous CR. At the Center for Allogeneic Stem Cell Transplantation (CAST) at Huddinge University Hospital, adoptive immunotherapy based on chimerism results have started to be a routine procedure, but the results are too preliminary to evaluate.

3.3.5 MRD and chimerism after nonmyeloablative SCT

In leukemia patients, the conditioning treatment given before SCT is meant to eradicate recipient hematopoietic cells, normal and malignant cells. Therefore, less intensive nonmyeloablative conditioning regimens are expected to give higher incidence of MC and MRD after SCT. Indeed, MC has been detected in most patients after nonmyeloablative SCT.[24,154,272-274] In most cases DC is obtained after a transient MC while in other cases further immunotherapy is needed for conversion from MC to DC.[273,275] While MRD data are rare in AML and ALL patients,[276] some studies have focused on chimerism and MRD monitoring in CML patients.[277-280] Following nonmyeloablative SCT (NST), we studied the kinetics of MRD and chimerism in CML patients.[278] These results were compared with those obtained from CML patients receiving conventional SCT (CST). In the early posttransplant period (<3 months), we found a higher incidence of MC and MRD in NST patients compared to CST patients. However, during the first year, most NST patients achieved DC and molecular remission. Similar MRD and chimerism results after NST have been reported by others.[277,279]

3.4 CONCLUSIONS

Leukemia relapse remains an obstacle to successful treatment with chemotherapy and SCT. Many patients who achieve remissions will still relapse, indicating the presence of leukemic cells not detected by morphological analysis. Therefore, different methods have been developed for MRD assessments. The most sensitive and widely used methods are based on PCR technology. These MRD methods depend on analysis of leukemia-specific translocations, antigen receptor rearrangements and chimerism analysis. In CML patients, the most widely used MRD method is RT-PCR analysis of BCR-ABL transcripts that can be applied in >95% of the cases. In ALL patients, analysis of antigen receptor rearrangements is possible in almost all patients. In addition, BCR-ABL transcript analysis can be performed in 5-30% of the ALL cases. AML patients are a heterogeneous group of patients, and there is yet no widely applicable MRD-PCR method for this patient group. Analysis of fusion gene transcripts can be applied in 30-40% of the AML cases.

Chimerism analysis can be performed in most patients after SCT. Although not leukemia specific, the sensitivity and specificity of this method to detect MRD can be enhanced by cell separation.

During the last decade, MRD analysis has become an important tool in the management of leukemia patients. In many studies, MRD detection has been shown to be an independent prognostic factor for patient outcome. However, there is still some controversial whether MRD results should be used in clinical decision-making. Different factors may contribute to the conflicting results found in different studies.

1. Patient population. The patient group under study may have an effect on the clinical outcome in relation to MRD results. For instance, adult patients with ALL respond to treatment more slowly than children and therefore the MRD status at later time-points have shown to be more predictive for relapse in adult ALL. The value of a MRD positive sample can also differ in subgroups of ALL - e.g., Ph-positive ALL.
2. Transplant regimens. The type of conditioning regimen and the type of graft given may affect the incidence of MRD and chimerism after SCT.[258,281,282] In ALL patients who are MRD positive at the time of SCT, T-cell depletion increases the risk of relapse considerably as compared to non-T-cell depleted graft. The use of PB as stem cell source has been associated with lower incidence of MC and MRD as compared to BM.[283,284]
3. Sensitivity of the method used may have a major impact on the predictive value of MRD detection. Using optimized methods, MRD has been detected in the majority of ALL patients in long-term clinical remission.[285] In addition, increased sensitivity may allow detection of fusion gene transcripts (Ph-chromosome and t(8;14)) in normal cells. A sensitivity of at least 10^{-4} is usually recommended for MRD analysis.
4. Qualitative *vs.* Quantitative MRD analysis. In CML patients, a qualitative MRD analysis is of limited value and does not allow identification of individual patients. BCR-ABL transcripts can be detected in virtually all patients after INF-alfa and imatinib treatment and in most patients during the early posttransplant period. Quantitative MRD analysis allows the identification of individual patients at high risk of relapse. In addition, quantitative analysis provides the possibility to find threshold values, which may differentiate between patients at high risk of relapse and those who will most probably remain in CR.
5. Time and frequency of sampling. MRD analysis at a single time point is usually not sufficient to identify patients with poor prognosis. A combined MRD information from different time points after treatment allows kinetic studies of tumor load and appears to be highly informative. In the early time period after SCT (~3 months), MRD can be detected in some ALL patients who will remain in clinical remission. Therefore, MRD positivity during this time period might not be associated with increased risk of relapse. Serial and quantitative MRD analysis is probably the best approach for identifying the majority of those patients who will relapse.

The currently used MRD assays are heterogenous as regards to the markers and techniques used in the analysis. In a routine laboratory, a combination of these methods is needed in order to make MRD analysis available for most patients. This requires skillful personal and different equipment and materials. Despite this, a sensitive MRD target will not be found in all patients. The search for new MRD markers may allow identification of markers that can be used across different leukemia types. In this sense, it has been suggested that WT1 may be a used as a panleukemic MRD marker. However, studies on

WT1 have produced conflicting results and the clinical significance of this marker is still controversial.

The development of cDNA array technology has enabled the study of expression of thousands of genes in a single experiment. This technique has the potential to identify novel markers for MRD analysis. Chen *et al* compared the gene profiles of normal and leukemic cells and found 7 proteins that were increased in B-ALL at higher levels than in normal B-cell progenitors.[286] Further analysis of one of the markers, CD58, showed that it may be useful as a MRD marker in immunophenotype analysis.[226]

The use of cDNA arrays has also produced clinically relevant results in leukemia patients. It has been demonstrated that AML and ALL can be distinguished by gene expression profiles.[287-289] In childhood ALL, it was even possible to correlate molecular aberrations (translocations) with distinct gene expression profiles.[290] It has also been shown that patients with resistance to STI571 can be identified exclusively according to their gene expression profile.[291] The cDNA array methodology may contribute to the identification of new prognostic factors but also potential targets for molecular therapies.

