Minimal Residual Disease Assessment in Childhood Acute Lymphoblastic Leukemia.

INGRID THÖRN
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Abstract

Traditionally, response to treatment in hematological malignancies is evaluated by light microscopy of bone marrow (BM) smears, but due to more effective therapies more sensitive methods are needed. Today, detection of minimal residual disease (MRD) using immunological and molecular techniques can be 100 times more sensitive than morphology. The main aim of this thesis was to compare and evaluate three currently available MRD methods in childhood acute lymphoblastic leukemia (ALL): (i) real-time quantitative PCR (RQ-PCR) of rearranged antigen receptor genes, (ii) multicolor flow cytometry (FCM) of leukemia-associated immunophenotypes and (iii) real-time quantitative PCR of fusion gene transcripts (RT-PCR).

In paper I, we assessed the applicability of RQ-PCR in a population-based cohort of childhood ALL diagnosed in Sweden between 2002-2006. Clonal IG/TCR rearrangements were identified in the 96% of the 279 ALL cases. Using RQ-PCR, the quantitative range of $10^3$ was reached in 93% of B-cell precursor (BCP) ALL and 86% of T-cell ALL (T-ALL) by at least one target gene. In paper II, we compared MRD detection using both RQ-PCR and FCM in the context of NOPHO ALL-2000 protocol. By applying the stratification threshold of $\geq 0.1\%$ MRD late during induction therapy (day 29), we could demonstrate that both methods can predict the risk of BM relapse but not extramedullary relapse. However, the threshold of $\geq 0.2\%$ MRD appears to be more optimal using RQ-PCR in BCP ALL, whilst in T-ALL, the results indicate that RQ-PCR is preferable for MRD assessment.

The stability of RNA in vitro is a critical factor when using sensitive molecular techniques such as MRD detection. In paper III, we evaluated the influence on MRD detection when blood is collected in tubes with RNA stabilization reagents (PAX gene Vacutainer®) compared to collection in EDTA-tubes (non-stabilized). We analyzed 68 matched samples from chronic myeloid leukemia patients and the results indicated that non-stabilized blood processed within 30 hours is preferable for MRD detection.

In paper IV, follow-up samples from eight children with Philadelphia positive (Ph+) ALL were evaluated with the three available MRD methods. MRD measured by the fusion gene transcripts (BCR-ABL1) appeared to be the most sensitive method, however, precise quantification can be difficult and the other methods are thus complementary.

In conclusion, all three applied MRD methods are useful and correlate to each other, although not necessary exchangeable in individual patients. We also conclude that MRD assessment by RQ-PCR, based on rearranged IG/TCR genes and multicolor FCM are predictive for identification of high risk childhood ALL patients.

Keywords: Childhood acute lymphoblastic leukemia, minimal residual disease, IG/TCR gene rearrangements, BCR-ABL1 fusion gene transcripts, real-time quantitative PCR, multicolor flow cytometry

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To Thea, Lukas and Nora
List of Papers

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

**Paper I**

**Paper II**
*IT and EF contributed equally to this work.*

**Paper III**

**Paper IV**
*Monitoring minimal residual disease with flow cytometry, antigen-receptor gene rearrangements and fusion transcript quantification in Philadelphia positive childhood acute lymphoblastic leukaemia.* Leukemia Research 2009 [Epub ahead of print].

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Related paper


*In vitro cellular drug sensitivity at diagnosis is correlated to minimal residual disease at end of induction therapy in childhood acute lymphoblastic leukemia.* Leukemia Research 2009;33(3) 46–53.
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ABL1</td>
<td>Abelson murine leukemia viral oncogene homolog 1</td>
</tr>
<tr>
<td>ALL</td>
<td>Acute lymphoblastic leukemia</td>
</tr>
<tr>
<td>ASO</td>
<td>Allele specific oligonucleotide</td>
</tr>
<tr>
<td>BCP ALL</td>
<td>B-cell precursor ALL</td>
</tr>
<tr>
<td>BCR</td>
<td>B-cell receptor</td>
</tr>
<tr>
<td>BCR</td>
<td>Breakpoint cluster region</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>BMR</td>
<td>Bone marrow relapse</td>
</tr>
<tr>
<td>BMT</td>
<td>Bone marrow transplantation</td>
</tr>
<tr>
<td>C</td>
<td>Constant</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CML</td>
<td>Chronic myeloid leukemia</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>Ct</td>
<td>Cycle threshold</td>
</tr>
<tr>
<td>D</td>
<td>Diversity</td>
</tr>
<tr>
<td>cytCD3</td>
<td>Cytoplasmatic CD3</td>
</tr>
<tr>
<td>EMR</td>
<td>Extramedullary relapse</td>
</tr>
<tr>
<td>FCM</td>
<td>Flow cytometry</td>
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<tr>
<td>FISH</td>
<td>Fluorescence in situ hybridization</td>
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<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
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<tr>
<td>IG/Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IGH</td>
<td>Immunoglobulin heavy</td>
</tr>
<tr>
<td>IGK</td>
<td>Immunoglobulin kappa</td>
</tr>
<tr>
<td>IGL</td>
<td>Immunoglobulin lambda</td>
</tr>
<tr>
<td>J</td>
<td>Joining</td>
</tr>
<tr>
<td>KDE</td>
<td>Kappa deleting element</td>
</tr>
<tr>
<td>LAI</td>
<td>Leukemia-associated immunophenotype</td>
</tr>
<tr>
<td>LC</td>
<td>Light chain</td>
</tr>
<tr>
<td>MAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MLL</td>
<td>Mixed lineage leukemia</td>
</tr>
<tr>
<td>MNC</td>
<td>Mononuclear cells</td>
</tr>
<tr>
<td>MRD</td>
<td>Minimal residual disease</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>NOPHO</td>
<td>Nordic Society for Pediatric Hematology and Oncology</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>Ph</td>
<td>Philadelphia chromosome</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>RAG</td>
<td>Recombination activating gene</td>
</tr>
<tr>
<td>RQ-PCR</td>
<td>Real time PCR</td>
</tr>
<tr>
<td>RSS</td>
<td>Recombination signal sequences</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase PCR</td>
</tr>
<tr>
<td>T-ALL</td>
<td>T-cell ALL</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>TdT</td>
<td>Terminal deoxynucleotidyl transferase</td>
</tr>
<tr>
<td>TKI</td>
<td>Tyrosine kinase inhibitor</td>
</tr>
<tr>
<td>V</td>
<td>Variable</td>
</tr>
<tr>
<td>WBC</td>
<td>White blood cell</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
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</table>
Introduction

As far back as the 19th century, European physicians described patients who had increased white cell counts in the peripheral blood. This disorder was consequently designated leukemia (Greek: leukos = white; haima = blood). Today, the term leukemia comprises a heterogeneous group of malignancies and is characterized by abnormal proliferation and/or defective apoptosis of leukocytes and their precursors that can be further classified according to clinical, morphological and molecular features. Traditionally, leukemias are subdivided clinically as either acute or chronic, where acute refers to a leukemia characterized by rapid tumor cell proliferation and a predominance of immature blast cells in blood and bone marrow. In general, compared to acute leukemias, chronic leukemias are characterized by more mature tumor cells with lower proliferation rate. In addition, leukemia is also defined as lymphoid or myeloid, depending on the immunophenotype of the malignant cell population. In lymphoid malignancies, it is also essential to distinguish between B-cell and T-cell lineage.

Despite being a rare disease, acute lymphoblastic leukemia (ALL) is the most common malignancy in childhood with a dominance of pre-B-cell origin (BCP ALL) compared to T-cell origin (T-ALL). Fortunately, the prognosis is favorable with >80% of patients achieving long term survival following modern risk-adapted therapy. During the last decades, methods have been developed which permit the leukemic clonal population to be followed even when reduced to levels below the sensitivity of light microscopy; i.e. minimal residual disease (MRD) detection. Currently three techniques for MRD detection are available and each of these methods has specific advantages and disadvantages.

1  Real-time quantitative polymerase chain reaction (RQ-PCR) of rearranged immunoglobulin (IG) and T-cell receptor (TCR) genes. In B- and T-cells, complex gene rearrangements result in the generation of a unique B-cell receptor (BCR/Ig) or TCR, respectively. The clonal concept implies that leukemia emanates from one cell, thus all daughter cells will carry the same rearrangements. These rearrangements, encoded by unique DNA sequences, will enable RQ-PCR to be used to follow MRD during malignant cell reduction.
II **Multicolor flow cytometry (FCM) of leukemia-associated immuno-phenotype.** During lymphoid cell development various surface (and intracellular) proteins are expressed at specific stages of maturation. Identification of aberrant protein expression of the leukemic cell clone at diagnosis enables residual leukemic cells to be monitored throughout treatment; i.e. MRD by FCM.

III **Real-time quantitative PCR of fusion gene transcripts (RT-PCR).** Chromosomal translocations can be identified at diagnosis in approximately 40% of childhood ALL cases. Further on, PCR amplification of the corresponding leukemia specific fusion gene transcript can be used to track MRD. One example is the translocation t(9;22)(q34;q11) which results in the characteristic Philadelphia chromosome (Ph+), and the *BCR-ABL1* fusion transcript.

The prognostic value of MRD assessment during early treatment phase in childhood ALL has previously been documented by several groups (1-11). However, variation exists with regard to the cut-off value for MRD positivity and the MRD level increasing the risk of relapse. The MRD levels are also highly dependent on the protocol of treatment and method of MRD detection chosen. Hence, within each protocol it is necessary to establish what constitutes a significant level of MRD, decide upon MRD method/s to be applied and also to determine which time point(s) to be included.

The aim of this thesis was to apply, compare and evaluate the pros and cons of the available methods for MRD detection in childhood ALL patients. Furthermore, the response to treatment was evaluated in relation to the MRD results in the context of NOPHO 2000-ALL protocol. However, we first need to recapitulate some basics of normal B-cell and T-cell development since the leukemic cells in ALL are generally regarded as the malignant counterparts of normal immature lymphoid cells.

**B-cell development**

The main players in the specific immune defense are specialized white blood cells, i.e. lymphocytes that can be further divided into; (i) B-cells producing antibodies and (ii) T-cells mediating cellular defense mechanisms. The principal biological function of B-cells is to recognize and eliminate foreign antigens. The ability to specifically recognize millions of different antigens is based on the enormous diversity of antigen receptor molecules, i.e. membrane bound and secreted Ig molecules. This diversity is created by specific rearrangement processes which occur during the maturation of each B-cell in the bone marrow, whereby different gene segments of the IG genes are joined together (12). Consequently each BCR differs; however, an individual B-cell expresses identical receptors with identical antigen specificity encoded by the unique rearrangement (13).
Lymphocyte development occurs in the bone marrow (BM) and starts with a pluripotent hematopoetic stem cell. This stem cell receives signals from BM stromal cells to begin lymphoid differentiation and ultimately B-lineage differentiation. During this development a number of different surface (and intracellular) proteins, e.g. CD (cluster of differentiation) markers, are expressed at specific developmental phases. The expression of these markers together with the different stages of IG gene rearrangement helps to define distinct stages of B-cell maturation (Figure 1). The first committed B-cell progenitor expresses CD34, CD10, cytoplasmatic CD22 and rearrangement at the IG heavy-chain (IGH) locus begins (more detailed information in section rearrangement of the IGH and IGK (IG kappa) and IGL (IG lambda) loci). In the early pre-B stage, surface expression of CD19 appears and it is between the pro- and pre-B cell stage that the IGH rearrangement is completed (14, 15).

