DISSERTATIONES MEDICINAE UNIVERSITATIS TARTUENSIS
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**EDWARD LAANE**

# Multiparameter flow cytometry in haematological malignancies

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*To Siim and Elise*

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I **Laane, E.**, Tani, E., Björklund, E., Elmberger, G., Everaus, H., Skoog, L. and Porwit-MacDonald, A. (2005) Flow cytometry immunophenotyping including Bcl-2 detection on fine needle aspirates in the diagnosis of reactive lymphadenopathy and non-Hodgkin's lymphoma. *Cytometry: Part B – Clinical Cytometry* 64, 34–42.

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I Collection, organization and analysis of flow cytometry data. Collection, organization and analysis of clinical data. Data preparation for statistical analysis. Data interpretation. Manuscript writing.

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# ABBREVIATIONS

| | | |
|---|---|---|
| 6-MP/MTX | – | 6-mercaptopurine/methotrexate |
| AL | – | acute leukemia |
| ALK | – | anaplastic lymphoma kinase |
| ALL | – | acute lymphoblastic leukemia |
| Allo-SCT | – | allogeneic stem cell transplantation |
| AML | – | acute myeloid leukemia |
| APC | – | allophycocyanin |
| Ara-C | – | arabinofuranosyl cytidine |
| AUL | – | acute undifferentiated leukemia |
| Auto-SCT | – | autologous stem cell transplantation |
| Bcl-2 | – | B-cell lymphoma 2 |
| BD | – | Becton Dickinson |
| BL | – | Burkitt lymphoma |
| BM | – | bone marrow |
| B-NHL | – | B-cell non-Hodgkin's lymphoma |
| B-NHL-UN | – | B-cell non-Hodgkin's lymphoma, unspecified |
| CD | – | cluster of differentiation |
| CLL | – | chronic lymphocytic leukemia |
| CNS | – | central nervous system |
| CR | – | complete remission |
| Cy-5 | – | indodicarbocyanine |
| DC | – | dendritic cells |
| Dexa/Vcr | – | dexamethasone/vincristine |
| DLBCL | – | diffuse large B-cell lymphoma |
| DNA | – | deoxyribonucleic acid |
| EBV | – | Epstein-Barr virus |
| FAB | – | French-American-British |
| FACS | – | fluorescent activating cell sorter |
| FCS | – | Flow Cytometry Standard |
| FCM | – | multiparameter flow cytometry |
| FISH | – | fluorescence in situ hybridization |
| FITC | – | fluorescein isothiocyanate |
| FL | – | follicular lymphoma |
| FNA | – | fine needle aspiration |
| FSC | – | forward side scatter |

| | | |
|---|---|---|
| Gr | – | group |
| Hb | – | hemoglobin |
| HC | – | hospital control |
| HHV8 | – | human herpesvirus-8 |
| HIV | – | human immunodeficiency virus |
| HR | – | high risk |
| K/L | – | kappa/lambda ratio |
| IC | – | immunocytochemistry |
| IC/LPL | – | immunocytoma/lymphoplasmocytic lymphoma |
| Ig | – | immunoglobulin |
| II | – | intermediate intensity |
| LAIP | – | leukemia-associated immunophenotype |
| LD | – | log difference |
| LG-NHL | – | low-grade non-Hodgkin's lymphoma |
| Lin | – | lineage cocktail |
| MALT | – | mucosa-associated lymphoid tissue |
| MCL | – | mantle cell lymphoma |
| mCR | – | morphological complete remission |
| mDC | – | myeloid dendritic cell subset |
| MDS | – | myelodysplastic syndrome |
| MFI | – | mean fluorescence intensity |
| moAb | – | monoclonal antibody |
| MRD | – | minimal residual disease |
| MTX | – | methotrexate |
| My | – | myeloid marker |
| NA | – | not available |
| NHL | – | non-Hodgkin's lymphoma |
| NIH | – | National Institute of Health, U.S.A. |
| NK | – | natural killer |
| NK-NHL | – | natural killer-cell non-Hodgkin's lymphoma |
| NMZL | – | nodal marginal zone B-cell lymphoma |
| NOPHO | – | Nordic Society of Pediatric Hematology and Oncology |
| OS | – | overall survival |
| PBS | – | phosphate buffered saline |
| PCR | – | polymerase chain reaction |
| pDC | – | plasmacytoid/lymphoid dendritic cell subset |
| PE | – | R-phycoerythrin |

| | | |
|---|---|---|
| PerCP | – | peridinin chlorophyll protein |
| preB-ALL | – | precursor B lymphoblastic leukemia |
| preT-ALL | – | precursor T lymphoblastic leukemia |
| pts | – | patients |
| RFS | – | relapse-free survival |
| RH | – | reactive hyperplasia |
| RNA | – | ribonucleic acid |
| RRR | – | relative risk of relapse |
| RT | – | room temperature |
| SCT | – | stem cell transplantation |
| SI | – | standard intensity |
| sIg | – | surface immunoglobulin |
| SR | – | standard risk |
| SSC | – | side-scatter |
| TCRBCL | – | T-cell rich B-cell lymphoma |
| TdT | – | terminal deoxynucleotidyl transferase |
| T-NHL | – | T-cell non-Hodgkin's lymphoma |
| WBC | – | white blood cell |
| WHO | – | World Health Organization |
| X-RT | – | radiation therapy |

# INTRODUCTION

Multiparameter flow cytometry (FCM) is a powerful laser-based technology that is used to characterize a cell and its characteristics. In haematology FCM is widely used and has become the integral part of diagnostic work-up of lymphoid as well as myeloid malignancies. Malignancies are classified into four major categories, according to the tissue and cell types from which they arise: malignancies derived from epithelial cells (carcinomas), from connective tissue or muscle cells (sarcomas), from haematopoietic tissue (leukemias and lymphomas) and malignancies derived from cells of the nervous system. By current concept, malignancies originate from a single cell that has undergone a specific genetic change that enables it to outgrow its neighbors (Hanahan and Weinberg, 2000). It also means that malignancies are clonal. Malignancies of haematopoietic system are different from other tumors as far as their evolution and dissemination are not as dependent on angiogenesis, architectural disruption and metastatic processes that are critically involved in most other malignancies (Graves et al., 1986). Haematological malignancies originate from cells of the bone marrow (BM) and the lymphatic system. Three major forms of haematological neoplasms are leukemias, lymphomas and plasma cell neoplasms. The number of new patients in Europe diagnosed with haematological malignancies can be estimated in approximately 230 000 patients. The new cases of leukemia, lymphoma and myeloma account for 8% of all the new cancer patients diagnosed in Europe and the estimated deaths from the haematological malignancies account for 7% of the cancer-related deaths. The overall incidence of haematological malignancies appears to be rising (Rodriguez-Abreu et al., 2007). Peripheral blood and BM are ideal sources of material for FCM studies due to natural suspension of cells. Lymphoid cells are also easily available from lymph nodes and aspiration material. This feature is the basis for popularity of FCM in haematology. Although FCM has been traditionally used to determine lymphoid cells of the immune system, the development of the method including the possibility of detecting intracellular antigens has broaden the applicability of FCM considerably. It is possible to detect by FCM intracellular proteins that regulate the cell homeostasis and critical biochemical pathways. Another excellent feature of FCM is the power to detect small cell populations at submicroscopic level and give quantitative results. In practical terms this allows more precise evaluation of treatment response and patient stratification in order to choose the most optimal treatment modality. Finally, with development of antibodies it is possible to explore new important cell types in haematopoietic system, which co-ordinate immune system and may even play an important role in tumor development and tumor control.

# REVIEW OF THE LITERATURE

## 1. Flow cytometry

Cytometry is defined as the measurement of cells. Nowadays FCM is capable of rapid, accurate and reproducible measurement of cell properties at high speed. The most widely used clinical application of FCM in haematology is the classification of haematopoietic cells by cell surface antigens. However, there are many other applications and approaches of FCM such as analysis of DNA, studies of cell proliferation and death, RNA and protein content, calcium ions, kinetics of intracellular enzymes, microbiology and biotechnology analysis. These applications are more specifically described in the "Bible of FCM" written by H.M. Shapiro (Shapiro, 2003).

### 1.1. History of flow cytometry

Significant achievements in the development of FCM capabilities are given in Table 1.