Finally, standardization of MRD protocols is necessary in order to come to a consensus on the significance of MRD detection for each type of disease and treatment. With the introduction of RQ-PCR, this may be easier to achieve than before. In Europe, several protocols have been established to develop common guidelines for MRD analysis. Some of these networks include the Europe Against Cancer Program (RQ-PCR analysis of fusion gene transcripts), the European Study on MRD Detection in ALL, the International Study Group on Standardization of Residual Disease Detection in BCR-ABL positive leukemias, and the European Study Group for MRD analysis in SCT for ALL.[213]

4 AIMS OF THE PRESENT STUDY

The overall aim was to analyze the clinical significance of MRD detection in leukemia patients treated with SCT. Some of the specific aims were:

- To investigate the predictive value of chimerism analysis for relapse after SCT.
- To analyze the clinical significance of MRD detection before and after SCT in patients with ALL.
- To analyze the kinetics of MRD and mixed chimerism in CML patients receiving a nonmyeloablative SCT.
- To evaluate WT1 as a MRD marker.

5 MATERIAL AND METHODS

In this section, different aspects of the methods used will be discussed. Details regarding the methodological protocols will only be described if they are not presented in the papers in which they are used.

5.1 PATIENTS AND TRANSPLANTATION

The local ethics committee at Huddinge University Hospital approved the studies in Papers I-V (DNR 63/96 and 194/01). Patient characteristics in are given in Table 5.

Table 5. Patient and donor characteristics in Papers I-V

Paper	**I**	**II**	**III**	**IV**		**V**
No. of patients	30	30	32	10 (CST)	15 (NST)	32
Females/males	19/11	13/17	13/19	4/6	10/5	17/15
Median age, years	33	13	11	42	51	38
Range	0.6-54	2-53	2-53	31-52	36-63	3-63
Median follow-up, months	30	39	65	25	20	16
Range	16-47	13-119	(37-144)	9-34	6-29	3-43
Diagnosis	AML 22 MDS 6 Others 2	ALL 30	ALL 32	CML 10	CML 15	CML 28 ALL 3 AML 1
Disease stage						
Early/Advanced [1]	9/21	16/14	15/17	10/0	15/0	27/5
Donors						
Females/Males	19/11	12/18	13/19	5/5	6/9	11/21
HLA-identical siblings	10	15	15	2	10	11
HLA-identical related	2					
Matched Unrelated	18	15	17	8	5	21
Conditioning						
Cy+TBI	22	26	29	5		11
Bu/Cy	8	4	3	5		9
Flu+Bu					15	10
Flu+TBI						2
ATG	20	16	18	8	15	27
GVHD prophylaxis						
CsA		2	2		3	
CsA+MTX	30	27	29	9	11	26
CsA+MMF				1	1	5
MTX		1	1			
+ T-cell depletion	3	2	2			1

[1]early = 1 CR/CP; CST = Conventional SCT; NST = Nonmyeloablative SCT

5.2 CHIMERISM ANALYSIS

For chimerism analysis, five different VNTRs were used. Primer sequences and other data concerning these VNTRs are shown in Table 2 (Paper I). Using these VNTRs, we were able to find an informative marker in all MUD transplants and ~90% of the sibling transplants. If several markers were informative, we usually selected the one that gave a shorter allele in the patient as compared to the donor. This is due to preferential PCR amplification of small allelic products relative to large ones.

DNA samples from donor and patient, taken before SCT, were used as markers and analyzed together with the post-SCT samples (Figure 6). Cell separation was performed in all samples taken after SCT using immunomagnetic beads. Cell separation of CD19+ and CD3+ cells was performed first and thereafter separation was performed according to the leukemia immunophenotype of each patient. The different cell fractions were subjected to cell lysis and chimerism analysis was performed directly on the cell lysate without prior DNA extraction because of the risk of loosing DNA material from the few patients cells present in the sample. Cell lysate DNA could be safely reanalyzed 4-5 times, including freezing-thawing in-between subsequent analyses. After 10-12 repeated freeze-thawings of the same lysate sample, fragmentation of template DNA was observed.

PCR amplified products were run on a ready-to-use polyacrylamide gel (PAGE) system (Pharmacia Biotech, Uppsala, Sweden) and analyzed after an automated silver staining procedure. This automated system gave high reproducibility. We used a semiquantitative estimation of mixed chimerism where recipient-band intensity and donor-band intensity were compared to a serial 10-step (nonmyeloblative SCT, 10%, 20%…100%) or 4-step (myeloablative SCT, 1%, 5%, 20% 50%) dilution assay by mixing patient and donor DNA.

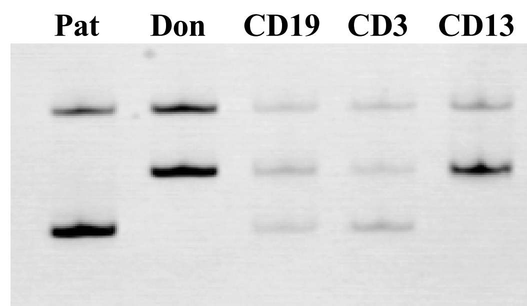

Figure 6. Chimerism analysis. Patient and Donor DNA samples were screened before SCT to find an informative marker. After SCT cell separation has been performed with immunomagnetic beads for CD19+, CD3+ and CD13+ cells. This patient show mixed chimerism in the CD19+ and CD3+ cell fractions but the CD13+ cell fraction is complete donor chimeric.

5.3 ANTIGEN RECEPTOR REARRANGEMENT ANALYSIS

PCR amplification of IgH genes in ALL patients was done using a degenerate primer complementary to framework three (FR3) of the variable (V_H) gene-segments (5'-ACA CGG CTG TGT ATT ACT GT-3'), together with a consensus joining $(J_H)_{1-6}$ gene-segment-primer (5'-AAC TGC AGA GGA GAC GGT GAC C-3'). Primers for other Ig and TcR genes were designed according to Pongers-Willemse *et al.*[133] DNA was extracted from leukemia cells obtained at diagnosis and screened for rearrangement targets. PCR products were electrophoresed and single bands were excised from preparative 2% GTG agarose gels. Excised bands were purified using the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany), according to the manufacturer's guidelines. Purified PCR products were ligated into TA vectors and subsequently transformed into competent cells as described in the pGEM-T Easy Vector System 1 protocol (Promega, Madison, WI, USA). Plasmids from 10 independent clones were purified using the Plasmid Mini Kit (QIAGEN, Hilden, Germany), and unidirectionally sequenced with a T7 vector-specific primer, using the ABI prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit

(Perkin Elmer, Branchburg, CA, USA). Sequences were analyzed on a 373A DNA sequencer (Applied Biosystems, Foster City, CA, USA). The predominant nucleotide sequence derived from the various plasmid clones of each patient was studied. In most cases, all 10 clones displayed identical sequences. The sequence from the N-regions of rearranged VDJ genes was used to accommodate the 3'-end of the patient-specific primer used for each patient. Patient-specific primers were then used in combination with one of the original primers used to identify leukemic DNA. PCR-amplified specific products of 80-120 bp size were analyzed on the same PAGE system as described for VNTR analysis. Quantification was performed by parallel amplification of 1 μg of sample DNA with a 10-fold serial dilution of leukemic cell DNA in mononuclear cell DNA from 5 healthy donors (Figure 7). All samples were amplified in duplicate together with negative controls.
Most of the DNA material used in the rearrangement analysis was extracted from archival BM slides by a salting-out procedure as described in Paper III. The simple and rapid extraction method we used in this study usually gave DNA of high quality. Purity and concentration was estimated from the optical density ratio (OD260/OD280) and DNA quality was analyzed by successful PCR amplification with a VNTR marker.