As the pre-B stage progresses, Ig μ-chains appear in the cytoplasm and are also weakly expressed on the surface together with one of the two pseudo-light chains, V-homologous sequence (V-pre-B) or C-homologous sequence (λ5), as the pre-BCR (16, 17). Expression of the pre-BCR triggers termination of further rearrangements at the IGH locus and initiates light chain (LC) rearrangement. Successful rearrangement and transcription of a LC leads to the formation of a complete Ig molecule. Due to the complexity of the rearrangement process, only about 20% of B-cells succeed in creating a functional Ig molecule with the ability to receive appropriate survival signals from the bone marrow environment. The appearance of membrane bound IgM and IgM/IgD defines the immature and mature (naïve) stages of B-cell development, respectively, and represents the end of the antigen independent phase.
The IGH locus is located on chromosome 14 and consists of clusters of variable (V), diversity (D), joining (J) and constant (C) germline gene segments spanning over 1100 kb (18, 19). The IGHV genes can be grouped into seven different subgroups (IGHV1-IGHV7) based on sequence homology, with at least 80% homology within each subgroup. This similarity among subgroup members is useful as it enables amplification of gene segments using con-
sensus primers (20-23). The IGH rearrangement follows a sequential order and begins with the joining of a D gene segment to a J gene segment followed by the joining of a V gene to the pre-arranged DJ segment (Figure 2) (14, 15, 24).

When a functional μ-chain is produced and expressed on the surface together with a pseudo LC, rearrangement at the LC IG loci begins. However, in BCP ALL rearrangement of the LC loci may also occur if a non-functional μ-chain is produced (25). Rearrangement of the LC loci is similar to the IGH rearrangement, with the only exception being the absence of D segments in the LC loci resulting in a direct VJ rearrangement (Figure 2) (12). LC rearrangement follows a hierarchical order; first one of the IGK alleles is epigenetically predetermined for rearrangement (26, 27) (28). If a functional kappa-chain is not synthesized the rearrangement can be deleted by rearrangement involving the kappa deleting element (KDE) (29). This occurs prior to rearrangement of the second IGK allele and if both IGK rearrangements are non-functional one of the IG lambda (IGL) alleles is rearranged. Approximately 60% of mature B-cells express Ig molecules with kappa-chains and 40% with lambda-chains. This expression is exclusive to each B-cell and can be used to detect clonality in mature B-cell malignancies.

**Figure 2.** Simplified model of the V(D)J rearrangement process. The left illustration represents rearrangements involving VDJ gene segments, e.g. IGH and TCRD (TCR delta) rearrangements. The right part illustrates rearrangement with VJ gene segments, e.g. IGK/L and TCRG (TCR gamma) rearrangements.
The KDE gene is located downstream of the IGKC gene segment. Two different types of KDE recombinations can inactivate non-functional IGK rearrangements. The KDE gene can be rearranged to the intron located between the IGKJ and IGKC gene segments resulting in deletion of the IGKC gene whilst the IGKV-J junction remains intact (30). In these cases it is possible to detect both an IGKV-J and an intron-KDE rearrangement on the same allele (31, 32). The second possible IGK-KDE rearrangement involves rearrangement of an IGKV gene segment upstream of the IGKV-J joint. This results in an IGKV-KDE rearrangement with a consequent loss of the IGKV-J junction.

V-(D-)J rearrangement process

Antigen receptor gene rearrangement is a complicated process involving several proteins, which together form the recombinase enzyme system complex (33, 34). This recombination can be separated into three stages: recognition (1), cleavage (2) and rejoining (3).

(1) Recognition is mediated by enzymes encoded by the recombination activating genes (RAG1 and RAG2) which recognize specific DNA sequences flanking the 3´side of the V gene segment, the 5´side of the J gene segment and both sides of the D gene segment, so called recombination signal sequences (RSS) (35). RSS are composed of conserved heptamers and nonamers separated by either 12 or 23 nucleotide spacer sequences. Only genes containing spacers of different lengths can efficiently recombine to each other, i.e. the 12/23 rule (12, 36, 37).

(2) Double strand breaks in the DNA are introduced between the RSS and the gene segment. The coding end is altered further due to the deletion of nucleotides by exonuclease activity and the random insertion of nucleotides (N-nucleotides). This insertion of nucleotides is mediated by the enzyme terminal deoxynucleotidyl transferase (TdT), and occurs in a template independent way (38). A lack or reduction in TdT activity during gene rearrangements coincides with an absence or limited number of N-nucleotides insertions (39, 40).

(3) Processing of the coding ends is followed by rejoining of the double strand break mediated by non-homologous end joining proteins (41).

In addition to the large number of combinatorial possibilities of different V/(D)/J gene segments, the deletion and insertion of N-nucleotides within the junctional regions further increases this diversity and generates an enormous repertoire of unique IG/TCR molecules. The number of available gene segments within IG/TCR gene loci is summarized in Table 1. The combinational diversity reported in the table does not include the diversity obtained by the randomly deleted and inserted nucleotides as described earlier, these alterations can increase the potential for variation 1000 fold (42). Therefore,
the chance of having the exact same functional rearrangement within an IGH locus is approximately 1 in $6 \times 10^6$.

Table 1. The number of functional V(D)J genes at different loci are listed and the potential combinational repertoire. Data adapted from IMGT®, the international ImMunoGeneTics information system® (19)

<table>
<thead>
<tr>
<th>Chromosome locus</th>
<th>No of functional V genes</th>
<th>No of functional D genes</th>
<th>No of functional J genes</th>
<th>Combinational diversity</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGH 14q32.33</td>
<td>38-46</td>
<td>23</td>
<td>6</td>
<td>5200-6300</td>
</tr>
<tr>
<td>IGK 2p11.2</td>
<td>31-35</td>
<td>--</td>
<td>5</td>
<td>155-175</td>
</tr>
<tr>
<td>IGL 22q11.2</td>
<td>29-33</td>
<td>--</td>
<td>4-5</td>
<td>116-165</td>
</tr>
<tr>
<td>TCRD 14q11.2</td>
<td>7-8</td>
<td>3</td>
<td>4</td>
<td>36-224</td>
</tr>
<tr>
<td>TCRG 7p14</td>
<td>4-6</td>
<td>--</td>
<td>5</td>
<td>20-30</td>
</tr>
<tr>
<td>TCRB 7q34</td>
<td>40-48</td>
<td>2</td>
<td>12-13</td>
<td>960-1248</td>
</tr>
<tr>
<td>TCRA 14q11.2</td>
<td>45-47</td>
<td>--</td>
<td>50</td>
<td>2200-2300</td>
</tr>
</tbody>
</table>


T-cell development

T-cells play a central role in cell-mediated immunity by means of a specific receptor on their surface for recognition of antigen i.e. the TCR, similar to the BCR. Similarly to the generation of unique BCRs, the TCR is encoded for by numerous rearranged gene segments, summarized in Table 1.

Development of an immuno-competent mature T-cell occurs in the cortex of the thymus and begins with an immature lymphoid progenitor cell derived from the BM (43, 44). The thymocyte (the immature T-cell) development can be divided into a number of maturational stages based on the expression of different surface and intracellular proteins and the progression of TCR gene rearrangements (Figure 3). Pro-thymocytes express CD34, CD7 and HLA-DR, but neither CD1 nor CD3, and maintain their TCR genes in germ-line configuration (45, 46). The thymic microenvironment directs the differentiation of immature T-cell by both negative and positive selection and the mature T-cell ultimately express either CD4 or CD8 and use either TCRα/β or TCRγ/δ chains for antigen recognition.
**Figure 3.** Simplified model of T-cell development in the cortex of thymus. CD, Cluster of differentiation, TCR, T-cell receptor, TCRD, TCR delta, TCRG, TCR gamma, TCRB, TCR beta, TCRA TCR alpha, TCR-G, TCR locus in germline configuration, TCR-R, TCR locus rearranged, TCR-D, TCR gene deleted.

**T-cell receptor rearrangement**

The TCR delta (TCRD) and TCR beta (TCRB) gene loci include V, D and J segments, whilst the TCR gamma (TCRG) and TCR alpha (TCRA) loci lack D gene segments. The rearrangement process of TCR genes occurs in a hierarchical order analogous to IG gene rearrangements in B-cells. The first locus to undergo recombination during T-cell development is the TCRD locus, followed by the TCRG and then the TCRB locus (46). Rearrangement of the TCRA gene is restricted to TCRα/β cells. Moreover, as the TCRD locus is
located within the TCRA locus, rearrangement of TCRA genes involves deletion of TCRD gene segments. As expected in TCRγ/δ T-ALLs, TCRG and TCRD genes are rearranged, however, perhaps unexpectedly, the majority also possess rearranged TCRB genes. All TCRα/β T-ALL have TCRB, TCRA and TCRG gene rearrangements and at least one deleted TCRD allele, however the second TCRD allele is also deleted in two-thirds of cases (47-49).

In addition, TCR genes may also be rearranged in normal pre-B cells, and even more frequently in the case of malignant cells (50, 51). These “illegitimate” cross lineage rearrangements are possibly a result of the BCP ALL cell arrest occurring at a stage of maturation where IG and TCR loci are in an accessible chromatin configuration and in the presence of activated recombinase enzymes (52).

Rearranged IG/TCR genes in childhood ALL

The enormous diversity within junctions of V(D)J gene segments of the IG and TCR loci creates unique “fingerprint-like” DNA-sequences, presumed to be different in each individual leukemia. Hence, these sequences can be used as leukemia specific targets for PCR analyses and detection of low levels of residual disease.

In childhood ALL rearranged IGH, IGK-KDE, TCRG, TCRD and also more recently TCRB genes are commonly used as MRD targets. The frequency of IG and TCR gene rearrangements in BCP ALL and T-ALL are listed in Table 2 (53-61). Rearrangements of the IGH locus can be detected in more than 80% of BCP ALL and many of them also have IGK rearrangements (30%) or KDE rearrangements (50%), together with cross lineage rearrangements of TCR genes (up to 70%).

In T-ALL the majority of cases have rearranged TCRG and TCRB genes (80-90%), however, approximately 10% of the CD3+ T-ALLs still have all their TCR genes in germline configuration (49, 62). TCRD rearrangements are identified in 40-60% of cases. Cross-lineage rearrangements of IG genes are relatively uncommon in T-ALL (10-20%) as compared to BCP ALL, and predominantly occur at the IGH locus (62, 63). However, the majority of IGH rearrangements observed in T-ALL only concern incomplete IGHD-IGHJ joining, and concurrent rearrangement of the TCRD gene and IGH loci in T-ALL has been shown (50).

Table 2. Frequencies of IG and TCR rearrangements in childhood BCP ALL and T-ALL.

<table>
<thead>
<tr>
<th></th>
<th>IGH</th>
<th>IGK a</th>
<th>IGL</th>
<th>TCRG</th>
<th>TCRD</th>
<th>TCRB</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCP ALL</td>
<td>80-90%</td>
<td>60%</td>
<td>20%</td>
<td>45-65%</td>
<td>45-65%</td>
<td>22-35%</td>
</tr>
<tr>
<td>T-ALL</td>
<td>10-20%</td>
<td></td>
<td></td>
<td>80-90%</td>
<td>40-50%</td>
<td>80-90%</td>
</tr>
</tbody>
</table>

a IGK-JK and IGK-KDE rearrangements.
Childhood acute lymphoblastic leukemia

Approximately 70-80 new cases of childhood (<15 year) ALL are diagnosed in Sweden each year and this incidence has remained stable for many decades (3.9 cases per 100 000 child per year) (64). Fortunately, the prognosis has improved and within the Nordic countries overall event-free survival in 1998 was 77% compared to 57% in 1981 (65, 66). In the NOPHO ALL-2000 protocol the probability of relapse free survival is 78% (67).