**Table 1.** A brief description of achievements in the development of FCM.

| Year | Development |
|---|---|
| 1934 | Photoelectric measurement of cells in a capillary |
| 1941 | Development of fluorescence antibody technique |
| 1947 | Photoelectric particle counting |
| 1949 | Particle counting by Coulter volume |
| 1953 | Hydrodynamic focusing for reproducible delivery of cells in a fluid |
| 1955 | Automated scanning instrument for screening cytological smears |
| 1956 | High speed automatic counting by Coulter volume |
| 1961 | First use of fluoresence for quantitation |
| 1964 | Electrostatic principle for ink jet<br>Acridine orange differentiation of leukocytes |
| 1965 | Cell sorting<br>Particle separator in principle capable of separating by volume, optical density, or fluoresence<br>Spectrophotometry of cells |
| 1967 | The 1'st paper of fluoresence flow cytometry |
| 1968 | Automated imaging<br>Fluorescence flow cytometry patent<br>Flow vs scanning for cancer cytology |
| 1969 | The 2nd, 3rd and 4th paper of fluoresence flow cytometry<br>The 1st paper describing light scatter |
| 1972 | Fluorescence-activated cell sorting |
| 1973 | Doublet discrimination patent |
| 1974 | Mathematical analysis of DNA distributions |
| 1975 | Invention of monoclonal antibodies |

| | |
|---|---|
| 1977 | The first use of monoclonal antibodies in flow cytometry<br>Two-color fluoresence compensation |
| 1978 | Radiation collector methods – three patents |
| 1979 | The second use of monoclonal antibodies in flow cytometry<br>Flow imaging<br>Patent of the radiant energy reradiating flow cell system |
| 1982 | Slit-scanning flow cytometer |
| 1984 | Convention of nomenclature for DNA cytometry – analysis guidelines<br>Proposal for data file standard (FCS 1.0)<br>Three-color immunofluorescence |
| 1987 | Time as a quality control parameter<br>Dual-beam high-speed sorting |
| 1988 | 4 pi light collection flow chamber – increased sensitivity |
| 1990 | Data file standard (FCS 2.0) |
| 1991 | Barcode reader – first automation of flow cytometry for clinical systems |
| 1995 | Five-color flow cytometry |
| 1997 | Eight-color, ten parameter flow cytometry |
| 1997 | Data file standard (FCS 3.0) |
| 2001 | Eleven-color, 13 parameter flow cytometry |
| 2004 | Seventeen-color flow cytometry |
| 2006 | Quantum dots in flow cytometry |
| 2010 | Data file standard (FCS 3.1) |

The first important publication, which could be argued as the foundation of cell analysis, was published by Moldovan (Moldovan, 1934). This was the first attempt to count or measure cells flowing in suspension. Basically he used microscope that was focused on a capillary glass tube through which a forced suspension of cells, recording the passage of each cell by a photoelectric device at the eyepiece. It is also believed that the basis of modern analytical cytology was established by Torbjorn Caspersson in 1941 in Stockholm who showed that nucleic acids were necessary prerequisites for the protein synthesis in the cell. These studies were performed by using cadmium spark source for ultraviolet light and electronic circuits for detection of signals. The next important discovery was made in 1940s were Coons and coworkers (Coons et al., 1941) developed the fluorescent antibody technique. Further, in 1950 Coon and Kaplan reported a new fluorochrome, fluorescein, conjugated in isocyanate. This was the first definitive fluorochrome used at present day. In 1953 P.J. Crosland-Taylor published the sheath-flow principle used to present day in almost all flow cytometers (Crosland-Taylor, 1953). He showed that the clogging could be prevented by using a large diameter channel and centering the cell stream in a fluid sheath using the principle of laminar flow. Probably the key inventor in flow cytometry was Wallace H. Coulter. His first commercial Coulter ® Counter was marketed in 1956 in which an electronic measurement for cell-counting and sizing – the Coulter Principle, was achieved (Coulter,

1956). This instrument was able to give an immediate blood cell count and utilized a very small volume of blood. Electrostatic cell sorter was invented by Mack Fulwyler in 1965 (Fulwyler, 1965). In 1965 Louis Kamentsky and coworkers were able to minimize the clogging by using a bowtie shaped channel and built the first multiparameter flow cytometry. As the next step, in 1967 Kamentsky and Melamed elaborated on Moldaven's method of forcing cells through a capillary tube and designed a sorting FCM (Kamentsky and Melamed, 1967). At the same time Marvin Van Dilla at Los Alamos was interested in cell cycle studies and identified fluorescence signals as a promising approach. He was the first who publicly reported on January 23, 1968 the results of fluorescence measurement including DNA histogram (Sack, 2009). Parallel in Europe, Wolfgang Göhde in Germany, inspired of the work of Caspersson, designed in 1969 the first commercially available flow Cytometer, the ICP-11 Impulscytophotometer sold by Phywe, Göttingen (Sack, 2009).

Another one of the most important cytometry's pioneers is Leonard Herzenberg at Stanford University. His work led to the commercialization of cell sorters by Becton Dickinson (BD). In short, Herzenberg had obtained an National Institute of Health, U.S.A. (NIH) grant to build up two machines, one for NIH and one for Stanford. Bernie Shoor, a long-time BD employee, worked out an agreement with Herzenberg to have BD actually build the instruments. BD negotiated a license and built both machines, called Fluorescence-Activated Cell Sorter (FACS)-1, which were delivered in early 1973 (Sack, 2009). The next important date is 1975 when Köhler and Milstein introduced monoclonal antibodies (moAb) technology, which provided the basis for highly specific immunological reagents for use in cell studies (Köhler and Milstein, 1975). At the same year, in early 1975, NIH ordered from Mack Fluwyler to build a large multiparameter sorter to the National Cancer Institute. This machine EPICS II (Electronically Programmable Individual Cell Sorter) was shipped in July 1975 (Sack, 2009).

It was a fundamental progress to the field of cytometry when Loken et al defined the process of spectral overlap using two-color analysis (Loken et al., 1977). This process was subsequently repeated as each color was added to rise the number of simultaneous colors up to 17 (Perfetto et al., 2004). However, in clinical samples the usage of panels with 8–10 colors seems to be sufficient.

An important milestone in the history of cytometry was the creation of the Flow Cytometry Standard (FCS) format opening thus the field to those outside of the major companies (Murphy and Chused, 1984). The FCS standard transformed the field of cytometry. Over the years FCS has been continuously updated from FSC 1.0 to FSC 3.1 protecting thus the stability and accuracy of the entire field (Spidlen et al., 2010).

The first International Workshop on Human Leukocyte Differentiation Antigens was held in 1984 (Bertrand et al., 1984). This and following workshops defined antigens on leukocytes and other cells and allocated cluster of differentiation (CD) numbers of them.

## 1.2. The beginning of clinical cytometry

DNA analysis was a natural application for clinical cytometry and it was assumed from early days with work of Van Dilla that this would have a great clinical significance. However, the majority of clinical applications were focused on lymphocyte and lately leukocyte phenotyping. The study of lymphocytes was heavily influenced by the discovery of moAbs in 1975 (Köhler and Milstein, 1975).

Over the past 40 years, it has been a well-defined periods of technology development. The 1950s was the decade of cell counting, the 1960s the emergence of cell sorting, the 1970s the fluorescence, the 1980s the second period of sorting and from the 1990s the era of multicolor fluorescence with re-emerge of high-speed cell sorting with low-cost analyzers. The next generation instruments will probably come a tool for the masses and this will be the ultimate success for flow cytometry (Sack, 2009).

## 1.3. The principles of flow cytometry

FCM scans single cells or particles as they flow in a liquid medium past an excitation light source. The basic principle of FCM is that light is scattered and fluorescence is emitted as light from the excitation source strikes the moving particles.

A basic FCM consists of a source of light, a flow cell, optical components to focus light of different colors on to the detectors, electronics to amplify and process the resulting signals digitally with a computer. This process can be described as four interrelated systems, shown in Figure 1.

In flow cytometer the cells are moving in a liquid linear stream through the laser beam, passing the beam one at the time. This is achieved by hydrodynamic focusing. As the cell passes through the laser beam, light is scattered in all directions and fluorescence signals are elicited. Cells are counted and partly identified by differential light scatter.

Computer analysis is based on size (FSC), granularity (SSC) and whether the cells are carrying fluorescent molecules either in forms of antibodies or dyes. Photo-detectors convert the light signals to electric impulses, which are processed and analyzed. Fluorescence occurs when a fluorescent molecule absorbs light at one wavelength, reaches an excited state and then returns to the ground state, emitting light at longer wavelength. Argon lasers, which are the most common light source in FCM, produce monochromatc light at 488 nm, which excites many fluorochromes.

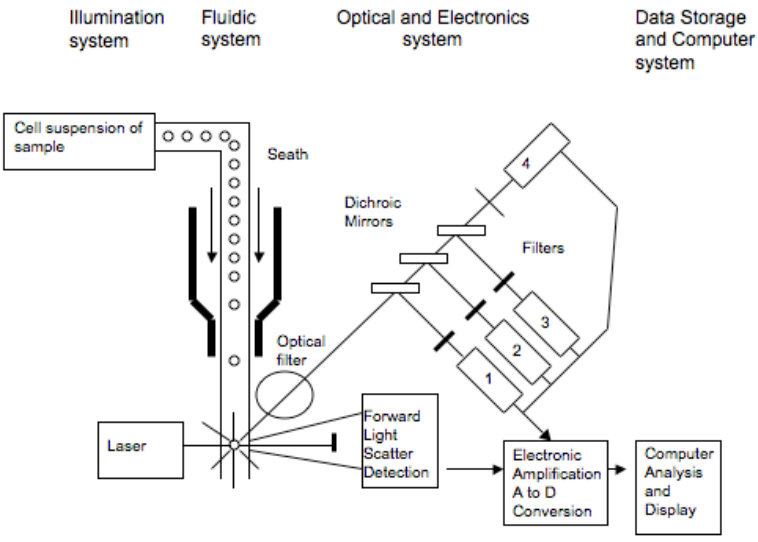


**Figure 1.** General structure of flow cytometer.

Adapted from Brown and Wittwer, 2000.

A single cell suspension is hydrodynamically focused with sheath fluid to intersect an argon-ion laser. Forward side scatter (FSC) is detected as a correlate of cell size. Dichroic mirrors and filters are spectrally separating side-scatter (SSC) as a measure of granularity (detector 1) and fluorescence signals (detectors 2–4). The light signals are amplified and data of each cell is converted to digital form by photomultipliers for analysis and display on a computer screen.