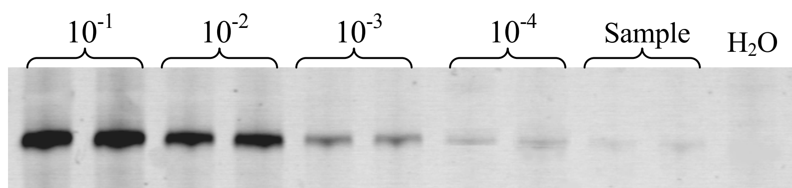

Figure 7. PCR analysis IgH gene rearrangement with patient-specific primers. Semiquantitative analysis with a 10-fold dilution series of leukemic DNA. The intensity of the sample bands are compared to the dilution series for quantification.

5.4 COMPETITIVE PCR FOR BCR-ABL

Before the introduction of RQ-PCR, quantitative analysis of BCR-ABL transcripts was performed by a competitive PCR approach (Paper IV). This method was adopted from Cross *et al*,[97] and the plasmids containing the competitor genes were kindly provided by the same group at Hammersmith Hospital, London.
The methodology of RNA preparation and reverse transcription (RT) is described in Paper V. However, details about the competitive PCR technique are not included in Paper IV due to a major revision and are therefore described below.
Qualitative PCR. 5 μl of cDNA (total volume of 50 μl) was used in a 25 μl PCR-reaction containing 1x PCR buffer (10mM Tris-HCl pH 8.3, 50mM KCl, 1.5mM $MgCl_2$, 0.001% gelatin), 200 μM of each dNTP (Applied Biosystems, Roche, Branchburg, NJ, USA), 5% glycerol (Sigma, St.Louis, MO, USA), 100 ng/μl cresol red (Sigma), 0.03 units/μl AmpliTaq polymerase (Applied Biosystems) and 0.5 μM of each primer. The primers amplified both b2a2 and b3a2 variants of the p210 fusion product. Primer sequences were B2A: 5'-TTC AGA AGC TTC TCC TGA CAT-3' and CA3-: 5'-TGT TGA CTG GCG TGA TGT AGT TGC TTG G-3'. Patients with the p190 fusion product was analyzed with the primers E1N+: 5'-AGA TCT GGC CCA ACG ATG ACG A-3' and CA3- (see above). After an initial 4 min hot-start/denaturation step at 94 ^{0}C, 40 PCR amplification cycles were carried out in a PTC-200 thermal cycler (MJ Research, Watertown, CA, USA). The first 10 cycles were done in a two-segment step at 94 ^{0}C for 30 s and at 61 ^{0}C for 1 min.

The following 30 cycles were done in a three-segment step at 94 ^{0}C for 15 s, 59 ^{0}C for 50 s and 72 ^{0}C for 30 s. The primers used to detect ABL transcripts were A4-: 5'-CGG CTC TCG GAG GAG ACG ATG A-3' and A2N: 5'- CCC AAC CTT TTC GTT GCA CTG T-3'. PCR conditions for ABL were 94 ^{0}C for 4 minutes followed by 35 PCR amplification cycles. The first 10 cycles were done in a two-segment step at 94 ^{0}C for 30 s and at 66 ^{0}C for 1 min. The following 25 cycles were done in a three-segment step at 94 ^{0}C for 15 s, 63 ^{0}C for 50 s and 72 ^{0}C for 30 s.

Five µl of the PCR products was run in a ready-to-use PAGE system as described above.

Competitive PCR. Quantification was done by a competitive PCR using plasmid constructs containing a modified BCR-ABL fusion gene that produces larger PCR products of ABL and BCR-ABL than the wild-type transcripts (Figure 8).

PCR reactions were performed as described above, except that 2.5 µl of cDNA and 2.5 µl of competitor were added to each reaction. Dilutions of competitor plasmids were done every half order of magnitude ranging from 10 to 10^7 copies per 2.5 µl. BCR-ABL and ABL transcript numbers were estimated by comparing the competitor and sample band intensity to find the equivalence point. Results were expressed as the ratio between BCR-ABL and ABL transcript numbers (BCR-ABL/ABL).

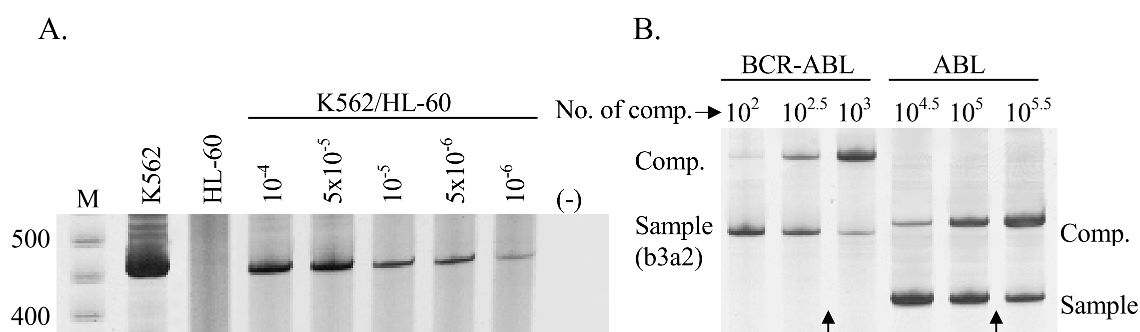

Figure 8. Sensitivity and methodology of the quantitative RT-PCR technique. **A.** RNA from K562 cells (b3a2) was serially diluted in RNA from HL-60 cells in a total amount of 20 µg RNA. After cDNA synthesis and 40 cycles of PCR amplification, PCR products were visualized after a silver staining procedure. M: 50bp marker, (-): Negative control included at the RNA extraction step. **B.** Samples were co-amplified with different amounts of the competitor plasmid (comp.) to estimate the number of BCR-ABL and ABL transcripts in the sample. Arrows indicate the equivalence point in band strength between sample and competitor bands. Thus, the BCR-ABL/ABL ratio is $(10^{2.6}x1.22)/(10^{5.2}x1.26)=0.002$ (0.2%). Multiplication by 1.22 and 1.26 is done to compensate for size differences between the competitor and sample PCR products.