ALL is a clonal disease arising from an immature lymphoid cell that has undergone mutations. The genetic aberration causing the leukemia are generally acquired, although inherited predisposition may occur e.g. Down Syndrome (DS) (68, 69). However, only a minority of the children with predisposing syndrome develop leukemia. The true etiology of ALL in children is unknown, although many environmental factors (i.e. ionizing radiation, exposure to organic solvents, infection) have been investigated as potential initiating factors (70). As with many other cancers, there is strong support for the “multiple hit theory”, which proposes that one mutation is not enough for leukemia development and rather an accumulation of mutations is required for cancer development (71). Furthermore, evidence suggests that at least in some leukemias a pre-leukemic phase exists and that the first mutation occurred in utero (71-76)

Diagnosis and classification

BM examination is essential when establishing a diagnosis of ALL, and up to 20% of children lack circulating blast cells at diagnosis (77). BM samples are usually obtained by aspiration and these aspirated cells provide material for numerous laboratory investigations. The routine diagnostic setting currently incorporates not only morphological evaluation and immunophenotyping by flow cytometry, but also conventional cytogenetics, molecular cytogenetics (fluorescence in situ hybridization, FISH) and various other molecular techniques. A diagnosis of ALL is based on an arbitrary cut off level of 20% blast cells in the BM sample and flow cytometry data supporting a lymphoid cell of origin. The World Health Organizations’ (WHO) classification of tumors from hematopoietic and lymphoid tissues specifies two distinct categories of ALL: BCP ALL and T-ALL (78).

In children, BCP ALL represents approximately 85% of leukemia cases with a peak incidence observed between the ages of 2-7 years (79). BCP ALLs can be further divided into three subgroups i.e. pro-B-ALL, common ALL and pre-B-ALL based on their protein expression profile which corresponds to different stages of B-cell development (Figure 1). Previous classification was based solely on morphological features (L1, L2 and L3 blast cells) (80, 81), however, in this thesis this sub-classification of BCP ALL will not be taken into account. T-ALL accounts for the remaining 15% of
ALL in children and is frequently associated with hyper-leukocytosis and the presence of mediastinal mass.

Accurate diagnosis and sub-classification are essential for further stratification within the treatment protocols.

Immunophenotypic classification

Immunological characterization of lymphoblastic leukemia was introduced during the mid-1970s. This testing initially used sheep erythrocyte forming rosettes to identify T-cells, and to detect B-cells a polyclonal antiserum with affinity to the common acute lymphoblastic leukemia antigen (CALLA) was used. Since then, immunophenotypic analysis has remained as a standard within the diagnostic setting both for classification of the leukemia and also to determine the stage of cell differentiation. Today, several hundred monoclonal antibodies (MAb) with over 300 CD-markers (cluster of differentiation grouping defined by the International Workshops of Leukocyte Differentiation Antigens) are available. The specificity of these MAbs, further enhanced by labeling with different fluorochromes, allows for simultaneous detection of cell lineage and differentiation markers. Modern automated flow cytometers can simultaneously detect several different colors and together with enhanced computer software provide rapid characterization of a large number of cells. Therefore, in the majority of cases the cell lineage classification of an acute leukemia can be determined within hours of BM sampling (82, 83).

ALL with a B-cell origin usually expresses the B-lineage antigens CD19, CD20, CD22 together with CD45 (common leukocyte antigen), CD10, CD34 and nuclear TdT. However, BCP ALL cells may lack one or several of these antigens compared to the normal B-cell counterpart as a result of altered/asynchronous antigen expression. In addition to these alterations, some BCP ALL cells aberrantly express myeloid-associated antigens, usually CD13, CD15 and/or CD33, although, the expression of these antigens does not appear to influence the outcome (84-86).

T-ALL cells express surface and intracellular proteins which correspond to various stages of thymocyte differentiation (87). The CD7 antigen is assumed to be the earliest sign of commitment to T-cell development, followed by expression of CD1 and then cytoplasmatic CD3 (cytCD3) expression (88). Typically, TdT and CD34 are expressed by the T-ALL cells although their presence is not mandatory (48, 82). Immunological markers can also be used in ALL to monitor the effectiveness of treatment by analyzing BM samples for MRD (described in more detail in the MRD section below).
Cytogenetics

Cytogenetic characteristics form the basis for subclassification of BCP ALL within the recent WHO classification of leukemia and lymphoma (78). Moreover, childhood ALL is managed using risk-adapted therapy where information regarding genetic abnormalities serve as an important factor in risk stratification (89, 90). Cytogenetic changes in leukemic blast cells can principally be divided into two major groups based on either chromosomal numbers or structural chromosomal alterations (91-93). The number of chromosomes present and structural alternations can be visualized by standard chromosome analysis of metaphase cells, however molecular methods such as FISH and RT-PCR are necessary to detect cryptic alterations such as the translocation t(12;21) and also to detect aberrations in non-mitotic cells (94).

The two most frequently occurring aberrations present in BCP ALL are hyperdiploidy and the translocation t(12;21) (79, 95, 96). Both alterations are frequently detected within the age peak between 2-7 years, with the high hyperdiploid subgroup being associated with a good prognosis (96). The prognostic significance of t(12;21) remains controversial, possibly due to the observation that relapses within this subgroup tend to occur at a late stage (94, 97).

Chromosomal abnormalities in T-ALL are detected at a lower frequency than in BCP ALL and any such findings are currently not used in risk stratification (98). However, more than 20% of T-ALL cases are reported to carry translocations at either the TCRA/D or TCRB loci (chromosome 14q11 and 7q32-q36) (99, 100). The TAL1 gene located at chromosome 1p33 may be translocated to the TCRA/D loci and this leads to an overexpression of the transcription factor encoded by the TAL1 gene. Another activating mechanism of the TAL1 gene is a cryptic interstitial deletion of an approximately 90 kb long DNA fragment. This deletion results in a SIL-TAL1 joining and is detected in 5-15% of childhood T-ALL patients (101, 102). Furthermore, this translocation can be used as a leukemia specific MRD target either by RT-PCR detection of transcripts or by DNA based real-time PCR methodology (102, 103).

Philadelphia chromosome t(9;22)

In Paper IV we focused on a minor subgroup of BCP ALL patients harboring the unfavorable reciprocal translocation t(9;22)(q34;q11). This translocation involves part of the long arms of chromosomes 9 and 22, and generates a small chromosome 22 derivative. Historically, this chromosome 22 derivative is called the Philadelphia chromosome (Ph) in honor of the city in which it was discovered (104). The result of this chromosomal translocation is a fusion of the ABL1 gene (Abelson murine leukemia viral oncogene homologue 1) on chromosome 9 to the BCR (breakpoint cluster region) gene on chromosome 22 (region q11) (Figure 4) (105).
The *BCR-ABL1* fusion gene was the first fusion oncogene to be identified in leukemia as well as in the context of cancer and can be regarded as a prototype. The Ph chromosome is present in approximately 3-5% of childhood ALL, 25% of adult ALL and virtually all cases of CML (106, 107). The breakpoints on chromosome 9 are usually scattered within the first intron of the *ABL1* gene whereas the breakpoints on chromosome 22 are clustered within two major areas: a major breakpoint cluster region (M-BCR) and a minor breakpoint cluster region (m-BCR). In childhood Ph+ ALL 80-90% of patients harbor the m-BCR breakpoint resulting in the formation of a 190-kDa fusion protein (p190) whilst in CML the M-BCR is dominant and results in a protein of 210 kDa. An in-frame *BCR–ABL1* fusion transcript encodes for a BCR-ABL1 fusion protein with deregulated *ABL1* tyrosine kinase activity and oncogenic potential. Consequently, cell proliferation increases, but other critical aspects of cell growth and survival such as apoptosis, differentiation and adhesion are also dysregulated (108, 109).

**Treatment and prognosis**

Since the early nineties all children diagnosed with ALL in Scandinavian countries are treated with protocols designed by the Nordic Society for Pediatric Hematology and Oncology (NOPHO). The NOPHO ALL-2000 protocol is based on a standardized multi-drug chemotherapy of 2-2½ years dura-
tion, and for a specific subgroup of patients also includes bone marrow transplantation (BMT) (Figure 5). All patients receive identical treatment during induction therapy, which consists of doxorubicin, prednisolone, vincristine and intra-thecal methotrexate.

The patients are stratified into two major risk groups according to the NOPHO ALL-2000 protocol, i.e. patients with or without unfavorable features at presentation. This stratification is based on clinical status, immunophenotype, cytogenetics and also molecular genetic analysis (Table 3). BCP ALLs without unfavorable features are treated according to standard intensity (SI) or intermediate intensity (II) protocols, whilst patients with unfavorable features are divided into three different groups and receive a more aggressive therapy. Age and white blood cell (WBC) count at time of diagnosis are prognostic factors in patients with BCP ALL and this can, at least in part, be explained by their association with specific genetic aberrations. T-cell immunophenotype is always considered to be an unfavorable feature.

**NOPHO ALL-2000**

![Diagram of NOPHO ALL-2000 therapy protocol](image)

*Figure 5. Simplified model of the NOPHO ALL-2000 therapy protocol. MRD time points stipulated in the protocol are indicated, day 15 for MRD assessment is optional. (VI) very intensive, (EI) extra intensive.*
Table 3 Stratification of childhood acute lymphoblastic leukemia into different therapy groups according to NOPHO ALL-2000 protocol

<table>
<thead>
<tr>
<th>Patients without unfavorable features</th>
<th>1-10 yr and WBC ≤10 x 10^9/l</th>
<th>BCP ALL immunophenotype</th>
<th>Good response to initial therapy*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard Intensive therapy (SI)</td>
<td>1-10 yr and WBC ≤10 x 10^9/l</td>
<td>BCP ALL immunophenotype</td>
<td>Good response to initial therapy*</td>
</tr>
<tr>
<td>Intermediate Intensive therapy (II)</td>
<td>10-50 x 10^9/l or ≥10 yr and WBC &lt; 50 x 10^9/l</td>
<td>BCP ALL immunophenotype</td>
<td>Good response to initial therapy*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Patients with unfavorable features</th>
<th>WBC ≥50 x 10^9/l and T-ALL and/or CNS leukemia and/or Testis leukemia and/or 11q23 (MLL), t(9;22)***, t(1;19) and/or Hypodiploidy (&lt;45 chromosomes) and/or Poor/slow responder</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intensive therapy (I)</td>
<td>Algorithm to stratify to different intensive protocols</td>
</tr>
<tr>
<td>Very Intensive therapy (VI)</td>
<td></td>
</tr>
<tr>
<td>Extra Intensive therapy (EI)</td>
<td></td>
</tr>
</tbody>
</table>

*Good response to initial therapy is defined as < 25 % lymphoid blast cells in a non-aplastic BM at day 15 and/or < 5 % at day 29.
** Poor responder to initial therapy if defined as > 25 % lymphoid blast cells in a non-aplastic BM at day 15, and/or 5-25 % at day 29
*** Patients with t(9;22) may be treated according to the European Childhood Ph+ ALL (EsPhALL) trial protocol.