## 1.4. Standardization in flow cytometry

The intra- and inter-laboratory reproducibility of a test must be achieved and optimized in order to become a diagnostic laboratory test. However, there are a wide range of different reagents and methods used for sample preparation, data acquisition and analysis of FCM immunophenotyping (Bènè et al., 1995; Hasset and Parker, 1995; Homburger et al., 1993; van'T Veer et al., 1992). Therefore consensus protocols, data interpretation and reporting for leukemia and lymphoma immunophenotyping are needed.

A Concerted Action in EU Biomedical and Health research program started in 1994 and was the first international study in the field of diagnosis of minimal residual disease (MRD), the disease level which is not detectable by conventional light microscopy. The following centers participated: Department of Immunology, Erasmus University and University Hospital, Rotterdam and Dutch Childhood Leukemia Study Group, The Hague, The Netherlands; Karolinska Hospital, Stockholm, Sweden; M Tettamanti Research Center, Monza, Italy and Portuguese Institute of Oncology, Lisbon, Portugal. The aims were an investigation of MRD in acute leukemias and development of standardized FCM and PCR-based MRD techniques (San Miguel et al., 1999).

At present, EuroFlow, a scientific consortium takes and effort to develop new tools for fast, sensitive and standardized tools for diagnostics of haematological malignancies. EuroFlow consortium consists of ten diagnostic research groups from the Netherlands, Spain, Portugal, Germany, France, Czech Republic, Poland and Belgium. The major aim of this project is to publish optimized and standardized immunostaining protocols for the diagnosis, classification, and prognostic subclassification of haematological malignancies as well as for detection of MRD during follow-up.

Another important institution, which has worked for FCM standardization is European Leukemia Net Foundation, Project 10: Diagnostics, lead by M.C. Béné and G. Zini. Several guidelines on normal BM analysis (Arnoulet et al., 2010), myelodysplastic syndromes (van de Loosdrecht et al., 2009) and general recommendations (Béné and Kaeda, 2009) are published.

### 1.5. How to use flow cytometry in practice to avoid errors

To get reliable FCM results, it is generally important:

- to maintain a clean fluidic system, drawing a 10–30% bleach solution followed by a distilled water to avoid carry-over effect
- to adjust sample flow rates since too high rates during acquisition can result in lower data resolution
- to avoid the production of aerosols in the fluidic system
- to ascertain that all MoAbs and other reagents are compatible with the FCM
- to select fluorochromes that can be detected using the optical configuration of the FCM and understanding that emission spectra overlap contributes to detection, which can be used to guide fluorochrome selection in multicolor analysis
- to determine which parameters will be used for acquisition, to decide which appropriate control samples will be used and what type of data analysis will be performed
- and most importantly to make well-labeled tubes and utilize well-defined protocol.

## 2. Classification of Haematopoietic malignancies

The the World Health Organization (WHO) classification of haematological malignancies stratifies neoplasmas primarily according to cell lineage: myeloid, lymphoid, histiocytic/dendritic cell and mast cell. According to the WHO, lymphomas are classified in the principle of distinct ckinical entities (Jaffe, 2001). For this all available information has generally to be used, including morphology, immunophenotype, genetic and clinical data. However, morphology and immunophenotype are sufficient for the diagnosis in most cases.

## 2.1. Lymphomas

Lymphomas are malignant clonal proliferation of lymphocytes. Lymphomas are divided into two major categories (Jaffe, 2001): Hogkin's lymphoma and non-Hodgkin's lymphoma (NHL).

Non-Hodgkin's lymphoma is divided:

- B-cell non-Hodgkin's lymphoma (B-NHL)
- T-cell non-Hodgkin's lymphoma (T-NHL) and natural killer (NK)-cell non-Hodgkin's lymphoma (NK-NHL).

### *2.1.1. B-NHL*

***Inicidence***

The most common lymphoma worldwide is B-NHL. B-cell lymphomas compromise over 85% of NHLs and approximately 4% of new cancers cases around the world. B-NHLs are more common in developed countries, particularly in the United States, Europe, Australia and New Zealand. The annual incidence of B-NHL ranges from 1.2/100 000 in China to over 15/100 000 in the United States, with intermediate incidence rates in South America, Africa and Japan. Two most common types are large B-NHL and follicular lymphoma (FL), which compromise 50% of all NHLs. The individual B-NHLs vary in their relative frequency in different areas of the world. FL is more common in developed countries, particularly in the United States and Western Europe, and is uncommon in Eastern Europe, Africa and Asia. Burkitt lymphoma (BL) is endemic in equatorial Africa, where it is the most common malignancy of childhood, but compromises only 1–2% of lymphomas in Western Europe and in the United States (Jaffe, 2001). The incidence of lymphoma in Lithuania as a representative of Baltic region is presented in Table 2 (Griškevičius, et al., 2007). The median age for all types of B-NHLs is 60–70 years, but BL has a median age in adults of 30 years.

**Table 2.** Lymphoma in Lithuania – retrospective analysis 2004–2006.

| Subtype | Proportion of cases (%) | Incidence /100 000 |
|---|---|---|
| Diffuse Large B Cell | 52.77 | 3.81 |
| Small lymphocytic | 9.61 | 0.69 |
| Mantle cell | 8.21 | 0.60 |
| Marginal zone | 6.63 | 0.48 |
| Peripheral T, unspecified | 5.28 | 0.38 |
| Follicular | 4.33 | 0.31 |
| Primary mediastinal | 2.44 | 0.18 |
| Burkitt lymphoma | 2.17 | 0.16 |
| Mycosis fungoides | 1.62 | 0.12 |
| Angioimmunoblastic T | 1.49 | 0.11 |
| Anaplastic large cell | 1.35 | 0.10 |
| Lymphoplasmocytic | 1.22 | 0.09 |

### *Aetiology*

Infectious agents are associated with the development of several types of B-NHLs. Epstein-Barr virus (EBV) is present in nearly 100% of endemic BL (Prevot et al., 1992) and in 40% of sporadic and HIV associated cases (Hamilton-Dutoit et al., 1993). EBV is commonly involved in the pathogenesis of the majority of B-NHLs arising in iatrogenically immunosupressed patients. Human herpesvirus-8 (HHV8) /Kaposi sarcoma herpesvirus is associated in the pathogenesis of primary effusion lymphoma and the lymphomas associated with Castelman disease in HIV-infected patients (Cesarman et al., 1995). Hepatitis C virus is implicated in lymphoplasmocytic lymphoma associated with type II cryoglobulinemia (Agnello et al., 1992) and with some lymphomas of the liver and salivary glands (Ascoli et al., 1998). Gastric MALT lymphomas with *H. pylori* infection depend on the presence of *H. pylori* activated T cells and these lymphomas may respond to antibacterial treatment (Wotherspoon et al., 1993). *B. burgdorferi* has been implicated in the pathogenesis of cutaneous MALT lymphoma (Cerroni et al., 1997).

The major risk factor for B-NHLs is considered an abnormality of the immune system, either immunodeficiency or autoimmune disease. Although in most patients with B-NHLs immune system abnormalities are not found, immunodeficient patients have a markedly increased incidence of B-cell neoplasia, particularly large B-cell lymphoma and BL. Major forms of immunodeficiency are at present infection with HIV, iatrogenic immuno-supression to prevent allograft rejection or graft versus host disease. Hashimoto thyroiditis may have association with extranodal marginal zone MALT lymphoma.

### *Genetics*

Three B-NHLs have characteristic genetic abnormalities that are important in determining their biologic features and that are useful for differential diagnosis. These include: t(14;18) in FL, t(11;14) in MCL and t(8;14) in BL. Characteristic for these translocations is that a cellular proto-oncogene is placed under the control of the immunoglobulin promotor on chromosome 14q, resulting in constitutive activation of the gene which in turn gives to the cell survival or proliferative advantage. In FL, t(14;18) translocation results in over-expression of an anti-apoptosis gene Bcl-2. In MCL and BL, the translocations result in over-expression of cell cycle genes associated with proliferation Cyclin D1 or Myc, respectively (Jaffe, 2001).

### *Diagnostics*

Immunophenotyping of lymphoma cells is essential in lymphoma diagnostics (Jaffe, 2001). However, no one antigen is specific for any of B-NHLs, and a

combination of morphologic features and a panel of antigens is necessary for the correct diagnosis. In some cases, clinical knowledge is essential, such as marginal zone lymphoma of MALT type versus nodal or splenic marginal zone lymphoma, and mediastinal large B-cell lymphoma (Jaffe, 2001). In difficult cases cytogenetic, molecular genetic and/or fluorescence in situ hybridization (FISH) analyses are mandatory. These techniques are used to determine clonality and specific genetic rearrangements characteristic to lymphoma subcategories.

Therefore correct lymphoma diagnosis is based on:

- Morphology
- Immunophenotype
- Cytogenetics and/or FISH
- Molecular genetics.

Immunophenotyping is used to distinguish the lymphomas of small cell types from reactive hyperplasia (RH) and the proliferating lymphomas from non-lymphoid tumors. Immunophenotyping is also used to subclassify morphologically overlapping lymphomas.