ABL transcript levels were quantified also in the BCR-ABL negative samples to ensure that the absence of BCR-ABL was not due to poor sample quality. ABL levels of $>10^{4.5}$ copies was considered to indicate good sample quality in BCR-ABL negative samples. In most cases, PB or BM samples were processed on the same day of or the day after sampling. Samples, which consisted of 5-10 ml of PB and 1-3 ml of BM, were usually sufficient except during the early posttransplant period when the cell yield was sometimes lower than desired.

5.5 REALTIME PCR

In Paper V, we used RQ-PCR to compare the kinetics of BCR-ABL with that of WT1 after SCT. Primers and protocols are described in this paper. The method was based on Taqman probe technology and performed on the ABI 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). FAM and TAMRA were used as reporter and quencher fluorochromes, respectively. Primers and probes were designed using the Primer Express software (Applied Biosystems) and the length of the PCR products were 80-100 bp. This method has worked very well without any major optimization efforts (Figure 2).

When the RQ-PCR method was optimized, we wanted to see whether the BCR-ABL/ABL ratios derived from this method were comparable with those obtained by the competitive PCR approach.[240,292] As shown in Figure 9A, there is a high degree of correlation between both methods. Some samples that were tested BCR-ABL negative by one method were found to be positive by the other method. This comparison is complicated by the fact that samples were not analyzed by both methods simultaneously. cDNA samples analyzed with the competitive PCR methods had in some cases been stored in the freezer for 2 years before they were analyzed by the RQ-PCR method.

In addition to the internal control gene ABL, we also quantified the level of G6PD transcripts by RQ-PCR. In 241 samples, the ABL and G6PD levels correlated with $r = 0.55$, $p<0.0001$ (Figure 9B). G6PD levels were a median of 8.2 times higher than ABL levels. This difference is higher than found by Emig *et al*, who reported a median G6PD/ABL ratio of 1.5.[240] The discrepancy between both studies might be due to differences in primer and/or probe efficiencies in one or more targets.

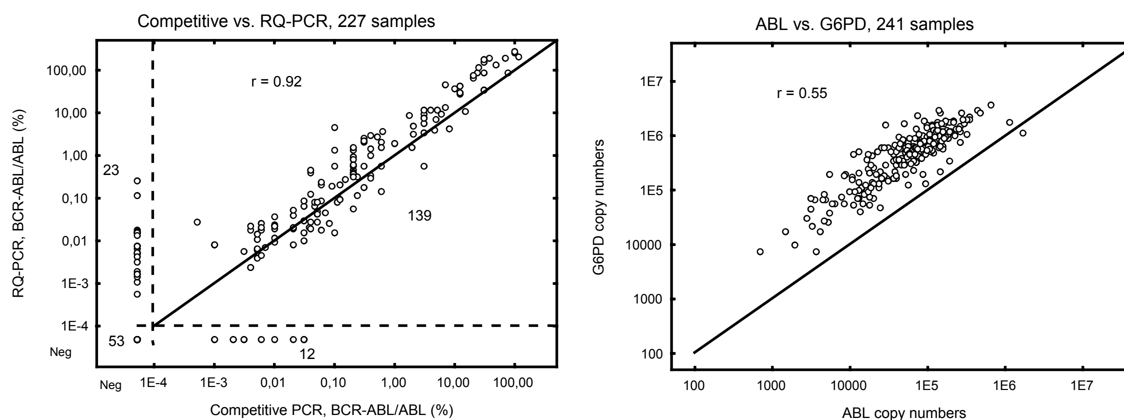

Figure 9. A. Comparison between BCR-ABL/ABL ratios obtained from competitive PCR and RQ-PCR. Broken lines indicate the threshold for MRD negative samples. Figures show the number of samples. **B.** Correlation between the internal control genes ABL and G6PD. Copy numbers were estimated from standard curves of each gene, generated during RQ-PCR.

6 RESULTS AND DISCUSSION

6.1 CHIMERISM ANALYSIS AFTER CELL SEPARATION (PAPER I)

In this paper, we prospectively analyzed the clinical effect of MC detection in PB and BM of 30 patients with AML and MDS after SCT. In all patients, cell separation, according to the leukemia phenotype expressed at diagnosis or relapse before SCT, was performed on samples after SCT. Twelve patients relapsed after SCT. Mixed chimerism in the leukemia-affected cell lineage was detected in 14 patients, of whom 10 relapsed compared to 2 of 16 DC patients ($p<0.01$). The four patients with MC and continuous complete remission showed only MC in BM. All eight patients with MC detected in PB relapsed compared to 4 of 22 DC patients ($p<0.001$). In this study, MC was detected median 66 (23-332) days before hematological relapse. No correlation was found between MC in CD3+ and CD19+ separated cells and relapse. At the time of reappearance or continuous MC in patients who later relapsed, all patients were considered to be in CR, according to morphological examination. This shows the low sensitivity of the latter method for predicting relapse in patients with AML and MDS after SCT.
Several studies have now shown that chimerism analysis in different leukemia-affected cell lineage is a sensitive method, which identifies patients at risk of a threatening relapse several months before the clinical relapse is verified morphologically.[156,157,159] We believe that chimerism analysis, taking samples at short intervals after SCT combined with accepted MRD methods, will provide the tools necessary for treatment with adoptive immunotherapy at an earlier time after SCT than today.