The improvement in overall survival observed throughout the last decades is primarily achieved in patients without unfavorable features (SI and II therapy groups), which encompasses about 2/3 of patients. However, the overall relapse rate among this group is still significant, approximately 20%. Furthermore in the remaining 1/3 of patients displaying unfavorable features, improvements in survival have been more modest (90). Children assigned to the extra intensive therapy group include those harboring Ph+ ALL and are clinically associated with an older age, a higher leukocyte count and more frequent involvement of the central nervous system (CNS) (110).

The introduction of novel molecular drugs targeting the BCR-ABL1 tyrosine kinase represents a promising treatment strategy for Ph+ leukemias. The prototype for these drugs is the small compound imatinib mesylate (Glivec). However, in Ph+ ALL imatinib treatment results in a predominantly transient response due to the development of drug resistance (111). Incorporation of this agent into multi-drug regimens may overcome this problem, and preliminary results of frontline combinations are very promising although most studies published concern adult Ph+ ALL patients (112, 113). However, the long term clinical impact of this unique targeted treatment in ALL is still under investigation (114).
Although childhood leukemias display high sensitivity to chemotherapeutic agents, drug resistance plays a central role in disease outcome and a correlation between drug resistance in vitro and MRD levels after induction therapy has been shown (115, 116). In the NOPHO ALL-2000 protocol specific time points for MRD sampling are stated and include days 15, 29, 50 and 106 (Figure 5).

A new protocol for childhood ALL is since July 1 2008 used within the Nordic countries, NOPHO ALL-2008, and in this protocol MRD has been introduced as a stratification parameter. Three different risk groups (standard, intermediate and high) are identified according to risk criteria present at diagnosis and during the first 3 months of therapy. MRD should be analyzed at two time points, late during induction therapy, protocol day 29, and after consolidation therapy/block B, protocol day 79. For instance, MRD levels ≥0.1% at day 29 exclude the patient from standard risk therapy, independent of all other risk criteria. In BCP ALL MRD levels should be determined by multicolor FCM (four- or six-color) and in T-ALL by IG/TCR based RQ-PCR.

Chronic myeloid leukemia (CML)

The main focus of this thesis is childhood ALL, however, in paper III we analyzed follow-up samples from CML patients where we evaluated the influence on MRD detection when blood is collected in tubes with RNA stabilization reagents.

CML is classified as a chronic disorder and the cell of origin is considered to be a stem cell (117). At presentation the WBC count is usually very high with dominance of granulocytes and their progenitors in the blood (78). Despite the use of the term “chronic”, the natural course of the disease involves progression through an accelerated phase into a blastic phase. During blast crisis, the immature cells can either express a lymphoid or myeloid immunophenotype, and this corroborates the notion that malignant transformation in CML occurs in an uncommitted hematopoietic stem cell. As mentioned before, the hallmark for CML diagnosis is the presence of the Philadelphia chromosome. Since the introduction of molecular drugs targeting the altered ABL1 tyrosine kinase (i.e. imatinib) as a therapy in CML, the survival has dramatically increased with 83% of patients estimated to have event-free survival after 6 years (118). This response to treatment is routinely followed by MRD detection of BCR/ABL1 transcripts (119, 120).
Minimal residual disease

Overview

Traditionally, therapy response in leukemia patients is evaluated by light microscopy of BM smears and the cytological detection of less than 5% blast cells is considered as a complete remission (CR). Development of new methods, mostly molecular techniques, during the last two decades has prompted the remission status of hematological malignancies to be redefined. In particular the detection of residual leukemic cells (i.e. MRD) during or after therapy has become a promising tool within clinical practice. MRD measurement is presumed to be a reflection of the cytoreduction in vivo in the individual patient and can provide information about the effectiveness of therapy (121). Both cellular and clinical variables as well as pharmacokinetic and pharmacogenetic factors influence the response to treatment (Figure 6).

Figure 6. Factors affecting response to treatment in childhood ALL patients; host features, leukemia cell characteristics and therapy doses/properties. Adapted from Campana D, Br J of Heamatology 2003 (121).

At the time of diagnosis, the bone marrow is highly infiltrated with leukemic cells and the tumor burden may exceed $10^{12}$ malignant cells. In contrast, after induction therapy the total number of malignant cells dramatically decreases. For this reason, techniques to detect MRD should be highly sensitive, ideally capable of detecting 1 leukemic cell within the range of 10 000 - 100 000 normal BM cells (0.01-0.001% = $10^{-4}$ - $10^{-5}$), thereby representing a
100-fold increased sensitivity compared to morphological examination (122). However, as BM aspirates represent only a fraction of the total number of BM cells, even with highly sensitive techniques malignant cells may escape detection.

Furthermore, within the clinical setting MRD testing has to fulfill diagnostic quality parameters such as reliability and reproducibility. Due to the potential serious clinical consequences of reporting false negative/positive results it is an absolute requirement that the MRD method must be accurately capable of discriminating between malignant and normal cells. Another important requirement for MRD techniques is the ability to apply the method to all (or at least the majority of) ALL patients. Different methods to measure MRD have been suggested, however, the three most commonly applied methods for MRD analysis include (i) RQ-PCR of rearranged antigen receptor genes, (ii) multicolor FCM, and (iii) RT-PCR amplification of fusion gene transcripts. The advantages and pitfalls of these techniques are summarized in Table 4.

Table 4 Characteristics of the different techniques used for MRD detection in childhood ALL. Modified from Szczepański T Leukemia 2007 (21) (123).

<table>
<thead>
<tr>
<th>Method</th>
<th>Applicability</th>
<th>Sensitivity</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>RQ-PCR of IG/TCR rearranged genes</td>
<td>90-95%</td>
<td>$10^{-4}$-$10^{-5}$</td>
<td>Sensitive</td>
<td>Laborious, time consuming at diagnosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.01-0.001%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flow cytometry, 3- or 4-color</td>
<td>90-95%</td>
<td>$10^{-3}$-$10^{-4}$</td>
<td>Rapid</td>
<td>Complex data analysis Limited sensitivity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.1-0.01%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RT-PCR of fusion gene transcripts</td>
<td>40-50%</td>
<td>$10^{-5}$-$10^{-6}$</td>
<td>High sensitivity</td>
<td>Only useful in a subpopulation of patients</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.001-0.0001%)</td>
<td>Consensus method</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Leukemia specific</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Method</th>
<th>False positive</th>
<th>False negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>RQ-PCR of IG/TCR rearranged genes</td>
<td>Below quantitative range?</td>
<td>Clonal evolution</td>
</tr>
<tr>
<td>Flow cytometry, 3 or 4 color</td>
<td>Regeneration of normal BM cells</td>
<td>Immunophenotypic switch (early phase)</td>
</tr>
<tr>
<td>RT-PCR of fusion gene transcripts</td>
<td>Cross contamination</td>
<td>RNA degradation</td>
</tr>
</tbody>
</table>
Real-time quantitative PCR

The basic theory behind a PCR reaction is the ability to exponentially and selectively amplify specific regions of genomic DNA or RNA. Real-time quantitative PCR (RQ-PCR) has the additional capability of accurately quantifying the nucleotide sequence of interest. For MRD analysis, the TaqMan® system is commonly utilized (124). The principle behind this technology includes amplification with consensus (and/or allele specific) primers followed by the detection of emitted fluorescence from the hydrolyzed Taq-Man® dual-labelled probe. RQ-PCR has a very large dynamic detection range, thus enabling standard curves to be constructed by serial dilution over five orders of magnitude. In the analysis either DNA or RNA can serve as a template. When using RNA as a template a pre-step is required whereby the RNA strand is reversed transcribed to complementary DNA (cDNA) prior to PCR amplification. Therefore, in this thesis, the term RT-PCR is used for real-time quantitative analysis when RNA is used. The quality of the isolated nucleic acids is of great importance, particularly in relation to RNA which is extremely susceptible to degradation, thus careful pre-analytical handling of the clinical sample is crucial as it can impact the results.

RQ-PCR of rearranged antigen receptor genes

In the past, semi-quantitative approaches such as the limited dilution technique were used for MRD detection of rearranged IG/TCR genes (125). However, the applicability of MRD detection has dramatically increased since the introduction of RQ-PCR and is today the most widely employed method in ALL (126, 127). As discussed, during B- and T-cell development the gene rearrangement process results in creation of unique IG/TCR junctional sequences (12). Since the clonal concept implies that leukemia emanates from one cell, all daughter cells harbor identical rearrangements. Therefore, the sequence of the junctional region from each rearranged gene allows the design of allele specific oligonucleotides (ASO), i.e. a junctional specific primer (128). ASO primer design plays a crucial role in the analysis since the specificity of the MRD analysis, to a large extent, is mediated by this primer. Due to the use of ASO primers, the RQ-PCR analysis of IG/TCR genes includes a set-up stage to verify the sensitivity level and quantitative range. Despite the laborious and time-consuming nature of this approach to MRD detection, it is considered to be the most reproducible method both within and between laboratories (123). Guidelines to ensure consistent interpretation of MRD data have been published by the European Study Group on MRD detection in ALL (ESG-MRD-ALL) (127, 129). Standard curves are constructed by serial dilution of the diagnostic sample in normal mononuclear cell DNA (MNC) and the diagnostic sample is considered to contain 100% of the actual target gene.
Aside from the technical difficulties mentioned above, clonal evolution and secondary rearrangements are considered to constitute the main drawback of MRD analysis of antigen receptor genes due to the possibility of obtaining false negative results (130-134). To circumvent this, it has been proposed that at least two different PCR targets should be followed to minimize the risk of false negative results (133, 135, 136). In addition, follow-up samples are always analyzed together with the standard curve and negative controls (non-template controls in duplicate and non-specific amplification (MNC) in six replicates). Two examples of MRD analysis are illustrated in Figure 7. The horizontal red line is the threshold against which cycle threshold (Ct) values are determined. The Ct values (x-axis) represent the number of PCR cycles necessary to detect a signal above the threshold and are inversely proportional to the amount of target present at the beginning of the reaction. Consequently, the more leukemic cells in the follow-up sample the lower Ct values, and standard dilutions (10^{-1}-10^{-4}, blue amplifications curves) enable quantification of unknown samples.

![Figure 7. Amplification plots from RQ-PCR analysis: standard dilutions 10^{-1}-10^{-4} (blue), two follow-up samples (red) and control samples (green). a) Analysis with quantitative range 0.01% (10^{-4}). Follow-up samples day 29; MRD= 0.05%, day 50; MRD= positive <0.01%. b) Quantitative range 0.1% (10^{-3}), follow-up samples day 29; MRD=0.2%, day 50; MRD <0.1%. y-axis; (RFU) relative fluorescence unit, x-axis; number of PCR cycles.](image)

**Multicolor flow cytometry**

The principle of immunological MRD detection is based on the leukemia-associated immunophenotype (LAI) as defined at diagnosis. The concept behind data evaluation involves the identification of “empty spaces” in flow cytometry dot plots, i.e. areas that are not occupied by lymphoid cells during normal B-cell development (137). Dissimilarity between surface and intracellular markers expressed by the leukemic blast cells compared to normal cells confirms the presence of ALL cells within these “empty spaces”. However, one drawback of flow cytometry analysis is “immunophenotypic shifts”, the
expression of one or more antigens may change during cytotoxic therapy and therefore LAIs should preferably be identified using two or more MAb combinations (138, 139). Within the European BIOMED-1 Concerted Action, a standard panel of antibodies using triple-color staining, has been suggested for MRD detection in ALL (Table 5) (87, 140, 141). Using these antibody combinations and also optional patient specific combination of MAbs’, FCM can be applied as a MRD method in 95% of leukemia cases. In FCM analysis there are two main variables that influence MRD detection: the number of cells analyzed and the degree of dissimilarity between the leukemic cells and the normal cells. The number of cells may constitute a problem in follow-up samples taken at early time points due to low cellularity in the BM. Differences between the leukemia cell antigen expression and the normal counterpart may be small and thus high levels of regenerating normal BM cells may be suspected as leukemic cells or hide the leukemic cells (142, 143).