***Clinical presentation***

In the WHO classification, B-NHLs are listed according to their major clinical presentations:

1) Predominantly disseminated lymphoma/leukemia
   - CLL/small lymphocytic lymphoma
   - Lymphoplasmocytic lymphoma/Waldenström's macroglobulinema
   - Hairy cell leukemia
   - Splenic marginal zone lymphoma
   - Plasma cell myeloma.

In general, these lymphomas have relatively indolent clinical course but present with disseminated disease. The usual sites of involvement are BM with or without peripheral blood and solid tissues such as spleen or lymph nodes.

2) Primary extranodal lymphomas
   - MALT lymphomas.

MALT lymphomas virtually always present in extranodal sites, and probably correspond to normal lymphoid cells specific for extranodal immunologic reactions. These lymphomas less likely disseminate, and if they do, the dissemination is more often to other extranodal sites than to lymph nodes or BM.

3) Predominantly nodal lymphomas

- FL (Figure 2)
- Mantle cell lymphoma (MCL)
- Nodal marginal zone lymphoma (NMZL)
- Diffuse large B-cell lymphoma (DLBCL)
- BL.

These lymphomas typically present with disseminated disease involving predominantly lymph nodes, but with frequent involvement of BM, spleen and liver. They also may involve other extranodal sites, but rarely present with localised extranodal disease. FL and NMZL are low-grade lymphomas with indolent clinical course. MCL has been recognized relatively recently. MCL can be classified as indolent lymphoma since its survival is in years, but its median survival is significantly shorter than that of FL.

DLBCL and BL are aggressive B-NHLs, which may present with either nodal or extranodal disease and may be either localized or disseminated. DLBCL is the most common lymphoma worldwide, accounting approximately 30% of the cases. Patients typically present with rapidly growing masses at a localized nodal or extranodal site. An important clinical subtype of DLBCL is primary mediastinal (thymic) large B-cell lymphoma. This is an aggressive lymphoma of young adults with a slight female predominance. Distinctive clinical entities of DLBCL are also primary effusion lymphoma and intravascular lymphoma. Morphological variants of DLBCL are: centroblastic, immunoblastic, T-cell rich and anaplastic.

BL is a highly aggressive lymphoma. Typically rapidly proliferating B cells are medium-sized and bearing translocations resulting in deregulation of the c-myc oncogene. Major clinical subtypes of BL are: endemic, sporadic and immunodeficiency-associated. Some cases that have morphologic features intermediate between typical BL and DLBCL are called “Burkitt-like”. In the WHO classification this category is a subtype of BL, so-called atypical Burkitt/Burkitt-like lymphoma (Jaffe, 2001).

Illustration of Follicular lymphoma. (A) Touch imprint cytology shows predominantly small to medium-sized cells centrocytes (➔) with cleaved nuclei and occasional large cells (⇨) called centroblasts (May-Grünwald-Giemsa, original magnification x 10). (B). Immunophenotype of kappa positive follicular lymphoma cells (red dots). Follicular lymphoma cells were positive for CD10, CD19, CD20, CD79b and CD45, but were negative for CD5 and CD23.

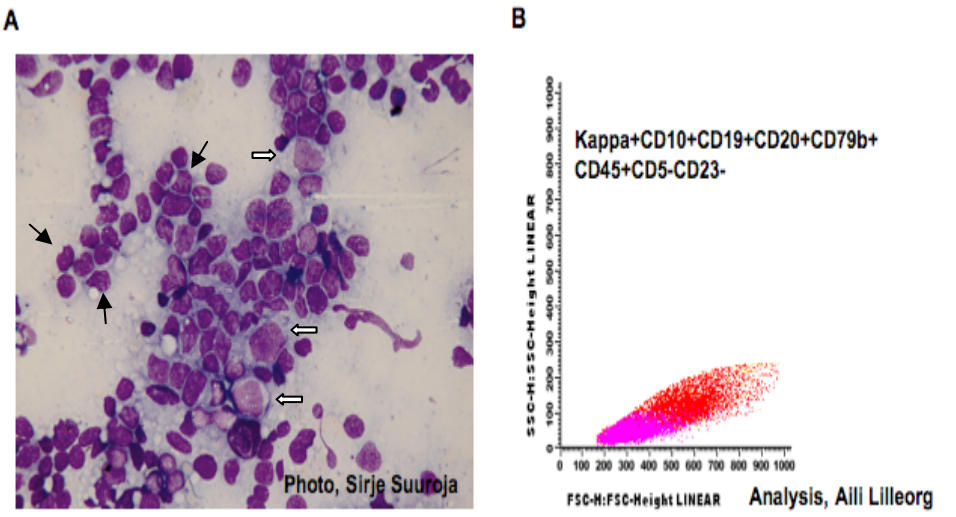


**Figure 2.** Cytology and immunophenotyping of Follicular lymphoma.

### *Treatment and outcome*

Natural history and sensitivity to treatment of the B-NHLs are extremely heterogeneous. Therefore the correct diagnosis is essential to predict clinical outcome and direct therapy. Patients with indolent lymphomas such as CLL, FL and plasma cell myeloma are considered incurable and may be observed without therapy until patients become symptomatic. The median survival exceeds generally five or more years.

Treatment options are: radiotherapy, chemotherapy, immunochemotherapy and radioimmunochemotherapy. Localized MALT lymphoma may be cured with radiotherapy. MCL combines features both of indolent and aggressive lymphomas and is considered at present incurable, with a median survival of three years. However, treatment protocols incorporating high dose arabino-furanosyl cytidine (Ara-C) and autologous transplantation seems promising (Geisler et al., 2008). In certain B-NHLs such as FL or hairy cell leukemia biological agent α-interferon could be effective. The exact cell-death mechanism of α-interferon is not fully elucidated since interferons have multiple actions including antiproliferative, apoptotic and anti-angiogenic effects. Furthermore, interferons have ability to modulate an immune response specifically activating cytolytic T cells and NK cells. Hairy cell leukemia is also responsive to pentostatin or cladribine with excellent treatment results. Long-term survival could be achieved in 80% of patients (Else et al., 2009). High rates (87%) of CR in FL have been achieved with radioimmunotherapy with yttrium-90 ibritumomab tiuxetan (Morschhauser et al., 2008). The most powerful treatment for CLL is chemoimmunotherapy regimen combining fludarabine with

cyclophosphamide and rituximab. Treatment response is achieved over 90% of patients (Hallek et al., 2010; Tam et al., 2008). Waldenström's macroglobulinemia is responsive to bendamustine (Treon et al., 2011).

In paradox, aggressive high-grade lymphomas are potentially curable. Currently approximately 40% of DLBCL are cured with aggressive chemo-immunotherapy containing adriamycin (Coiffier et al., 2010). The back-bone of chemotherpay is usually CHOP regimen consisting of cyclophosphamide, adriamycin, vincristine and prednisolone. If lymphoma cells are CD20 positive, then monoclonal antibody against CD20, rituximab is usually added to CHOP. This immunochemotherapy regimen, R-CHOP is administrated every three or two weeks, named R-CHOP 21 or R-CHOP14, respectively. If lymphoma cells are CD20 negative, adding of etoposide (CHOEP) is an option (Wunderlich et al., 2003). BL is highly aggressive and is treated with more aggressive chemotherapeutic regimens, including rituximab (Oriol et al., 2008).

The future is directed to investigate potential genetic targets (Alizadeh et al., 2000). Close cooperation between haematologists and pathologists is essential for the future progress in the cure of lymphomas.

### *2.1.2. T-NHL and NK-NHL*

T-NHLs are derived from mature or post-thymic T cells. Because NK cells are closely related and share some functional and immunopheotypic properties with T cells, WHO classification considers NK-NHL together with T-NHL (Jaffe, 2001). These lymphomas are relatively uncommon, comprising approximately 12% of all lymphomas (Anon et al., 1997). Clinically, T-NHLs and NK-NHLs are one of the most aggressive lymphomas with some exceptions such as Mycosis fungoides or ALK-positive anaplastic large cell lymphoma. Aggressive clinical behavior is related to advanced clinical stage and resistance to common chemotherapy.

## 2.2. Acute leukemias

Acute leukemia (AL) is a malignant disorder of haematopoietic cell precursors, which manifest as a clonal expansion of malignant blasts in BM, blood or other tissues. AL is divided into two categories depending on the origin of the neoplastic cells: acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML). ALL is characterized by proliferation of lymphoblasts that originate from a lymphocyte progenitor cell. AML originates from the myeloid haematopoietic progenitors, which include myeloblasts, monoblasts, erythroblasts and megakaryoblasts. The proliferative process may involve one or several of these precursor cells.

Worldwide, the overall incidence of AL is approximately 4/100 000 per year, with 70% of cases being AML. The majority of AML cases occur in adults

at median age 60 years and with an incidence of 10/100 000 per year in individuals older than 60 years. ALL is predominantly a disease of children, 75% of cases are diagnosed below 6 years of age (Jaffe, 2001). The incidence peak of ALL is at age 2–7 years, where the incidence is as high as 10/100 000 children per year (Schmiegelow and Gustafsson, 2005). The incidence of ALL is highest in the developed countries. The most common subgroups are B-cell precursor ALL with a t(12;21)(p13;q22) or a high-hyperdiploid genotype. The outcome for children is superior at the peak incidence years age 2–7 when compared to children in older or younger age groups (Schmiegelow and Gustafsson, 2005).