6.2 MRD IN ACUTE LYMPHOBLASTIC LEUKEMIA (PAPERS II & III)

In Paper II, we retrospectively analyzed MRD in 30 patients with ALL.The aim was to determine whether the level of MRD before SCT was correlated with outcome after SCT. For MRD detection, the junctional regions of Ig and TcR gene rearrangements were amplified and sequenced, and patient-specific primers were constructed for each patient. Quantification was performed by parallel amplification of pre-SCT DNA with a 10-fold serial dilution of leukemic cell DNA in normal DNA. Fifteen patients had high-level MRD (10^{-2}-10^{-3}), 10 low-level MRD (10^{-4}-10^{-5}) and 5 were MRD negative. The number of relapses in the three groups were 8 (53%), 5 (50%), and 0, respectively. Among patients with both acute and chronic GVHD, only 2 of 15 relapsed compared with 11 of 15 in patients without or only acute and chronic GVHD ($p< 0.003$).
Previous to our study, Knechtli *et al* had reported a relapse incidence of 100% in patients with high-level MRD, about 50% in those with low-level MRD and about 20% in MRD negative patiens.[209] The higher relapse incidence found among patients with high level MRD in their study may be because most of their patients received a T-cell depleted graft. GVHD, which is a T-cell mediated disease, was shown to protect against relapse in our study. In a study by Bader *et al*, it was also shown that not all patients with high level MRD before SCT had a relapse after SCT.[211] This was probably due to a GVL effect.[220]

In paper III, MRD was analyzed after SCT in 32 patients with ALL. MRD detection and quantification were performed in the same way as in Paper II. Twenty-seven patients from Paper II were also included in this study. MRD after SCT was detected in 9 patients of which 8 relapsed as compared to 6 relapses among 23 MRD negative patients ($p< 0.01$). In

the 8 patients with positive MRD results before relapse, the median time between first MRD detection and relapse was 5.5 (range 0.5-30) months.
In this study, MRD detection after SCT was highly correlated with relapse, 8 of 9 MRD positive patients relapsed. While most studies have found a strong correlation between MRD positivity and relapse,[83,214,215] regardless of the MRD quantity, some studies report a high frequency of MRD positive patients who do not relapse.[82,120,121,217] Some explanations to this difference may be differences in patient population, transplant regimens, and sensitivity of the MRD method applied. In 6 patients, no MRD was detected prior to relapse. Some possible explanations to the false negative results were lower sensitivity of the patient-specific primers (10^{-3} in 2 patients), CNS relapse (one patient), only blood samples available for one patient and clonal exchange (one patient). Some of the approaches to increase the predictive role of MRD results for relapse in ALL patients after SCT include quantitative and sensitive MRD analysis using at least two Ig/TcR rearrangement targets. By frequent sampling, patients at high risk of relapse can be identified and immunotherapeutic interventions can be started at an early stage when the tumor burden is still low. The median time interval between a positive MRD signal and relapse has varied between 1 month and 5.5 months in different studies.[83,214,216] This time interval may be sufficient in some cases to eradicate residual disease by the use of antileukemic interventions.[82,83]

6.3 NONMYELOABLATIVE SCT *VS.* MYELOABLATIVE SCT (PAPER IV)

In Paper IV, the kinetics of MRD and chimerism were studied in 15 patients with CML after nonmyeloablative SCT (NST) and in 10 patients after conventional SCT (CST). Chimerism analysis was performed in different cell populations and quantitative, competitive PCR was performed for BCR-ABL transcripts. All 15 NST patients showed T-cell mixed chimerism (MC) as compared to 5 of 10 CST patients. Granulocyte and B-cell MC was also more frequently detected in the NST patients. All NST patients also showed MRD positivity after SCT. The BCR-ABL/ABL ratio during the first 3 months was with a median of 0.2% in the NST patients signifcantly higher than 0.01% in CST patients. Eleven NST patients became MRD negative after a median time of 3.5 (range 1-7) months. MRD and chimerism kinetics are shown in Figure 10.
A high incidence of MC after NST has been detected in many studies.[24,154,272,273] High incidences of MC and MRD in CML patients have also been reported by others.[277,279] In the study by Kreuzer *et al*, 10 of 14 patients achieved a molecular remission after a median time of 9 (range 3-22) weeks.[279] This is in line with our findings. High molecular remission rates in CML has also been reported by Or *et al*, although details concerning chimerism and MRD data were not presented.[280]