Table 5. Antibody combinations and frequencies of leukemia-associated immunophenotypes in childhood ALL (78,135).

<table>
<thead>
<tr>
<th>Monoclonal antibodies</th>
<th>Frequency of phenotypic aberration</th>
<th>Immununphenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD19/CD34/CD45</td>
<td>22%</td>
<td>BCP ALL</td>
</tr>
<tr>
<td>CD10/CD20/CD19</td>
<td>64%</td>
<td></td>
</tr>
<tr>
<td>CD34/CD22/CD19</td>
<td>46%</td>
<td></td>
</tr>
<tr>
<td>CD34/CD38/CD19</td>
<td>56%</td>
<td></td>
</tr>
<tr>
<td>CD19/CD34/CD45</td>
<td>78%</td>
<td></td>
</tr>
<tr>
<td>TdT/CD7/cyCD3</td>
<td>91%</td>
<td>T-ALL</td>
</tr>
<tr>
<td>CD7/D5/D3</td>
<td>61%</td>
<td></td>
</tr>
<tr>
<td>CD7/CD4/CD8</td>
<td>38%</td>
<td></td>
</tr>
<tr>
<td>CD7/CD2/CD3</td>
<td>48%</td>
<td></td>
</tr>
<tr>
<td>CD7/CD34/CD38</td>
<td>40%</td>
<td></td>
</tr>
<tr>
<td>CD7/Cd13/CD33</td>
<td>12%</td>
<td></td>
</tr>
</tbody>
</table>

RT- PCR of fusion gene transcripts

MRD detection of fusion gene transcripts has major the advantage of having a strong association between the molecular aberration and the leukemic clone, irrespective of the presence of subclone formations and cellular changes caused by therapy. In childhood ALL approximately 40% of patients can be followed using fusion gene transcripts as MRD targets. Today the fusion gene transcript BCR-ABL1 is commonly used to evaluate the therapeutic response in Ph+ALL cases as well as in CML. Additionally, quantitative methods to follow MRD in subgroups of ALL patients based on cytogenetics features such as t(12;21), t(4;11), t(1;19) and SIL-TAL1 by RT-PCR are also available (103).
With quantitative RT-PCR the choice of control gene is of particular importance as the MRD level is determined in relation to the expression of the control gene. A suitable control gene should be expressed constitutively during the cell cycle, degraded to the same extent, expressed at an equal or a lower level than the target gene and without pseudo-genes (144). Three control genes were selected within the European Against Cancer program: Abelson (ABL), beta-2-microglobulin (B2M) and beta-glucuronidase (GUS) (145). The use of control gene in the MRD analysis has the potential to i) detect poor-quality samples, ii) correct for cDNA synthesis efficiency and iii) enable calculation of MRD results and experimental sensitivity. Standard curves of both target and control genes are obtained by serial dilution of plasmid DNA. However, a disadvantage of RT-PCR as an MRD method is that it provides no information about the number of leukemic cells, but instead enables the average expression of the fusion gene in the sample to be determined.
Aims

The overall aim of this thesis was to apply and compare MRD methods in childhood ALL. Furthermore, the response to treatment was evaluated in relation to MRD results in the context of NOPHO 2000-ALL protocol. In addition, we evaluated the influence of RNA stabilization on MRD quantification in blood samples from CML patients. The specific aim for each paper was:

I To screen for IG/TCR gene rearrangements in a cohort of Swedish childhood ALL cases diagnosed between 2002-2006. Identified rearrangements were then assessed as potential targets for MRD detection by real-time PCR. These analyses were performed at five different centres throughout the country.

II To compare MRD results obtained by RQ-PCR and FCM within a multi-centre setting strategy and evaluate response to treatment in relation to these MRD values in a Swedish cohort of childhood ALL.

III To compare MRD levels as well as RNA quality and quantity using PAXgene Vacutainers® (RNA stabilization reagents) with non-stabilized blood collection.

IV To apply, compare and evaluate the three currently available MRD methods in eight cases of Ph+ ALL cases in a single centre study.
Patient material and methods

Patient material

**Paper I and II** included patients from a Swedish cohort of childhood ALL cases diagnosed between January 1st 2002 and December 31st 2006. A total of 334 children (≤17 years of age) were diagnosed at six different centres throughout Sweden; Lund (Centre 1, n=49), Göteborg (Centre 2, n=75), Stockholm (Centre 3, n=79), Uppsala (Centre 4, n=66), Umeå (Centre 5, n=34) and Linköping (Centre 6, n=31). Patients were treated and stratified to different therapy intensities according to the NOPHO 2000-ALL protocol based on age at diagnosis, WBC count, immunophenotype, and cytogenetic aberrations present at diagnosis. The distribution between immunophenotype and therapy intensity protocol is presented in Table 6 together with the number of BMR and EMR. BM samples were collected at diagnosis and at the four follow-up time points stipulated by the NOPHO therapy protocol for MRD analysis. This study was approved by the local ethical committees.

Table 6. Characteristics of childhood ALL cases diagnosed 2002-2006 (all patients), and patients included in paper I and paper II. The number of cases is indicated and within brackets () the percentage of case.

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>BCP ALL</th>
<th>T-ALL</th>
<th>SI</th>
<th>II</th>
<th>HI</th>
<th>Other protocol</th>
<th>BMR</th>
<th>EMR</th>
</tr>
</thead>
<tbody>
<tr>
<td>All patients</td>
<td>334</td>
<td>291(87)</td>
<td>42(13)</td>
<td>106(32)</td>
<td>108(32)</td>
<td>101(30)</td>
<td>19(6)</td>
<td>50(15)</td>
<td>17(5)</td>
</tr>
<tr>
<td>Paper I</td>
<td>279</td>
<td>244(87)</td>
<td>35(13)</td>
<td>84(30)</td>
<td>91(33)</td>
<td>89(32)</td>
<td>15(5)</td>
<td>38(14)</td>
<td>14(5)</td>
</tr>
<tr>
<td>Paper II</td>
<td>228</td>
<td>204(89)</td>
<td>24(11)</td>
<td>73(32)</td>
<td>85(37)</td>
<td>70(31)</td>
<td>0(0)</td>
<td>24(11)</td>
<td>10(4)</td>
</tr>
</tbody>
</table>

Therapy NOPHO ALL-200 protocol: SI; standard intensity, II; intermediate intensity, HI; High intensity. BMR; bone marrow relapse, EMR; extramedullary relapse.

In **paper I**, diagnostic samples from 279 (244 BCP ALL and 35 T-ALL) of the 334 ALL cases (84%) were screened for the presence of IG/TCR gene rearrangements (Figure 8). Forty-five patients were not included in the analysis due to insufficient amount of diagnostic material, and another 10 patients were excluded due to resistant disease (7 cases) or lack of follow-up samples (3 cases). No significant differences in immunophenotype, sex distribution or WBC counts at diagnosis were observed between cases included and those excluded from the analyses. In **paper II** the presence of MRD was examined...
by RQ-PCR and FCM in the previously described cohort. BM follow-up samples were collected at day 15, 29, 50 and 106 following the start of therapy. 228 patients, and 726 paired MRD values, were included in the MRD method comparison and response to treatment evaluation (Figure 8).

**ALL study cohort Sweden 2002-2006**

<table>
<thead>
<tr>
<th>Paper I</th>
<th>Paper II</th>
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<tbody>
<tr>
<td>Included</td>
<td>Protocol patient</td>
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55
(46 BCP ALL, 7 T-ALL, 1 failed, 1 mature B)
45 no diagnostic sample
7 resistant disease
3 no follow-up samples

279
(244 BCP ALL, 35 T-ALL)

334
(290 BCP ALL, 42 T-ALL+ 1 mature B, 1 failed)

20
(15 BCP ALL, 4 T-ALL, 1 mature B)
2 secondary malignant neoplasms
3 resistant disease
1 death during induction therapy
3 (t(9;22)) other protocol
2 Down's syndrome

314
(275 BCP ALL, 38 T-ALL, 1 failed)

11
(8 BCP ALL, 3 T-ALL)

303
(267 BCP ALL, 31 T-ALL, 1 failed)

228
(204 BCP ALL, 24 T-ALL)

23
(20 BCP ALL, 3 T-ALL)

205
(184 BCP ALL, 21 T-ALL)

75
(63 BCP ALL, 7 T-ALL, 1 failed)

205
(184 BCP ALL, 21 T-ALL)

Figure 8. Schematic diagram of the patient cohort of childhood acute lymphoblastic leukemia (ALL) included in paper I and II. BCP ALL: B-cell precursor ALL, T-ALL: T-cell ALL, MRD: minimal residual disease

In paper III peripheral blood samples from 30 patients with CML were collected between May 2002 and May 2003 for quantification of MRD using the leukemia specific \( BCR-ABL1 \) transcript. Blood from these patients was drawn into two different collection tubes, conventional 7 ml EDTA Vacutainer® tubes and 5 ml PAXgene Vacutainers®. In total 68 paired samples were collected. Additionally, 33 blood samples from another cohort of CML patients were collected in EDTA tubes only and sent to the laboratory by mail for MRD analysis. For these samples, Ficoll separation was performed 20-30 hrs after sample collection.

**Paper IV** focused on childhood Ph+ ALL cases. During the period August 2001 to August 2006, eight consecutive patients with this particular translocation were diagnosed at Uppsala University Hospital, Sweden. The median age at diagnosis was 9.8 years (range, 5.8-13.6) and the median follow-up was 42 months (range, 20-62 months). Patients were treated accord-
ing to NOPHO ALL-2000 extra intensive protocol and four patients switch-
ed to the European Ph+ childhood ALL trial protocol (EsPhALL) following
50 days of induction therapy. With the exception of one patient, all patients
underwent BMT approximately 6 months after diagnosis. MRD values were
monitored in 140 bone marrow follow-up samples collected pre- and post-
bone marrow transplantation.

Methods
In this thesis three different methods were applied to measure MRD; (i) RQ-
PCR of rearranged antigen receptor genes (IG/TCR genes), (ii) multicolor
flow cytometry (FCM) of LAI and (iii) real-time quantitative PCR of fusion
gene transcripts (RT-PCR). These three methods detect residual leukemic
burden at three “levels”; RQ-PCR detects clone-specific DNA sequences,
FCM measures cells with aberrant surface and intracellular protein expres-
sion and RT-PCR amplifies messenger RNA (mRNA) from leukemia-
specific fusion genes.