In Estonia, during 1982–1996 the age standardized incidence rate regarding total acute *de novo* leukemias was 1.49/100 000 inhabitants for patients aged 16–64 years and 5.31/100 000 for patients ≥65 years (Wennström et al., 2004; Luik et al., 2004).

### 2.2.1. Aetiology

The aetiology and pathogenetic mechanisms of AL remain unknown in the vast majority of cases. The possible triggering factors associated with AML include ionizing radiation, previous cytotoxic chemotherapy with chlorambucil, cyclophosphamide, melphalan, thiotepa, treosulphan, etoposide and benzene. Cigarette smoking may increase the risk by two-fold. However, only 1–2% of the diagnosed AML can be related to these genotoxic agents (Jaffe, 2001). The aetiology of ALL is still largely unknown. Autosomal dominant and recessive cases of familial AL, in addition to associations with a variety of genetic syndromes including trisomy 21, Fanconi's anaemia, ataxia teleangiectasia and Bloom's syndrome are rare (Horowitz, 1997).

### 2.2.2. Classification of acute leukemia

The first widely used classification of AL was introduced in 1976 by the French-American-British (FAB) Cooperative group (Bennett et al., 1976) with modification in 1982 (Bennett et al., 1982) and 1985 (Bennett et al., 1985a; Bennett et al., 1985b). The FAB classification is based on morphological characteristics of the malignant cells in association with cytochemical stainings, immunophenotype and ultrastructural features.

In purpose to highlight the prognostic relevance of the chromosomal abnormalities and the biologic features, the introduced WHO classification recognizes four AML categories (Jaffe, 2001):

1) AML with recurrent genetic abnormalities
2) AML with multilineage dysplasia
3) Therapy-related AML and myelodysplastic syndrome (MDS)
4) AML not otherwise classified.

According to the FAB classification, ALL is divided into three morphologic types: L1, L2 and L3, defined by the cytologic features of the lymphoblasts in BM smears.

According to the WHO classification, ALL is classified into two categories based on postulated cell of origin and malignant cell immunophenotype.

1) Precursor B lymphoblastic leukemia/lymphoblastic lymphoma
2) Precursor T lymphoblastic leukemia/lymphoblastic lymphoma

Because of the biologic unity of ALL and lymphoblastic lymphoma, the use of one or the other term is in some occasions arbitrary. If the patients present with a mass lesion and 25% or fewer lymphoblasts in BM, the designation of lymphoma is preferred (Jaffe, 2001). Importantly, at present the cut-off limit for blast count for AML diagnosis is ≥20% according to WHO classification, as opposed to ≥30% required by historic FAB system.

### *2.2.3. Prognostic factors in acute leukemia*

Many clinical features and laboratory parameters have been reported to have prognostic value in AL. At present, the most important prognostic factors both in AML and ALL are: (1) age of the patient, (2) the karyotype, (3) initial response to treatment and (4) WBC count at diagnosis (Bassan et al., 2004; Litzow, 2004; Schmiegelow and Gustafsson 2005). Recognition of further prognostic variables is important scientific task and will make possible to tailor treatment according to different risk-categories, to improve survival and to reduce treatment-related toxicity and mortality.

#### *Prognostic factors specific for AML*

According to Grimwade et al (Grimwade et al., 1998): AML could be divided into three prognostic categories by cytogentic abnormalities detected at diagnosis:

- a favorable prognostic group – t(8;21), t(15;17), t(16;16) or inv(16);
- an unfavorable prognostic group – complex karyotype, –5, del(5q), –7 or abnormalities of 3q;
- an intermediate group – either normal karyotype or abnormalities not included in the other subgroups.

In some studies abnormalities of 9q, 11q, 20q, 21q and t(9;22) or abnormality of 7p are associated with a poor prognosis (Litzow, 2004). The good prognosis group is uniform but which cytogenetic abnormalities are included in the intermediate or poor risk categories, varies somewhat in various studies (Litzow, 2004). AML patients can be divided into highly predictive prognostic

groups, based on cytogenetics and early treatment response (Wheatley et al., 1999). However, the majority of AML patients display intermediate prognostic features with variable clinical outcome (Grimwade et al., 1998). Moreover, AML patients are considered to be in morphological complete remission (mCR) when BM samples contain ≤5% blasts, based on light microscope examination. In summary, there is an obvious need for more accurate treatment-related prognostic factors, which would help to improve outcome in AML patients.

***Prognostic factors specific for ALL***

The important feature is the extent of the disease – measured as adenopathy, organmegaly, CNS involvement or the presence of mediastinal mass (Borowitz and DiGiuseppe, 2001). Specific chromosomal aberrations are also associated with poor outcome (Schmiegelow and Gustafsson, 2005). According to the Nordic Society of Pediatric Hematology and Oncology (NOPHO) 2000-ALL study group, the following features defined poor prognosis (Schmiegelow and Gustafsson, 2005):

1) WBC ≥50.1 x $10^9$/l
2) T-cell ALL
3) CNS and/or testis involvement by leukemia
4) The presence of 11q23, t(9;22), t(1;19) and/or hypodiploidy (<45 chromosomes)
5) poor/slow response to the induction treatment.

Prognostic factors in adult ALL are less well defined. However, older age, high WBC count, lack of initial response to treatment and the presence of t(9;22) or t(4;11) are also associated with poor prognosis in adult ALL (Bassan et al., 2004).

### 2.2.4. AML treatment and clinical outcome

Combination of Ara-C and the anthracycline daunorubicin introduced in the 1960s and 1970s is considered as back-bone of AML treatment. At present, AML treatment could be divided into two phases (Stone, 2002):

1) induction therapy
2) post-induction therapy.

Standard induction therapy consists of seven days Ara-C and three days of anthracycline, usually daunorubicin, but also with idarubicin or mitoxantrone. In some studies the third drug etoposide or 6-thioguanine is added (Kimby et al., 2001). The aim of induction treatment is to achieve CR, but to maintain CR, post-induction therapy is necessary. For patients younger than 60 years, available options include allogeneic or autologous stem cell transplantation

(SCT) and intensive chemotherapy (Kimby et al., 2001). The optimal duration and number of cycles given in post-remission therapy is not clear. The latest data indicates that in younger adult patients who are not candidates for allogeneic SCT (allo-SCT), treatment by four courses of high-dose chemotherapy or by one course of high dose chemotherapy followed by autologous SCT (auto-SCT) results in improved survival rates (Farag et al., 2005). Allo-SCT is considered as standard post-remission therapy only in poor risk patients (Kimby et al., 2001; Smith et al., 2004). With current induction treatment, CR achieved in 70–80% of adult patients younger than 60 years and five-year relapse free survival reach up to 40% (Stone, 2002; Löwenberg et al., 2011). In older adults, CR is achieved in 30–50% of patients and median relapse-free survival (RFS) is only 9–12 months (Stone, 2002; Burnett et al., 2011). This data suggests that AML in older adults is intrinsically more resistant to standard chemotherapy. Older patients have a greater incidence of secondary AML related to MDS or chemotherapy. They do have also greater incidence of unfavorable chromosomal abnormalities such as –5 and –7 and multi-drug resistant glucoprotein expression (Stone, 2002).

Children with AML have more commonly favorable cytogenetic aberrations (Burnett and Eden, 1997; Lie et al., 2005). This feature together with impact of young age makes the prognosis for children relatively better than adults. CR is achieved in 70–90% of children with AML and the long-term survival rates reach up to 60% (Creutzig et al., 2001; Lie et al., 2005; Ravindranath, 2003).

### *2.2.5 ALL treatment and clinical outcome*

The treatment of precursor B-cell and T-cell ALL both in children (Schmiegelow and Gustafsson, 2005) and adults (Bassan et al., 2004) are closely related and include four main components:

1) induction therapy
2) consolidation (re-induction or delayed intensification) therapy
3) CNS prophylaxis
4) maintenance therapy.

The main components of the induction treatment are daily glucocorticoid (prednisolone or dexamethasone), weekly vincristine, and repeated doses of L-aspariginase and/or anthracycline together with intrathecal methotrexate (MTX). The post-remission consolidation or re-intensification generally includes alkylating agents and/or epipodophyllotoxins, repeated doses of L-asparaginase and high-dose MTX with or without concurrent thiopurines. CNS-directed therapy consists of irradiation and/or high-dose MTX and/or Ara-C with intrathecal MTX or triple therapy (MTX, Ara-C and glucocorticoids). The maintenance therapy consists of oral 6-mercaptopurine and oral or parental MTX at 1- to 2-weeks intervals and is given for two or three years. High-dose treatment in the first CR with auto-SCT or allo-SCT is justified only for high-

risk patients (Bassan et al., 2004; Schmiegelow and Gustafsson, 2005) due to the excess risk of transplant-related toxicity and mortality in standard-risk patients.