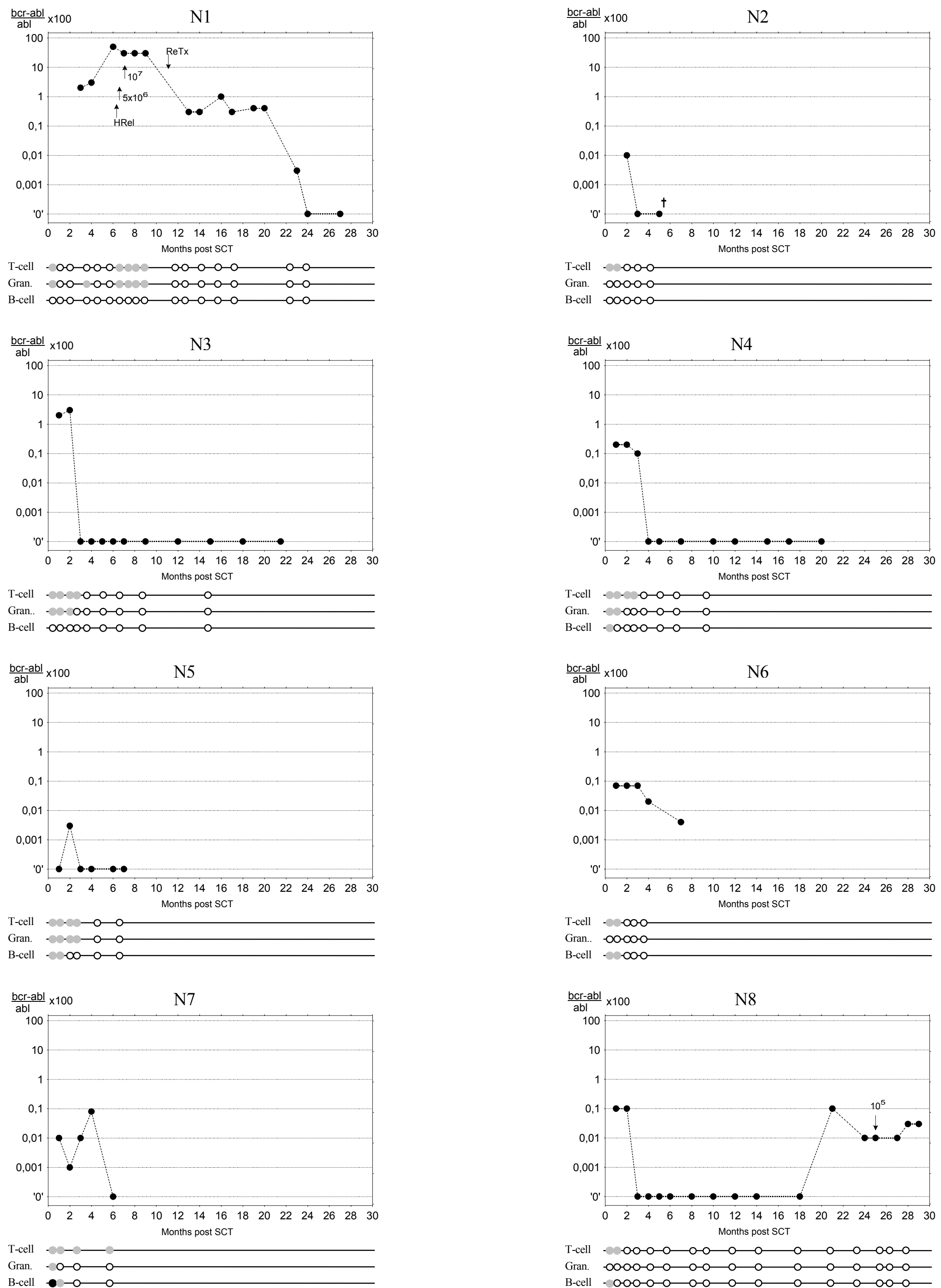

Figure 10. Kinetics of MRD and chimerism over time after allogeneic SCT. Results are shown for all 15 patients with non-myeloablative conditioning (N1-N15) and 2 patients with conventional conditioning (C9 and C10). Chimerism results in different cell populations are shown by circles (black circle: mixed chimerism with more than 50% recipient cells, grey circle: mixed chimerism with less than 50% recipient cells, open circle: donor chimerism). Arrows indicate the time of donor lymphocyte infusions (T-cells/kg), a second transplantation (ReTx) or Hematological relapse (HRel). Gran.: granulocytes.

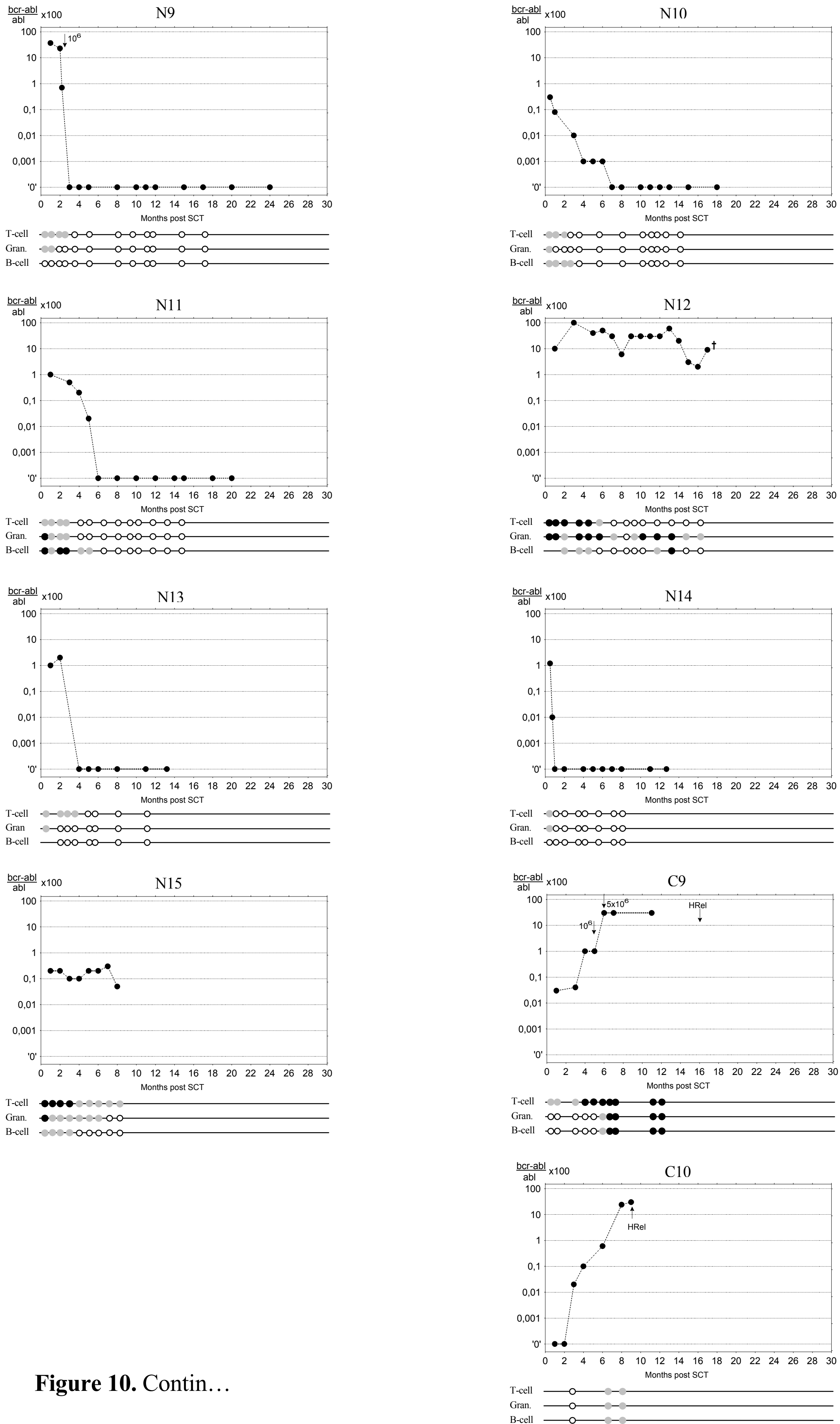

Figure 10. Contin…

6.4 WT1 AS A MRD MARKER? (PAPER V)

In Paper V, the value of WT1 as a MRD marker was evaluated by comparing the kinetics of WT1 levels with that of BCR-ABL. In 32 Ph-positive patients (28 CML, 3 ALL, 1 AML), RQ-PCR was applied to monitor the kinetics of WT1 and BCR-ABL after SCT. A background expression of WT1 was detected in PB of 13 healthy controls and in BCR-ABL negative samples (n=48). Kinetic studies of WT1 showed that an increase of WT1 above the background level was usually detected at the time of relapse. However an increase of WT1 before relapse was only detected in 2 of 6 patients with relapse. Furthermore, the highest WT1 values found at the time of relapse were about two logs higher than the background level, which indicates a sensitivity of only 10^{-2}. Thus, the sensitivity and ability of WT1 to predict a relapse was poor in this study.
Studies on WT1 as a MRD marker have produced conflicting results. While many studies have reported an association between WT1 expression and relapse,[179,180,182,183,293] others have failed to find such a correlation.[184-186] These differences can partly be explained by differences in sensitivities and the use of qualititative *vs.* quantitative analysis. WT1 expression in the PB of normal healthy controls have previously been reported in only a few studies, and usually in a fraction of the controls.[178,293,294] However, we could detect WT1 expression in all 13 controls and all MRD negative samples. According to our data, a qualitative analysis of WT1 is of limited value. A quantitative analysis may be useful in some patients. However, in most cases, the level and the time of increase are not sufficient for predicting a relapse.