Detection of MRD by IG/TCR based RQ-PCR
DNA from mononuclear BM cells was prepared using standard techniques
and in the diagnostic samples clonal IG/TCR rearrangement(s) were subse-
quently identified by PCR amplification using consensus primers and se-
quencing of the junctional region. Papers I and II were multi-centre studies
and all centres used consensus primers for detection of IGH, TCRG and
TCRD rearranged genes, whilst IGK-KDE and incomplete IGH rearrange-
ments were investigated in 3 and 2 of the 5 participating centres, respec-
tively. For MRD detection using clone-specific RQ-PCR (paper I, II and IV)
a consensus TaqMan-probe and reverse primers were used together with the
“fingerprint-like” ASO. In brief, standard curves were constructed by serial
dilutions of DNA from the diagnostic samples in normal DNA (using MNC
from ten healthy donors). Where possible two rearrangements were used per
leukemia case and typically more than one ASO primer was designed for
each rearrangement. The primer set giving the highest quantitative range and
sensitivity level was used for the detection of residual leukemic cells in fol-
low-up samples (127, 129). To detect MRD, 500 ng of follow-up DNA was
analyzed in triplicate together with negative controls (MNC in 4-6 replicates,
non-template control in duplicate). Thermal cyclers from three different
companies were utilized; the ABI 7700 and 7900 Prism Sequence Detection
System (Applied Biosystems), I-cycler (Bio-Rad) and Rotor-Gene RG3000
(Corbett Research). For quality assurance purposes two rounds of sample
exchange were performed (see paper I).
Detection of MRD by multicolor FCM

At diagnosis, the immunophenotype of the leukemic cells was determined by standard combinations of MAbs using three- or four-color FCM. In addition, to enable detection of the leukemic clone during treatment, appropriate antibody combinations were tailored to each patient (87, 140, 141).

Immunophenotypic analysis was performed using the FACSScan, FACSCalibur or FACSCanto flow cytometers (Becton Dickinson, San José, CA). Throughout the study, various software programs such as Cell Quest, Paint a Gate or Diva were used to evaluate the data. This staining and data acquisition was performed as previously described (146). Briefly, a stain and lyse/wash technique was used for surface markers and intracellular markers were analyzed after fixation and permeabilization procedure. FCM analysis was always performed within 24 hrs of BM sampling. To detect MRD using FCM a cluster of cells (at least 10 events) with LAI and with adequate scatter properties had to be identified. Preferably, the leukemic clone should be detected in two or more antibody combinations. In follow-up samples where less than 100 000 living cells per MAb combination were acquired, the sensitivity corresponded to 0.1% (1 leukemic cell in 1000 BM cells). If between 100 000 and 1 000 000 living cells were acquired, the sensitivity was 0.01% (1 leukemic cell in 10 000 BM cells). To ensure that consistency was maintained between centres, data files were exchanged and evaluated (Björklund et al JPHO 2009 in press).

RNA extraction and cDNA synthesis

In paper III we evaluated the quality and quantity of RNA from blood collected in PAXgene Vacutainer® tubes compared to conventional EDTA Vacutainer® tubes, and any subsequent difference in MRD quantification. Mononuclear cells from EDTA tubes were density separated within 2-5 hrs of collection, lyzed in Trizol® and stored at -20°C until RNA extraction was performed (EDTA/Trizol). PAXgene tubes contain an RNA preservation and stabilization solution which enables storage for many days prior to RNA extraction. Samples processed using the PAXgene system were stored at 4°C for 1-16 days before being processed according to the PAXgene Blood RNA Kit® protocol (year 2002). RNA concentrations were determined spectrophotometrically and whenever possible, 1 μg of RNA was reverse transcribed according to the guidelines published by Gabert et al (103). However, the RNA concentration in 18/68 PAXgene samples was too low to include 1 μg of RNA in a 20 μl reverse transcription reaction and thus less RNA was used. For these cases equal amounts of RNA were used from the parallel Trizol® extraction. RNA integrity was investigated for ten representative paired samples using the Bioanalyzer as described in the RNA 6000 Nano Assay (Agilent Technologies, Santa Clara, CA,USA).
Paper IV utilized the same method of RNA extraction and cDNA synthesis from mononuclear cells (EDTA tubes) as described above.

Detection of MRD by RT-PCR of BCR-ABL1 mRNA

The third method for MRD detection utilizes the presence of a leukemia specific fusion gene transcript, which was reverse transcribed to cDNA and then measured by RT-PCR. All PCR amplifications were performed using the ABI 7700 instrument (Applied Biosystems, Foster City, CA, USA). The BCR-ABL1 transcripts together with transcripts from control genes (ABL, GAPDH or GUS) were amplified and quantified as previously described (103). Results are expressed as cycle threshold (Ct) values, which represent the number of PCR cycles necessary to detect a signal above the threshold and are inversely proportional to the amount of target present at the beginning of the reaction (also true for RQ-PCR analysis with IG/TCR rearranged genes) (147). Commercially available plasmids carrying the fusion genes and control genes, respectively, were used to construct the standard curve and to calculate the copy number of the respective target as described previously (103, 148).

BCR-ABL1 mutation analysis

In paper IV the patients treated with tyrosine kinase inhibitors (TKI) during relapses were screened for mutations in the BCR-ABL1 tyrosine kinase domain by hemi-nested PCR (149). PCR products were sequenced in both directions using universal primers. In addition, the presence of the T315I mutation in the BCR-ABL1 kinase domain was investigated by quantitative real-time RT-PCR as described in Gruber et al. 2005 (150).

Statistical analysis

In paper I Chi square test was used to compare frequencies, whereas a two-tailed independent t-test was applied for quantitative data. Statistical analyses were performed using the SPSS Software 16.0. In paper II, correlations between MRD values, were tested with the Spearman’s rank correlation test and Pearson’s correlation coefficient. Differences between median MRD values in follow-up groups were tested with the Mann-Whitney U test. Prism 4 for Macintosh was used for median value plots. The probability of relapse free survival and construction of survival curves was calculated using the Kaplan-Meier method and the different subgroups were compared using the Log Rank test. Statistical Package SPSS Version 11.0 and 13.0 for Macintosh was used for the statistical analyses. In paper III the statistical significance was determined using a t-test for independent samples using Statistica 6. The significance limit for p-values was set to p<0.05 in all tests.
Results and discussion

IG/TCR rearrangements and RQ-PCR in ALL (Paper I)

Within the population based cohort of 334 childhoods ALL patients, we were able to analyze diagnostic samples from 84% (279 of 334 cases). In BCP ALL, rearranged IG/TCR genes were identified in 236/244 (97%) of these cases, which is in line with several other studies (56, 58, 59, 61). In our study the observed proportion of BCP ALL with IGH and IGK-KDE rearrangements was comparable to frequencies reported in other studies, 82% and 42%, respectively (25, 52, 56, 61). However, cross-lineage rearrangements (TCRG and TCRDV2-D3 genes) were detected in a lower proportion of BCP ALL cases, with TCRG rearrangements only detected in 39% (94/244) of BCP ALL cases. This is in contrast to other studies which detected TCRG rearrangements in 53-61% of cases (56, 58, 59, 61). Furthermore, when TCRG gene rearrangement frequencies were analyzed according to individual centres, data obtained in two of our centres correlated with previous reports (47% and 54%) whilst the other three centres only identified TCRG rearrangements in 23-31% of cases. This disparity between centres may be attributed to the primer sets chosen and also slight variation in the detection methods applied at the participating centres. For TCRG gene rearrangements, three different primer sets were used, whilst only centre 4 (which used BIOMED-1 primers) detected sequences involving the TCRG JP1/JP2 gene segment (102). Consequently, there was a shift towards the BIOMED-1 primers in all centres during the study period.

As evidenced both in this study and others, IGH genes are frequently rearranged within BCP ALL and serve as very suitable targets for RQ-PCR analysis. Here we report that analysis of IGH genes reached a sensitive level ($\leq 10^{-4}$) in 93% (232/249) of cases. In contrast to IGH rearrangement, when using IGK-KDE, TCRG and TCRD (V2-D3) as target genes, this yielded a sensitive analysis ($\leq 10^{-4}$) in 73%, 72% and 77% of BCP ALL cases, respectively. However, approximately 50% of these gene rearrangements were not selected as potential MRD targets for two reasons; either due to the presence of more suitable markers or due to a quantitative range less than $10^{-3}$.

Clonal evolution is a potential pitfall in childhood ALL, primarily due to ongoing or secondary rearrangements of the IG/TCR genes, therefore it is suggested that at least two target genes should be followed per patient (132,
Two or more rearrangements were identified in 179 of 244 BCP ALL cases (73%) and RQ-PCR assays of ≥2 target genes (sensitivity at least ≤10⁻³) were established in 146 of these 244 cases (60%). In our study the frequencies of patients that could potentially be followed with two target genes is lower compared to some other studies (61, 151). The reasons for this may be the inability to detect two target genes in a proportion of cases (27%) but also difficulty to obtain quantitative range of 10⁻³ using IGK-KD, TCRG and TCRD rearrangement as target genes. We also observed differences in obtaining sensitive RQ-PCR assay with the different PCR instruments. For instance, a higher span of Ct values between intercept and amplification of the nonspecific amplification control was observed in centre 2 which utilized the RotorGene RQ-PCR machine. (Definition of intercept in this aspect: the Ct value where undiluted diagnostic sample cross the threshold). This larger span appeared to positively influence achievement of a sensitive analysis, at least in RQ-PCR analysis of rearranged genes lacking D segments.

Of the 42 childhood T-ALLs diagnosed within the Swedish cohort 35 were included in the study and in 33 of 35 cases (94%) at least one TCR rearrangement was detected. Comparable to other studies TCRG rearrangements were detected within the majority of cases (91%) and TCRD rearrangements in approximately 50% of cases (2, 58, 61, 152).

A sensitive RQ-PCR analysis (≤10⁻⁴) was established in 74% (26/35) of T-ALLs, and in 86% (30/35) the quantitative range reached 10⁻³. Thus, it was more difficult to achieve a sensitive RQ-PCR analysis in T-ALL patients compared to BCP ALL patients as described by others (61, 153, 154). One explanation is that the most suitable target gene for RQ-PCR analysis in T-ALL (i.e. TCRD) is only rearranged in approximately 50% of cases, compared to IGH rearrangements in 80-90% of BCP ALL cases. In addition, this lower frequency may also reflect the absence of TCRB as a target in our screening program. Notably, TCRB rearrangements are currently included in T-ALL screening in Sweden (57, 155).

In summary, clonal IG/TCR rearrangements were detected in 96% of ALL cases and a sensitive RQ-PCR (≤10⁻⁴) was defined in 242/279 cases (87%) analyzed. With the stratification threshold defined as 10⁻³ for identification of childhood ALLs that are at a higher risk of having relapse, it would be possible to follow 93% of the BCP ALL and 86% of the T-ALLs cases in this study with at least one target gene. Hence, this national multi-centre study supports the use of RQ-PCR analysis as a robust method for MRD detection in the majority of childhood ALL cases.
MRD assessment in childhood ALL (Paper II)

In paper I we demonstrated the applicability of IG/TCR rearranged genes as MRD targets in a multi-centre study. In paper II, follow-up samples from the same cohort as in paper I were analyzed for presence of MRD both by RQ-PCR, based on IG/TCR genes as targets, and multicolor FCM, based on LAI. A total of 228 patients were included in the study, with the inclusion criterion being the availability of follow-up samples in order to perform paired MRD analysis (Figure 8). The cohort consisted of 204 BCP ALL cases and 24 T-ALL cases. MRD values were obtained from 726 follow-up samples with both methods at day 15 (n=118), day 29 (n=205), day 50 (n=202) and day 106 (n=201). At day 15 and 29, the median MRD values obtained by RQ-PCR (0.83% and 0.02%, respectively) were higher compared to the median FCM values (0.01% and <0.01%/negative, respectively), whereas at day 50 and 106 the median was <0.01%/negative with both methods. A higher median MRD value with RQ-PCR is however logical since the leukemic cell clone is enriched by density centrifugation.