The outcome of childhood ALL has improved dramatically since 1972 when the "Total therapy" concept – the combination of all known effective agents given during induction, intensification and maintenance of remission (Pinkel et al., 1972). With current risk-adapted therapy protocols, cure can be achieved in 70–80% of children (Gustafsson et al., 2000; Schrappe et al., 2000). However, survival has improved mostly in children with good-risk features. Since these patients constitute almost 70% of all children, the improvement in prognosis in this group has strong impact on overall survival (OS) (Gustafsson et al., 2000). Children with high-risk ALL have inferior prognosis with five-year event-free survival of 50–70% (Schmiegelow and Gustafsson, 2005). In adult ALL, CR can be achieved close to 70–85% of patients, but more than half of those patients suffer a relapse and the cure rate is only about 20–40% of patients (Bassan et al., 2004). ALL patients with the t(9;22) translocation have the poorest survival with two-year survival rates <10–25%. The only curative option in this group of patients is allo-SCT (Bassan et al., 2004). The outcome of the t(9;22) ALL may improve by incorporating imatinib into treatment program (Delannoy et al., 2006; Wassmann et al., 2006).

## 3. Minimal residual disease in acute leukemia

It has been estimated that AL patients at diagnosis are burdened by approximately $10^{12}$ malignant cells and those who achieve mCR may carry between 0 and $10^{9}$ residual leukemia cells (Sievers and Radich, 2000). Minimal residual disease (MRD) is defined as persistence of leukemia cells at submicroscopic level in mCR patients. This means that in MRD patients the small number of leukemia cells still present in the body in an amount which is undetectable by conventional light microscopy. The final goal of detecting MRD is to obtain a more precise evaluation of the effectiveness of the treatment in order to:

- design patient-adapted post-remission therapies which would reduce both the risk of relapse and over-treatment
- predict impending relapses prior to clinical manifestations
- make a better assessment of the quality of the stem cells harvested from auto-SCT
- facilitate early therapeutic interventions such as donor lymphocyte infusions following allo-SCT.

The detection of MRD is based on either immunophenotypical or molecular features that are present in leukemia cells, but not in normal haematopoietic cells (Campana and Pui, 1995). Molecular methods based on polymerase chain reaction (PCR), including real-time quantitative PCR, are highly sensitive and

are able to detect one leukemia cell in $10^5 – 10^6$ normal cells (van der Velden et al., 2003). However, this methodology can currently applied in only 20–30% of AML patients who have defined molecular targets, especially t(15;17), t(8;21) or inv(16) (Schoch et al., 2001; Yin and Grimwade, 2002). Even when patients with FLT3 gene length mutations and MLL gene internal tandem duplications are included, less than 50% of AML patients can be followed by PCR technique for MRD. In contrast to AML, MRD could be followed at least 80% of B-cell ALL and over 95% of T-cell ALL patients using patient-specific sequences of immunoglobulin or T-cell receptor rearrangements. However, the need to develop individual probes may cause limitations for the widespread use of MRD detection by PCR in ALL patients (Campana, 2003).

Immunophenotyping by FCM is an attractive alternative for distinguishing small number of leukemia cells from normal counterparts. This method is based on aberrant, leukemia-associated immunophenotypes (LAIPs) that differ from normal antigen expression during haematopoiesis (Campana et al., 1990; Drach et al., 1992; San Miguel et al., 1999). FCM evaluates several antigens on one cell, gives quantitative results and can detect small abnormal cell population against a reactive background. The sensitivity of MRD detection by FCM depends on the number of analyzed cells, the type of phenotypic aberrations, and the combination of monoclonal MoAb reagents (Campana and Coustan-Smith, 2004). By using live-gate strategy, in which at least $10^6$ cells are acquired, a sensitivity of detection of one aberrant cell per $10^4$ to $10^5$ BM cells can be achieved (Campana and Coustan-Smith, 2004; San Miguel et al., 1999).

## 3.1. LAIPs

According to the BIOMED-1 Concerted Action report, 88% of AML, 85% of B-cell ALL and 100% of T-cell ALL patients display aberrant phenotypes at diagnosis, thus making MRD follow-up by FCM feasible (San Miguel et al., 1999). FCM could be used for all AML patients via the application of a greater variety of fluorochromes and by improving the panel of antibodies, but a lower sensitivity due to the increased application of less aberrant LAIPs may limit this approach (Kern et al., 2003). Highly aberrant LAIPs in AML, which are not detectable in normal human adult BM at 0.01% level include: co-expression of CD34 together with CD2, CD7, CD14, CD56 or CD65 and triple-marker combination CD34+/CD15+/HLA-DR- (Macedo et al., 1995). The presence of highly aberrant LAIPs has been reported in 48% of childhood AML cases (Coustan-Smith et al., 2003). The most useful highly aberrant LAIPs in pre-cursor T lymphoblastic leukemia (preT-ALL) are co-expression of T-cell markers CD3 and CD5 with TdT or CD34 and in precursor B lymphoblastic leukemia (preB-ALL) co-expression of myeloid-associated markers CD13, CD15, CD33, CD65, CD66c with CD19+/CD34+ leukemia cells (Campana and Coustan-Smith, 2004). The most frequently used marker combinations to detect MRD in ALL and AML are shown in Table 3.

**Table 3.** FCM marker combination for MRD detection in acute leukemias.

| Leukemia cell lineage | Marker combination |
|---|---|
| AML | (CD34,CD117)/CD4/My |
| | (CD34,CD117)/CD7/My |
| | (CD34,CD117)/CD11b/My |
| | (CD34,CD117)/CD15/CD33 |
| | (CD34,CD117)/CD19/My |
| | (CD34,CD117)/CD56/My |
| | (CD34,CD117)/HLA-DR-/My |
| | CD13/CD133/CD34/CD33 |
| | |
| preT-ALL | TdT/CD5/CD3/CD7/CD2 |
| | CD34/CD5/CD3/CD7/CD1a |
| | |
| preB-ALL | CD19/CD34/CD10/CD13 |
| | CD19/CD34/CD10/CD15 |
| | CD19/CD34/CD10/CD21 |
| | CD19/CD34/CD10/CD22 |
| | CD19/CD34/CD10/CD33 |
| | CD19/CD34/CD10/CD38 |
| | CD19/CD34/CD10/CD45 |
| | CD19/CD34/CD10/CD58 |
| | CD19/CD34/CD10/CD65 |
| | CD19/CD34/CD10/CD66c |
| | CD19/CD34/CD10/NG-2 |
| | CD19/CD34/CD10/TdT |
| | CD19/CD34/TdT/IgM |

Abbreviations: My, myeloid marker

## 3.2. Minimal residual disease as a predictor of prognosis

Several studies have demonstrated that the level of MRD detected by either PCR or FCM based methods is a powerful predictor of prognosis in childhood ALL (Biondi et al., 2000; Björklund et al., 2003; Coustan-Smith et al., 2000; Willemse et al., 2002). Therefore MRD monitoring was incorporated into the NOPHO ALL-2000 protocol to form a basis for refined treatment stratification for the next protocol (Gustafsson et al., 2000). Less data is available on the prognostic significance of MRD evaluation in patients with AML. PCR based MRD monitoring is useful only in patients with t(15;17) where an increased level of t(15;17) transcripts are predictive for molecular and subsequent clinical relapse (Sanz et al., 2005). The clinical value of monitoring t(8;21) or inv(16) is still controversial.

**Table 4.** Summary of results of MRD studies in adult AML.

| Refe-rences | No of pts | Age, y (median) | Treatment-protocol | Time-points | Conclusions |
|---|---|---|---|---|---|
| Venditti et al., 2000 | 56 | 18–78 (54) | GIMEMA AML-10, 13 | 1) After induction<br>2) After consolidation | 1) After induction MRD cut-off level 4,5 x$10^{-4}$ associated with outcome, but not significant<br>2) After consolidation MRD cut-off level 3,5 x$10^{-4}$ predictive for RFS, OS |
| San Miguel et al., 2001 | 126 | 43 ± 18 (42) | PETHEMA 1991, 1996 | 1) After induction | 4 different risk categories according to MRD levels:<br>1) very low risk (<$10^{-4}$), no relapse<br>2) low risk ($10^{-4}$ – $10^{-3}$), relapse rate 14%<br>3) intermediate risk ($10^{-3}$ – $10^{-2}$), relapse rate 50%<br>4) high risk (≥$10^{-2}$), relapse rate 84%<br>MRD levels are predictive also for OS |
| Feller et al., 2004 | 72 | 18–76 (50) | HOVON 29, 42 or 32 | 1) After 1'st cycle (n=51)<br>2) After 2'nd cycle (n=52)<br>3) After 3'd cycle (n=39) | 1) After 1'st cycle MRD cut-off level 1%, RRR 6.1<br>2) After 2'nd cycle MRD cut-off level 0.14%, RRR 3.4<br>3) After 3'd cycle MRD cut-off level 0.11%, RRR 7.2<br>MRD frequently correlated with RFS and OS in all measured time-points |
| Kern et al., 2004 | 93;<br>Gr-1 58<br>Gr-2 62 | Gr-1<br>18–77 (51)<br>Gr-2<br>18–71 (52) | German AML 1999 | 1) Gr-1 After induction<br>2) Gr-2 After consolidation | 1) LD as a continuous variable after induction; significantly related to RFS, but not OS.<br>2) LD as a continuous variable after consolidation; significantly related to RFS and OS. |

Abbreviations: pts, patients; RFS, relapse-free survival; OS, overall survival; RRR, relative risk of relapse; NA, not available; Gr, group; LD log difference, the degree of reduction of malignant blasts between diagnosis and follow-up checkpoints.