7 CONCLUSIONS

- Leukemia lineage-specific chimerism analysis is a sensitive predictor of relapse in patients with AML.

- MRD detection prior to SCT is associated with high risk of relapse in patients with ALL.

- MRD detection after SCT is associated with high risk of relapse in patients with ALL.

- MRD positive ALL patients may benefit from the GVL effect of GVHD.

- Higher incidences of MRD and MC are detected early after transplant in CML patients receiving a nonmyeloablative conditioning as compared to those receiving a conventional coditioning.

- High rates of molecular remissions can be achieved after nonmyeloablative SCT in patients with CML.

- WT1 transcript analysis is of limited value for predicting relapse in BCR-ABL positive patients.

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10 SAMMANFATTNING PÅ SVENSKA

Benmärgen bildar mängder av vita blodkroppar som bygger upp vårt immunförsvar, röda blodkroppar som transporterar syre i kroppen samt blodplättar som hjälper blodet att koagulera. På ytan av en människas celler finns det vävnadsmarkörer (proteiner) som kan variera från en individ till en annan, dessa kallas för MHC. Man kan säga att varje individ har sin specifika "namnteckning" på ytan av cellerna. Dessa molekyler kan exempelvis presentera delar av ett virus eller en bakterie som sedan andra celler i immunförsvaret kan reagera på. Det är även dessa molekyler som gör att ett organ, exempelvis njure eller benmärg, från en annan individ uppfattas som främmande. Cancer kan uppstå i de vita blodkropparna och kallas då för leukemi eller på svenska blodcancer (från grekiskan, *leukos* "vit" och *haima* "blod"). Leukemin yttrar sig med en ohämmad celltillväxt av cancercellerna vilket leder till blodbrist och nedsatt immunförsvar med svåra infektioner som följd. Leukemierna definieras som antingen akuta eller kroniska, där de kroniska har ett långsammare förlopp. I huvudsak är det tre olika patientgrupper som har varit föremål för den här avhandlingen; Akut Lymfatisk Leukemi (ALL), Akut Myeloisk Leukemi (AML) och Kronisk Myeloisk Leukemi (KML). Standardbehandling av leukemi utgörs av cellgiftsbehandling. De patienter som inte svarar på denna behandling alternativt har s.k. högriskkriterier vid tidpunkten för diagnos är kandidater för benmärgstransplantation (BMT). Benmärgstransplantation kallas idag även hematopoetisk stamcellstransplantation (SCT). Benmärgstransplantation är sedan flera decennier en accepterad behandlingsmetod för patienter med leukemi, blod- och immunbristsjukdomar samt vissa mer ovanliga enzymbristsjukdomar. Vid benmärgstransplantation kan antingen benmärg användas från patienten själv (autolog transplantation) eller från någon annan individ (allogen transplantation). Andra individer som donerar benmärg utgörs antingen av syskon eller obesläktade frivilliga givare som passar vävnadstypmässigt (dvs. MHC överensstämmer) med patienten. I första hand försöker man hitta ett syskon som passar. Endast var tredje patient har dock tillgång till ett syskon som passar vävnadstypmässigt. I de fall där det inte finns något syskon som passar försöker vi istället hitta en lämplig obesläktad givare. Idag finns det mer än 8 miljoner frivilliga givare i olika register i världen som kan fungera som donatorer av benmärg, om MHC passar mellan givaren och patienten. Benmärgscellerna tas antingen ut från höftbenet med hjälp av speciella nålar eller renas från blodet, efter det att givaren har stimulerats med en tillväxtfaktor i ett par dagar.

Innan själva transplantationen behandlas alla patienter med cellgiftsbehandling och/eller strålning, där syftet är att slå ut patientens benmärg för att sedan ersätta den med en ny, frisk benmärg. I många år har man ansett detta vara grunden till framgången med BMT. Sedan många år är det dock bevisat att den nya benmärgen, dvs det nya immunförsvaret, själv har en kraftfull effekt mot leukemin. Detta beror på att det finns skillnader mellan patienten och givaren trots att MHC är lika. Detta gör att det nya immunförsvaret uppfattar patienten som "främmande" vilket framkallar en immunologisk attack. De celler som attackeras först är kvarvarande blodceller från patienten. I och med att även leukemiceller har patientens "namnteckning" på sin yta, uppfattas dessa som "främmande" och kommer därför att dödas. Den här transplantat-kontra-värd reaktionen kan även drabba kroppens övriga celler. Om reaktionen blir alltför kraftfull kan den bli livshotande. Benmärgstransplantation med benmärg från en annan individ utgör därför en svår balansgång, där vi eftersträvar en viss reaktion från givarens celler mot patienten men samtidigt får denna reaktion absolut inte bli för kraftfull. Efter BMT får alla patienter

immunförvarshämmande läkemedel som syftar till att minska risken för en svår transplantat-kontra-värd reaktion. Alla patienter riskerar efter BMT att drabbas av infektioner pga. avsaknaden av vita blodkroppar, innan den nya benmärgen börjar fungera (drygt två veckor efter BMT). Det tar dock tid för det nya immunförsvaret att mogna ut, varför många patienter har en ökad infektionsrisk i flera år efter behandlingen.
De största komplikationerna efter BMT utgörs av: återfall av grundsjukdomen, svår transplantat-kontra-värd reaktion samt infektioner.
Hos patienter med leukemi utgör återfall av leukemin det största hotet, trots den intensiva förbehandlingen och den nya benmärgens anti-leukemi effekt. Det är ett välkänt faktum att resultaten vid behandling av leukemier och annan typ av cancer är mycket mer framgångsrik om behandlingen sätts in tidigt, innan cancercellerna har blivit alltför många. Från studier på djur samt människa har man uppskattat antalet leukemiceller vid diagnos alternativt återfall till 1000 miljarder totalt i kroppen. Dagens rutinmässiga metoder som används för att avgöra om en patient har kvar leukemiceller eller ej, har en känslighet på 10^{-2} (1%). Detta innebär att en patient som sägs vara fri från leukemi, trots detta kan ha 10 miljarder leukemiceller i sin kropp! Det är med andra ord inte konstigt att patienterna riskerar att få sin sjukdom tillbaka trots att testerna har visat på "grönt ljus". Benämningen MRD (Minimal Residual Disease) som använts mycket i denna avhandling, syftar till att upptäcka leukemiceller under den analysnivå som dagens rutinmässiga metoder är begränsade till, dvs 1-5%. Det finns olika metoder för MRD analyser.