Using a threshold of 0.1% to compare the concordance between the RQ-PCR and FCM MRD results, 91% of the 650 paired follow-up samples from BCP ALL cases were consistent. For T-ALL cases the corresponding proportion was 86% in the 76 paired follow-up samples analyzed. At the later time points (day 50 and 106) 95% of the samples had MRD levels below 0.1% by both methods and consequently, a higher degree of concordance was observed compared to the first two time points (day 15 and 29), 96% and 82% respectively (p<0.001). When comparing the two methods, the possibility of achieving concordance is highly dependent on which time-points are included in the study. In a study by Neale et al 2004, comparing RQ-PCR and FCM MRD results in 1375 follow-up samples from 227 childhood BCP ALL patients, half of the samples were collected at time points late in therapy (> 46 days of therapy) and included both BM and blood samples (156). The majority (87%) of follow-up samples had no detectable MRD (<0.01%) using either of the methods and the concordance between methods using a 0.01% cut-off value was 97%. Of note, FCM MRD analyses were performed using enriched MNC cells, not all leukocytes as in our study. Using the 0.01% cut-off value in our series, 374 of the 651 (57%) of the paired follow-up samples were MRD negative (<0.01%) and we obtained concordance in 83% of follow-up samples.

In the paired samples where both methods reached the sensitivity level of 0.01%, 167 (26%) samples were positive and quantified by both methods (r²= 0.67) (Figure 9). One hundred and ten (17%) samples were above detection level only with one of the methods and in accordance with the enrichment of leukemic cells for samples analyzed by RQ-PCR, the majority of the discordant follow-up samples were PCR⁺/FCM⁻ (n=82, 75%). However, this discrepancy may not solely be explained by the cell source difference. We
scrutinized and re-evaluated 12 paired samples displaying ≥1 log difference and at least one MRD value ≥0.1%, interestingly, all of these samples with highest values were determined by RQ-PCR analysis. Different explanations are plausible: 1) different samples tubes were delivered to the PCR- and FCM laboratories with higher content of peripheral blood contamination in one of the tubes, 2) a subclone was followed with the PCR method, and 3) immunophenotypic modulation during therapy affected the ability of FCM to identify the leukemic cells. For instance, in one case (patient 4030), two clones were followed by RQ-PCR and the MRD level differed between the target genes by a factor of 100, clearly indicating that different subclones were followed. To evaluate if immunophenotypic modulation could be a reason for discrepant results, an extended re-evaluation is needed. The use of at least two target genes by RQ-PCR and for FCM analysis the use of two or more informative MAb combinations may reduce the risk of discrepant MRD values like this, for individual patients.

Figure 9. Correlation between 651 paired MRD values obtained by FCM and RQ-PCR and with the sensitivity level of ≤0.01% reached by both methods. The follow-up samples were collected during induction therapy (d15=108, d29=178) and during/after consolidation therapy (d50=181, d106=184). 374 samples were ≤ 0.01%/negative by both methods

• (diamond) MRD values ≥0.01% measured by both methods, n=167, R²=0.67, ▲ (triangle) MRD values ≥0.01% measured by one method and ≤0.01% by the other method, n=110, ■ (square) ≥1 log difference between FCM and RQ-PCR MRD values day 29, n=12.
For the analysis of response to treatment, we focused on MRD values obtained at protocol day 29. One hundred and eighty four BCP ALL patients were included in this analysis and within this group 15 patients had suffered from BMR and in 10 cases EMR had occurred. Regardless of whether the MRD value was obtained by RQ-PCR or FCM, our analysis showed that a MRD value ≥0.1% was a significant predictor of shorter BMR free survival. However, FCM proved to be a better predictor with regards to specificity; 9 of the 15 BMRs were in the group of 44 patients with MRD ≥0.1% day 29. By RQ-PCR, 10 of the 15 BMRs showed MRD levels ≥0.1% at day 29, however the group of patients with MRD ≥0.1% was in total 65 patients, which resulted in lower specificity compared to FCM. Due to the higher median MRD value measured by RQ-PCR than with FCM, we also applied an alternative cut-off value of ≥0.2% for RQ-PCR analysis and this improved the predictive capacity of BMR among BCP ALL cases. That notwithstanding, EMRs could not be predicted by MRD detection using either of the methods and this may relate to different biological mechanisms involved in BMR and EMR.

In T-ALL, MRD measured with RQ-PCR displayed a high sensitivity with 5/6 BMR cases having MRD values ≥0.1% compared to 3/6 cases a measured by FCM. Although both methods significantly predicted the increased risk of having relapses in T-ALL, RQ-PCR appears to be preferable for MRD assessment. Using the alternative cut-off value of MRD ≥0.2% did not alter the capacity to predict relapses in T-ALL.

Cut-off values chosen for risk stratification are dependent on both the sample time point and the therapy given prior to MRD sampling. The Dana Farber Cancer Institute (DFCI) consortium reported that an MRD level of 0.1% with RQ-PCR was the best discriminating cut-off level (day 30) when treating according to the DFCI protocol 95-01 using an induction therapy more intensive than in the NOPHO ALL-2000 protocol (10, 157). In other protocols, such as the Berlin-Frankfurt-Münster (BFM) protocol, in addition to identifying high risk patients with MRD ≥ 0.1%, a lower level of MRD (<0.01%) was found to identify a group of patients with very low risk of relapse (4, 61). In the present study, 56% and 39% of the paired MRD values were below 0.01% as measured by FCM and RQ-PCR, with a corresponding BM relapse risk of 9% by both methods. In a previous study of ALL patients treated according to the very similar NOPHO ALL-92 protocol, 40% were MRD negative (<0.01%) at day 29 measured with a sensitive competitive PCR techniques and these patients had an excellent outcome (9). That notwithstanding, in our cohort, the predictive strength using the 0.01% cut-off was less significant compared to the 0.1% level. The difference between the BFM study and ours probably is a reflection of the more intensive therapy given before their MRD measurement at day 33. At that time point the patients had received asparaginase in addition to the three-drug induction ther-
apy with prednisolone, antracyklins and vincristine, whilst only the latter are used in the NOPHO protocol prior the MRD sampling at day 29.

In conclusion, this study demonstrates that both multicolor FCM and RQ-PCR detection of rearranged IG/TCR genes are suitable as clinical tools for monitoring MRD with the aim of identifying childhood ALL cases that are at an increased risk of BMR. This was true both for BCP ALL and T-ALL analyzed with either FCM or RQ-PCR, although in T-ALL MRD assessment by RQ-PCR appears to be preferable. In addition, the cut-off value of 0.2% appears to be a more optimal threshold for MRD when measured by RQ-PCR in BCP ALL.

The influence of RNA stabilization (paper III)

Pre-analytical factors are not so often the subject of investigations but their effect on the analysis results should not be underestimated. In paper III we studied pre-analytical factors that may affect MRD results when using quantitative RT-PCR to detect fusion gene transcripts. One crucial factor is sample quality since the risk of obtaining false negatives increases when the RNA quality is suboptimal. This has led to the development of RNA stabilization systems to circumvent the problem of RNA degradation, especially if the samples do not reach the laboratory the same day. For example, previous studies have demonstrated that use of PAXgene Blood RNA Kit® for blood sampling and RNA extraction was superior for MRD detection when compared to non-stabilized blood samples stored for 72 hrs (158).

In our study we decided to compare the use of the PAXgene® system with non-stabilized blood sample collection (i.e. EDTA tubes with subsequently extraction of RNA using Trizol) in which RNA was extracted within 2-5 hrs of collection. Of note, RNA was extracted from the total leukocyte fraction in the PAX gene system, whereas the RNA from non-stabilized cells was derived from the mononuclear cell fraction and this difference in cell source may have influenced results. In the 68 paired blood samples from CML patients included in our study, a lower RNA concentration was obtained from the PAXgene tubes compared to the RNA concentration derived from non-stabilized cells. In general, lower RNA concentrations were also obtained from samples prepared from PAXgene tubes that had been stored for longer than 5 days. Two features that may have negatively influenced the RNA concentration are the larger elution volumes and smaller blood volumes used in the PAXgene system, even if the yield per ml blood was superior (due to RNA extraction from all blood leukocytes). Indeed, RNA concentration is an important consideration since the guidelines for MRD detection (103) recommend use of 1μg RNA in the cDNA synthesis reactions. In this respect a quarter of the PAXgene samples had a suboptimal RNA concentration.
Despite equivalent RNA input, the crossing points of the amplification curves (Ct values) of the housekeeping genes, \textit{ABL1} and \textit{GAPDH}, were on average 1-2 Ct values higher in the PAXgene samples compared to the corresponding EDTA/Trizol samples. This result indicates that a more efficient PCR amplification was achieved using blood collected in EDTA tubes followed by Trizol RNA extraction. Inhibitors present in the PAXgene system may be responsible for the reduced PCR amplification and a subsequent decrease in sensitivity observed during MRD assessment of the PAXgene samples. In total, 19 of the 68 parallel samples displayed discordant results between the two methods (Table 7). These differences were most pronounced among PAXgene samples processed 6-16 days after blood collection. The false negative samples did have a low \textit{BCR-ABL1} mRNA copy number and stochastic sampling effects may explain some of the negative results. However, the PAXgene method displayed a higher ratio of false negative samples, probably due to less efficient amplification.

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<th>EDTA/Trizol sample positive</th>
<th>EDTA/Trizol sample negative</th>
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<tr>
<td>PAXgene ((\leq 5) days) positive</td>
<td>12</td>
<td>1</td>
</tr>
<tr>
<td>PAXgene ((\leq 5) days) negative</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td>PAXgene (&gt; 5 days) positive</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>PAXgene (&gt;5 days) negative</td>
<td>12</td>
<td>15</td>
</tr>
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</table>

The PAXgene method results were analyzed separately for samples processed within 5 days and 6 to 16 days after blood collection. Samples were considered positive when at least one of two replicates had a Ct value <45.

It has been reported that 5 to 10 ml of blood are required to detect low levels of MRD (159), alternatively, a more stringent definition states that a minimum number of nucleated cells (1-2 \times 10^{-7}) should be used for cDNA synthesis (160). The EDTA Vacutainer® tubes draws 7 ml of blood whilst the PAXgene Vacutainer® tube is limited to withdrawal of 2.5 ml of blood. Therefore, in our comparison 14 ml vs 5 ml of blood were used in the EDTA/Trizol and PAXgene methods, respectively. MRD was detected using both type of collection tubes in 12 of the 30 paired samples (PAXgene samples processed within 5 days) and the \textit{BCR-ABL1} to housekeeping gene ratio showed a good correlation. This suggests that in CML both mononuclear cells and total white blood cells can be used for MRD analysis. Hence, extraction of RNA from total leukocytes following red cell lysis has recently been recommended for MRD detection of \textit{BCR-ABL1} fusion transcripts in CML patients as the use of mononuclear cells was deemed to be less sensitive (160).