The prognostic significance of MRD levels detected by FCM has only been investigated in two childhood AML studies. These studies demonstrate that the presence of MRD is related to a poor outcome (Coustan-Smith et al., 2003; Sievers et al., 2003). In adult AML, four groups have shown that MRD, especially above 0.1% of BM cells, detected either after induction treatment or at the end of post-remission chemotherapy, is highly predictive for subsequent relapse and shorter survival (Feller et al., 2004; Kern et al., 2004; San Miguel et al., 2001; Venditti et al., 2000). Results of these studies are summarized in Table 4. Unfortunately, there are still no guidelines on how to integrate the results of MRD analysis in treatment planning. In addition, the impact of MRD as a prognostic marker in AML patients treated by allo-SCT or auto-SCT has not yet been fully evaluated.

During recent years European Leukemia Net has undertaken the task to harmonize MRD detection either by PCR or FCM, which should lead the way for development of common protocols for future co-operative studies (Cilloni et al., 2009; Béné and Kaeda, 2009).

## 4. Late effects of acute leukemia therapy

Even, if long-term remission is achieved, many patients need continuous medical attention due to complications imposed by leukemia or its treatment. The side effects of CNS irradiation include neurophysiologic deficits, endocrine disturbances and obesity. In children there is a risk of short stature. Patients who are cured from AL may have reduced fertility, develop cataract due SCT conditioning treatment with alkylating agents and total body irradiation, develop cardiomyopathy due to high doses of anthracyclines, develop osteoporosis or second cancer and may have reduced lung function (Schmiegelow and Gustafsson, 2005; Smith et al., 2004). The most common secondary malignancies are brain tumors, MDS or secondary AML usually associated with previous CNS irradiation and genotoxic chemotherapy (Schmiegelow and Gustafsson, 2005; Bassan et al., 2004).

Current treatment protocols also induce a profound impairment of the immune system such as decreased immunoglobulin levels (Kristinsson et al., 2001; Reid et al., 1981), loss of protective antibodies against measles, rubella and polioviruses (Nilsson et al., 2002; Smith et al., 1995) and persistent abnormalities in T-, B- and NK-cell subsets (Ek et al., 2005). Therefore, immunospression is a major side effect of anti-leukemia therapy.

## 5. Dendritic cells

Dendritic cells (DCs) play a central role in coordinating immune system response (Banchereau et al., 2000). DCs were first identified in 1868 by Paul Langerhans as ‚dendritic-shaped' cells in the skin. DCs were ‚rediscovered' by Steinman and colleagues in 1973 in mouse spleen (Steinman and Cohn, 1973). However, a major interest in DC research begun after the discovery of their antigen-presenting function (Klareskog et al., 1977; Rowden et al., 1977).

DCs are uniquely able to induce primary immune responses and are the most potent antigen-presenting cells that recognize, process and present antigens to naïve, resting T-cells. Only DCs are able to stimulate naïve T-cells. In addition, DCs are the most effective cells that activate secondary T-cell responses and have the capacity to directly regulate B-cell responses as well (Banchereau et al., 2000). DCs develop from the BM haematopoietic progenitors under the control of soluble growth factors released from BM stroma cells and also direct cell-cell contact with BM stroma cells. DCs that leave BM are defined as DC precursors (preDCs) or immature DCs (iDCs) (Liu, 2001).

Three subtypes of HLA-DR+ preDCs have been described in human blood. The first are monocytes, which depending on cytokines, can differentiate into iDCs or macrophages (Gordon and Taylor, 2005). Two other preDCs subtypes lack the expression of lineage markers such as CD3, CD14, CD19, CD20 and CD56, but express high levels of HLA-DR and either high levels of CD11c, which is integrin-α or CD123 {the receptor for Interleukin (IL)-3}. PreDCs, which express high levels of CD11c and do not express CD123, are defined as myeloid DCs (mDCs) and they belong to the same lineage as monocyte-derived DCs (O'Doherty et al., 1994; Sallusto and Lanzavecchia, 1994). PreDCs that express high levels of CD123 and low levels of CD11c are defined as lymphoid or plasmacytoid DCs (pDCs) (Grouard et al., 1997; Olweus et al., 1997). It has been suggested that both DC subtypes can drive either Th-1 or Th-2 types of response or promote antigen-specific tolerance depending on the stage of differentiation and activation (Langenkamp et al., 2000; Mahnke et al., 2002).

At present, mDCs and pDCs are thought to preferentially recognize different antigens or pathogens. In response to viruses (Kadowaki et al., 2000) and to bacterial DNA rich in unmethylated CpG motifs (Hartmann et al., 1999; Hemmi et al., 2000), pDCs produce large amounts of IFN-α. Myeloid DCs produce large amounts of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-12 in response to glycoproteins from gram-positive bacteria (Lien et al., 1999) and lipoproteins from gram-negative bacteria (Poltorak et al., 1998). Furthermore, DCs are pivotal for the induction and maintenance of anti-tumor immune responses. Accumulating data indicates severe deficiencies in DC numbers and functions in cancer patients (Gabrilovich, 2004). Defects in DC function have been demonstrated in AML (Mohty et al., 2001), chronic myeloid leukemia (Mohty et al., 2002), hairy cell leukemia (Bourguin-Plonquet et al., 2002) and myeloma (Brimmes et al., 2006) and in patients with solid tumors (Gabrilovich, 2004). Two studies have demonstrated severe deficiency of pDCs and mDCs in peripheral blood of children with preB-ALL, but not with preT-ALL at diagnosis (Maecker et al. 2006; Mami et al., 2004). Collectively this data indicate that the impairment of the DC system may be one of the key factors in the late immune dysfunction observed in patients with ALL and may even have a role in tumor control. Therefore, there is an obvious need to study DCs regeneration pattern during acute leukemia therapy in different patients' subgroups and estimate DCs levels at the end of treatment.

# AIMS OF THE STUDY

The major aims of the thesis were to evaluate the usefulness of FCM in haematological malignancies. More specifically, the aims were:

- To identify the value of FCM in lymphoma diagnosis in FNA material (Paper I)
- To determine the prognostic relevance of the MRD in young adult AML patients detected by FCM in relation to SCT (Paper II)
- To determine the DCs levels at diagnosis and their pattern of regeneration evaluated by FCM in BM of children treated for ALL according to NOPHO ALL-2000 protocol (Paper III)

# MATERIALS AND METHODS

## 1. Patients

This study included in total 526 patients (256 male and 270 female), ranging in age from 2 months to 94 years, who were investigated or treated in Karolinska Hospital, Astrid Lindgren Children's Hospital in Stockholm and at University Children's Hospital in Uppsala, Sweden during 1994–2005.

The patient group of FCM study on FNA diagnosis of lymphoma (Paper I) consisted of 396 patients (187 male and 209 female), in age 4–94 years, median 60 years.

The patient group of MRD study in AML by FCM (Paper II) included 45 patients (23 males and 22 females) in age range 19–60 years, median 47 years.

The patient group of DCs study in ALL treated according to NOPHO ALL-200 protocol (Paper III) was formed of 76 children (42 boys, 34 girls). Median age at diagnosis was six years (1–17 years).

Nine children without haematological diseases formed a small hospital control (HC) group for establishing reference levels for DC subsets in the BM (Paper III). HC group consisted of four boys and five girls with median age four years (range 2 months – 13 years). Seven children had reactive BM, one had isolated neutropenia, and one patient rhabdomyosarcoma without BM involvement.

### 1.1. Ethics

The study was conducted according to the Helsinki Declaration and was approved by the local ethics committee.

### 1.2. Patients' selection and definition of diagnosis

***Patients' selection to study FCM usfulness on FNA diagnosis of lymphoma (Paper I)***

To study the value of FCM in diagnosis of lymphoma we prospectively included samples from 3038 patients with suspect lymphoma referred to the Division of Clinical Cytology, Karolinska Hospital, Stockholm, Sweden, for FNA between May 1999 and October 2001. From a total of 3038 FNAs performed during that period, IC was performed in 1088 samples. Samples with cell concentrations of less than $5\times10^5$ cells/ml, with large cells suggesting Hodgkin's lymphoma, metastasis of other tumors and from lymph node smaller than 1 cm in diameter from children were excluded from the study. In total 424 FNA samples obtained from 396 patients were analyzed by FCM. Of 396 investigated patients, 369 had a single FNA sample obtained. Twenty-six

patients had FNA twice and one patient had FNA three times taken. In these patients, FNA were performed at different lesions and/or different occasions. Of the 424 FNAs studied by FCM, 352 samples (83%) were obtained from lymph nodes and 72 (17%) from extranodal tissue (17 from the parotis, 14 from subcutaneous tissue, nine from breast tissue, seven from the orbita, seven from the tonsil, five from the spleen, four from skin lesions, three from submandibular glands, two from kidney tumors, two from the thyreoidea, one from the gum and one from the liver). Reactive hyperplasia of lymphoid tissue (RH) was diagnosed in 172 samples from 164 patients and NHL in 239 samples from 228 patients. Primary lymphoma was diagnosed in 141 patients. Relapse of lymphoma was found in 97 samples from 87 patients. Thirteen samples from 13 patients (seven cases of Hodgkin's lymphoma, two cases of angioimmunoblastic lymphadenopathy, two cases of dermatopathic lymphadenopathy, one case of lateral neck cyst and one case of seroma) were excluded from data analysis.