Den här avhandlingen har handlat om att etablera och utvärdera känsliga DNA-tekniker, för att tidigare än idag kunna identifiera vilka patienter som löper risk att få sin sjukdom tillbaka. I så fall kan behandling insättas tidigare med exempelvis vita blodkroppar från den som donerade benmärg inför själva transplantationen. Detta har visat sig vara en mycket effektiv och framgångsrik behandling hos patienter med kronisk leukemi. Det är också visat att denna behandling är mycket mer effektiv om den sätts in på ett tidigare stadium när leukemicellerna är färre.
För detta syfte har vi utvärderat olika metoder. Dessa metoder bygger på användningen av en s.k. PCR-teknik (från engelskans Polymerase Chain Reaction). PCR går ut på att kopiera upp en känd DNA sekvens som sedan kan analyseras på olika sätt. Med denna metod kan man hitta en leukemisk cell bland 1 miljon vanliga celler.
1. Chimärismmetoden. Ordet chimärism, som har sitt ursprung i den grekiska mytologin, har inom medicinen använts för att beskriva tillstånd där cellpopulationer från olika individer förekommer samtidigt hos en individ, exempelvis efter en transplantation. Vi har studerat anslaget av donatorsceller samt förekomst av eventuellt kvarvarande patientceller (blandat chimärism) efter BMT. PCR-tekniken har vi använt för att amplifiera s.k. minisatelliter. De sistnämnda finns hos alla människor i DNA:t men skiljer sig i *storlek* mellan olika individer. Dessa kan därför användas för att separera olika individer åt. Denna teknik har visat sig mycket värdefull för att förutsäga avstötning av den nya benmärgen. Om tekniken även är användbar för att tidigt upptäcka leukemi återfall har varit omdiskuterat. Fördelen med denna metod är att den i stort sätt kan användas för alla patienter. Chimärismtekniken används nu rutinmässigt för att följa upp patienter efter BMT.
2. Rearrangemangtekniken. Den patientspecifika leukemi tekniken bygger på det faktum att leukemi uppstår i *en* cell som sedan ger upphov till en oerhörd mängd kopior. De *lymfatiska* vita blodkropparna, som förenklat framförallt skyddar oss mot virusinfektioner, har på sin yta receptorer som exempelvis kan reagera på delar av ett virus. Varje enskild

lymfatisk cell har sin specifika receptor. Genom att med DNA teknik studera "DNA sekvensen" för denna receptor, kan denna sekvens användas som en "namnteckning" för patientens leukemi. Denna sekvens kommer att vara densamma för alla leukemiceller hos patienten. Eftersom denna metod är svår och tidsödande har den ännu inte satts i kliniskt bruk. Den är begränsad till ALL patienter.

3. Leukemispecifika tekniken. Cancer uppkommer oftast pga att det sker mutationer (ändringar) i vårt DNA. Eftersom dessa mutationer bara finns i leukemiceller kan de användas som markörer (namnteckningar) för att hitta kvarvarande leukemiceller efter transplantation. Den mutation som vi analyserat involverar två kromosomer, nr 9 och 22. Genom att ett DNA utbyte sker mellan dessa kromosomer så bildas det en ny gen, BCR-ABL genen. Med PCR tekniken har vi letat efter denna mutation som bevis på att det finns kvarvarande leukemiceller i blodet. Även denna metod har satts i kliniskt bruk. Metoden används främst hos KML patienter.

I delarbete I studerade vi betydelsen av chimärismtekniken hos patienter med AML efter BMT. Tekniken förutsåg återfall av leukemi flera månader innan dessa patienter fick ett återfall. Från dessa studier har vi dragit slutsatsen att chimärism metoden kan användas för att tidigt upptäcka patienter som löper risk att få sin leukemi tillbaka. Vi har nu börjat behandla patienterna på grundval av chimärism resultaten.

I delarbete II och III studerade vi patienter med ALL. Med rearrangemang tekniken letade vi efter leukemiceller precis innan BMT och vid flera tidpunkter efter BMT. Patienter som uppvisade spår av leukemiceller både innan och efter BMT hade ökad risk för att få ett återfall. Däremot såg vi att patienter som hade tecken på transplantat-kontra-värd reaktionen kunde klara sig undan ett återfall. Även här kunde vi förutsäga ett återfall flera månader innan det inträffade.

I delarbete IV studerade vi KML patienter som fått betydligt mindre doser cellgift innan BMT. Tanken är att det nya immunförvaret ska göra jobbet och bekämpa grundsjukdomen. Dessutom möjliggör denna behandling att fler och äldre patienter kan komma ifråga för BMT, i och med att förbehandlingen är betydligt lägre och därmed även risken för toxiska skador. Vi har använt oss av både chimärismtekniken och leukemispecifika tekniken för att följa patienter som fått en dennna nya behandling. Dessa resultat jämfördes med de som erhölls från patienter som hade fått en vanlig kraftfull förbehandling. Som väntat hade patienter med lägre förbehandling betydligt mer kvarvarande leukemiceller efter BMT. Däremot försvann leukemicellerna hos de flesta patienter, vilket tyder på att den nya behandlingen är användbar hos KML patienter.

I det sista delarbetet ville vi utvärdera en ny markör som heter WT1. De metoder som används idag för att hitta hitta leukemiceller är antingen för okänsliga eller så är användningen begränsad till specika patientgrupper. På senare år har flera studier visat att WT1 är en universal markör som kan användas för att leta efter leukemiceller hos alla patientgrupper. När vi studerade denna gen och jämförde med BCR-ABL genen (som är en pålitlig leukemispecifik markör) kunde vi konstatera att WT1 är inte tillräckligt känslig för att förutsäga ett återfall. Dessutom såg vi att genen även fanns i friska celler.

Sammantaget visar dessa studier att de metoder vi har satt upp är viktiga för att följa upp patienter efter BMT. Metoderna möjliggör att leukemiåterfall tidigare kan förutsägas och därmed att tidigare behandling kan sättas in än idag. Förhoppningsvis kommer detta leda till mindre återfall av leukemi efter BMT i framtiden.

11 PAPERS