As a control, we studied the effect of a 20-30 hrs delay, when processing blood collected in EDTA tubes, on RNA quality and quantity as well as
MRD quantification. For these 33 samples from CML patients, the RNA yield was significantly reduced compared to the EDTA/Trizol method processed within 2-5 hrs. Furthermore, the Ct values of the housekeeping genes were higher indicating less target templates. On average, the Ct values were comparable to the Ct values obtained from PAXgene samples processed within 5 days.

We conclude that optimal MRD results were achieved using EDTA samples processed within 5 hrs followed by the Trizol method for RNA extraction. However, if transit time to laboratory is expected to exceed 30 hrs from blood collection the PAXgene system is recommended.

MRD detection in Ph+ childhood ALL (paper IV)

As mentioned, many studies on childhood ALL have shown a significant correlation between the presence of MRD during treatment and clinical outcome (1-11). However, in a small subset of childhood ALL patients (~3-5%) carrying the Philadelphia chromosome, the impact of MRD monitoring has not been extensively investigated (107, 161).

In this study, a total of 140 samples from eight Ph+ ALL cases were collected for MRD assessment by RQ-PCR of IG/TCR rearranged genes, multicolor FCM as well as RT-PCR detecting BCR-ABL1 transcript. During the observation period four of the eight children relapsed as defined by morphological criteria and all open relapses were detected by the three MRD methods. However, MRD analysis failed to predict future relapses possibly due to long intervals between sampling, but also due to a very quick expansion of the leukemic clone. It has been suggested that a high levels of MRD at the time of BMT is associated with a high probability of relapse (162, 163) however in our limited series only one of the children that relapsed had high MRD level at the time of BMT, whereas the other two (patient 2 and 6) had very low or undetectable MRD at the time of BMT.

Overall, a comparable pattern of MRD kinetics was obtained in six of the eight cases. In one case (patient 7), MRD levels during induction therapy were only detected by m-BCR-ABL1 transcript analysis. This discrepancy may be explained by the persistence of a leukemic Ph+ stem cell lacking rearranged genes and CD19 expression (164). Recently, Castor et al 2005 have suggested that ALL cases expressing m-BCR-ABL1 (minor break-point) transcripts, in contrast to M-BCL-ALB1 (major break-point) expressing ALL cases, are associated with a lymphoid committed progenitor cell expressing CD34+/CD38+ or CD34+/CD38-/CD19+, but not CD34+/CD38-/CD19- expressing cells (165). However, re-evaluation of FCM plots did not reveal any CD34+, CD19- cells in this particular patient. Nevertheless, an argument supporting this theory of a leukemic Ph+ stem cell in patient 7 is the detection of a novel TCRG rearrangement at day 173 concomitant with
increased levels of BCR-ABL1 mRNA and 10% blast cells in the BM biopsy. Despite our best efforts it proved impossible to trace this new TCRG rearrangement back to the diagnostic sample, possibly due to limited sensitivity in the RQ-PCR analysis. In a study published recently by Zaliova et al 2009, 2/17 analyzed Ph+ childhood ALL showed the same pattern as patient 7 in our study (166). In addition, Zaliova et al used cell sorting (malignant cells, normal B-cells and myeloid cells) in one of their cases and detected BCR-ABL1 transcripts but not IG/TCR rearranged genes in all three cell compartments. This indicates that the translocation, t(9;22), occurs at an earlier stage in cell development compared to IG/TCR rearrangements.

The second patient with a discordant pattern of MRD was patient 3 where MRD was detected at day 29 and day 50 by FCM and IG/TCR rearrangement analysis, but without any detectable BCR-ABL1 transcripts. The reason for the differences between MRD levels was, however, in this case clearly established. Sequence analysis revealed a rare variant of the BCR-ABL1 fusion transcript joining exon 13 of BCR and exon 3 of ABL1 (b2a3). Therefore, since this rare fusion variant is not amplified by the standard assay, the BCR-ABL1 fusion transcript was not detected (103). This finding highlights the importance of performing RT-PCR of the fusion gene transcripts at diagnosis to verify the applicability of this assay. The rare BCR-ABL1 fusion lacking ABL1 exon 2 has previously been reported in a few cases of CML and ALL and is associated with a benign course, at least in CML (167-171).

Mutations in the tyrosine kinase domain were detected in two out of three patients receiving additional TKI therapy. The clone with the T315I gatekeeper mutation was selected in patient 1 during dasatinib treatment and in patient 2 after a short period of imatinib treatment. Both patients 1 and 2 suffered several relapses. These relapses in conjunction with TKI mutations and additional chromosomal aberrations (patient 1), illustrate the genetic instability of the leukemic clone and the selection of resistant clones by TKI treatments. Indeed, other studies suggest a high risk for development of TKI resistance in patients with Ph+ ALL although the role of TKI treatment in childhood ALL is still under investigation (111, 172).

One disadvantage with the RT-PCR method is that it provides no information about the number of leukemic cells, in contrast to FCM or RQ-PCR of IG/TCR genes. In these methods MRD is expressed as a percent (%) of leukemic cells/event against a background of normal cells/events, e.g. in FCM, 10 leukemic cells generate 10 signals (dots in the scatter diagram) and in RQ-PCR analysis 10 DNA copies of the leukemia specific rearrangement generate the same Ct value as 10 copies in the standard curve constructed. However, the difference between the methods are related to the background, in FCM analysis MRD is measured within all viable BM cells whilst in RQ-PCR the background is the mononuclear fraction of the BM sample. Using RT-PCR of fusion gene transcripts, we do not know anything about the relationship between the number of fusion gene transcripts or control gene tran-
scripts per cell. Nor do we know if the expression of fusion gene and/or control gene is affected by treatment. RT-PCR analysis also uses Ficoll separated cells and thereby the MRD level is measured amongst a background of mononuclear cells. Despite that, MRD is expressed as percentage (%) in all methods and Table 8 summarizes factors affecting the MRD results described.

Table 8 Description of factors affecting the quantification of MRD

<table>
<thead>
<tr>
<th>MRD method</th>
<th>Cells analyzed</th>
<th>Standard curve</th>
<th>Quantification</th>
</tr>
</thead>
<tbody>
<tr>
<td>RQ-PCR of IG/TCR genes</td>
<td>Mono nucleated cells</td>
<td>Yes, allele specific, dilution of diagnostic DNA and reference gene</td>
<td>Relative accurate quantification (fixed number of target per cells)</td>
</tr>
<tr>
<td>Flow cytometry of aberrant antigens</td>
<td>All nucleated cells</td>
<td>No</td>
<td>Accurate quantification</td>
</tr>
<tr>
<td>RT-PCR of fusion gene transcripts</td>
<td>Mono nucleated cells</td>
<td>Yes, fusion gene and control gene, plasmid dilutions</td>
<td>Relative quantification to control gene expression</td>
</tr>
</tbody>
</table>

In summary, in six of the eight Ph+ ALL cases the patterns of MRD kinetics were comparable between the three methods. Detection of fusion gene transcripts appears to be the most sensitive MRD method and proved to be the only one that detected residual leukemic cells in one of the patients. However, *BCR-ABL1* expression may not reflect the percentage of leukemic cells as the other methods do and these methods are thus complementary.
Conclusions

Tracing residual leukemic cells i.e. MRD assessment has been proven to be a powerful prognostic marker in childhood ALL during early phases of therapy (2, 4-6, 10, 173, 174). Despite the current evidence supporting the applicability of MRD detection, each therapy protocol needs to (i) establish the threshold level of MRD which identifies high-risk and low-risk patients, (ii) decide upon MRD method/s to be applied within the protocol and (iii) choose sampling time point(s) for measurement. The papers presented in this thesis detail the first national studies of MRD assessment in Sweden.

In paper I, we verified the applicability of RQ-PCR as a MRD detection method in a multi-centre study of childhood ALL. We could identify clonal IG/TCR rearrangements and establish a sensitive RQ-PCR assay ($\leq 10^{-4}$) for at least one target gene in the vast majority of cases. With the stratification threshold of $\geq 10^{-3}$ for identification of high-risk patients, 93% of BCP ALL and 86% of T-ALL reached this quantitative range with at least one target gene. Hence, this study supports the use of RQ-PCR as a robust method for MRD detection in the majority of childhood ALL cases.

In paper II, we compared RQ-PCR and FCM MRD values in follow-up samples from childhood ALL patients (the same cohort as in paper I). We also evaluated the response to treatment in the context of the NOPHO ALL-2000 protocol. Using the cut-off value of 0.1%, the concordance between RQ-PCR and FCM was 90% in the 726 paired samples analyzed. By applying the threshold value MRD $\geq 0.1\%$ late during induction therapy (day 29), we showed that MRD analysis using either multicolor FCM or RQ-PCR based on IGH/TCR rearrangements can predict the risk of BMR but not EMR. However, the median MRD level as measured by RQ-PCR was higher compared to FCM. Instead, using a cut-off value of $\geq 0.2\%$ for RQ-PCR analysis, the specificity increased in BCP ALL without affecting the sensitivity. Furthermore, in T-ALL the results indicate that RQ-PCR gave better discrimination of high-risk patients compared to FCM.

For all MRD assessments, sample quality is important to ensure reliable MRD values. The stability of RNA in vitro is of particular importance and degradation may be prevented using RNA stabilization systems, such as the PAXgene Vacutainer®. In paper III we amplified and quantified the BCR-ALB1 transcript in paired blood samples from patients with CML collected in parallel in EDTA tubes (non-stabilized blood, followed by Trizol® RNA extraction) or PAXgene tubes (stabilized blood, followed by PAXgene Sys-
tem® RNA extraction). In short, RNA extracted from PAXgene tubes generated higher Ct values, corresponding to less amplified target genes compared to the RNA extracted from EDTA tubes. We concluded that more optimal MRD results were achieved using non-stabilized blood (EDTA tubes) and with RNA extraction initiated within 5 hrs of blood collection.

In paper IV, eight childhood Ph+ ALL patients treated at our hospital were monitored using the three currently available MRD methods. Overall, a comparable pattern of MRD kinetics was obtained in six of the eight cases. In one case an unusual chromosomal break-point was identified, and this transcript could not be amplified using the standardized RT-PCR method. Another patient displayed high MRD levels when analyzed by RT-PCR, however neither RQ-PCR of rearranged IG/TCR genes nor FCM analysis could detect residual leukemic cells. One plausible explanation is the presence of an immature leukemic stem cell producing BCR-ABL1 transcripts but not rearranged IG/TCR genes or CD19 expression. Nevertheless, in this limited series detection of fusion gene transcripts appears to be the most sensitive MRD method. However, if possible, the other methods should also be utilized, since they provide valuable, complementary information.

In conclusion, all three applied MRD method within this thesis are useful and correlate well to each other, although not necessary exchangeable in each individual patient. In the majority of childhood ALL patients both RQ-PCR based on IG/TCR rearrangements and multicolor FCM are suitable as clinical tools for identification of childhood ALL cases with increased risk of BMR within the NOPHO ALL protocols.
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References


40. Landau NR, Schatz DG, Rosa M, Baltimore D. Increased frequency of N-region insertion in a murine pre-B-cell line infected with a terminal deoxynucleotidyl transferase retroviral expression vector. Mol Cell Biol 1987;7:3237-43.


131. Rosenquist R, Thunberg U, Li AH, et al. Clonal evolution as judged by immunoglobulin heavy chain gene rearrangements in relapsing precursor-B acute lymphoblastic leuke-


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