The initial diagnosis on FNA was made by two cytopathologist. Cytologic and immunologic criteria for the classification of NHL and RH were based on the Revised European-American Classification of Lymphoid Neoplasms (Harris et al., 1994). Primary lymphoma was defined as a previously undiagnosed lymphoma in which the FNA was the primary diagnostic test. Recurrent lymphoma was defined as one in which the diagnosis of lymphoma had been made by a previous biopsy. For the purpose of the study, the same observers reviewed all samples with discrepant diagnoses. For histopathologic diagnosis, Revised European-American Classification of Lymphoid Neoplasms and WHO classifications were used (Jaffe, 2001). The final diagnosis was recorded according to histopathologic examination or by cytology report if the biopsy was not performed.

### *Patients' selection to study MRD by FCM in young adult AML (Paper II)*

To study the value of FCM in MRD detection in patients with AML we analyzed 62 young adults in age 19–60 years in whom non-promyelocytic AML was diagnosed between July 1994 and June 2001 at Karolinska University Hospital Solna, (Stockholm, Sweden). mCR was achieved in 53 of 62 patients (85%). Eight patients were excluded due to:

- uninformative phenotypes (n=3),
- lack of sufficient clinical data (n=2)
- lack of sufficient FCM information (n=3).

Follow-up MRD information was available in 45 mCR patients. At diagnosis the following data was recorded {median (range)}: percentage of BM blasts 71% (34–96%), total WBC count 23 (0.5–237) $x10^9$/L, hemoglobin (Hb) level 89 (44–128) g/L, and platelet count 50 (15–263) $x10^9$/L. The median follow-up

time of surviving patients was five years (range 2–9 years). Most of the MRD data was collected during 1994 – 2000 and integrated in the European BIOMED-1 Concerted Action (BMH-CMT 94–1675) (San Miguel et al., 1999). The treating physicians were not aware of the MRD results.

The AML diagnosis was based on morphology and cytochemistry following the FAB classification (Bennett et al., 1976): three M0, 10 M1, nine M2, 11 M4, 10 M5, one M6, and one case of acute undifferentiated leukemia (AUL). All samples were reviewed and reclassified according to the WHO classification (Jaffe, 2001): three AML with recurrent genetic abnormalities {one AML with t(8;21)(q22;q22), one AML with inv(16)(p13q22) or t(16;16)(p13;q22) and one AML with 11q23 (MLL) abnormality}, four therapy related AML, 38 AML not otherwise categorized (four minimally differentiated AML, 10 AML without maturation, six AML with maturation, eight acute myelomonocytic leukemias, eight acute monoblastic and monocytic leukemias, one acute erythroid leukemia, and one bilineal acute leukemia).

Morphological CR was defined as less than 5% blasts without detectable Auer rods in BM samples displaying ≥20% cellularity with the maturation of all haematopoietic cell lines in the BM aspirate.

### *Patients' selection to study DCs levels in BM in childhood ALL (Paper III)*

The study on DCs levels in BM in childhood ALL was based on 111 children over one year old, who were diagnosed with *de novo* ALL at Astrid Lindgren Children's Hospital in Stockholm and at University Children's Hospital in Uppsala, Sweden between February 2002 and October 2005. Of these, 76 patients (42 boys, 34 girls) fulfilled the following inclusion criteria:

- treatment according to NOPHO ALL-2000 protocol (Table 5; Figure 3);
- included in NOPHO ALL-2000 MRD study;
- DCs levels measured at least twice during therapy.

The ALL diagnosis was based on WHO classification. Sixty-seven children had preB-ALL and nine had preT-ALL (Anon, 1998; Jaffe, 2001).

In the HC group, BM cellularity and cell-lineage maturation were normal for the children age.

**Table 5.** Distribution of patients within NOPHO-ALL 2000 protocol for ALL >1 years.

| Standard- and intermediate-risk group (N = 54)<br>ALL patients without unfavorable features:<br>WBC ≤ 50.0 x $10^9$/l and B-precursor ALL and no CNS – leukemia and no testis – leukemia and no 11q23, t(9;22), t(1;19) and no hypodiploidy (<45) and good response* | | |
|---|---|---|
| Therapy group | Patient number | Criteria |
| Standard Intensive therapy (Protocol SI) | N = 24 | 1) B-precursor ALL and<br>2) Age 1 to < 10 years of age and<br>3) WBC≤10.0 x $10^9$/l and<br>4) No unfavorable features and<br>5) Good response to initial therapy |
| Intermediate Intensive therapy (Protocol II) | N = 30 | 1) B-precursor ALL and<br>2) Age 1 to < 10 years and WBC 10.1 to < 50.0 x $10^9$/l or<br>3) Age ≥ 10 years and WBC < 50.0 x $10^9$/l and<br>4) No unfavorable features and<br>5) Good response to initial therapy |
| High-risk group: (N =22)<br>ALL patients with unfavorable features:<br>WBC ≥50.1 x $10^9$/l and T-cell ALL and/or CNS – leukemia and/or testis – leukemia and/or 11q23, t(9;22), t(1;19) and/or hypodiploidy<br>(<45) and/or poor response | | |
| Therapy group | Patient number | Criteria |
| Intensive therapy (Protocol I) | N = 8 | 1) WBC 50–200.0 x $10^9$/l and age < 5 years or WBC 50–100.0 x$10^9$/l and age ≥ 5 years and/or<br>2) T cell ALL without mediastinal mass or T cell ALL with mediastinal mass and age < 5 years and/or<br>3) 11q23/MLL-rearrangements and age < 10 years (optional) and/or<br>4) CNS involvement and age < 5 years and/or<br>5) No t(9;22)(q34;q11)/BCR-ABL fusion and<br>6) Chromosomal G-banding analyses show 34–<45 chromosomes or DNA-index < 0.95<br>7) Patients on Intermediate Intensity therapy and MRD ≥ $10^{-3}$ on day 106 |
| Very intensive therapy (Protocol VI) | N = 8 | Age ≥ 5 years at diagnosis and<br>1) T cell ALL and a mediastinal mass and/or<br>2) WBC 100–200.0 x$10^9$/l and/or<br>3) CNS-involvement at diagnosis (to receive cranial radio-therapy with therapeutic doses) |
| Extra intensive therapy (Protocol EI) | N = 6 | 1) Very slow response, defined as M3 status (>25% lymphoblasts) in the BM on day 29 and/or<br>2) 11q23/MLL-rearrangements and age ≥ 10 years (age 2–9 years: BMT optional) and/or<br>3) t(9;22)(q34;q11)/BCR-ABL fusion and/or<br>4) Hyperhaploid karyotype with modal number <34 and/or<br>5) WBC ≥ 200.1 x$10^9$/l |

*Response to therapy is defined as follows:

Good response to initial therapy:

A bone marrow on day 15 with < 25% blasts (= M1 or M2 BM) in a non-aplastic bone marrow

A bone marrow on day 29 with < 5% blasts (= M1 BM) in a non-aplastic bone marrow

Poor response to initial therapy:

A bone marrow on day 15 with > 25 % blasts (=M3 BM) in a non-aplastic bone marrow

A bone marrow on day 29 with > 25% (= M3 BM) or 5–25 % (=M2) in a non-aplastic bone marrow

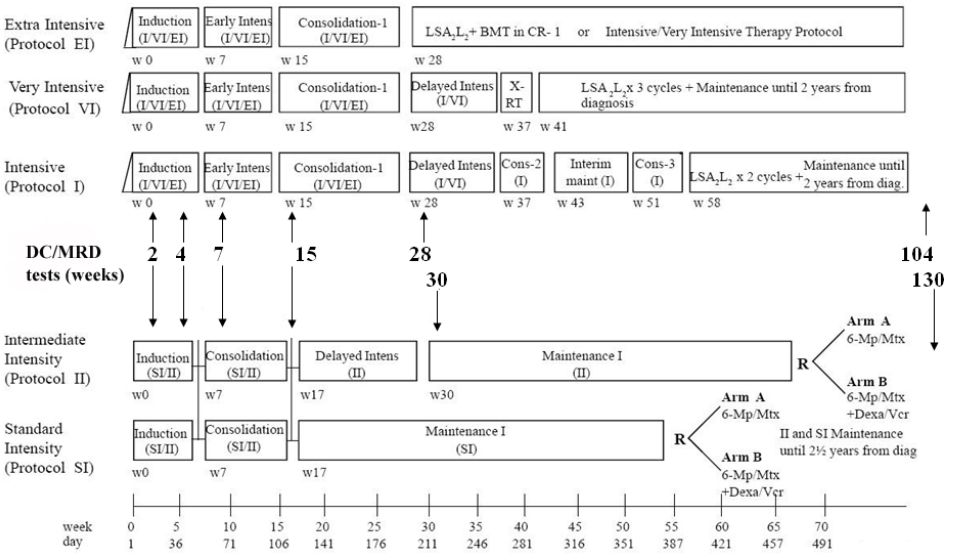


**Figure 3.** Dendritic cell (DC) and minimal residual disease (MRD) measurement during NOPHO ALL-2000 treatment protocol.

Patients were stratified into different therapy groups according to the risk criteria present at diagnosis (see Table 5). Time-points of DC detection and MRD tests are indicated by arrows in an overview of NOPHO ALL-2000 protocol (Gustafsson et al., 2000; Schmiegelow and Gustafsson, 2005).

# 2. FNA cytology

All FNA biopsies were performed by an experienced cytopathologist with a needle 0.4 to 0.6 mm in diameter, according to the procedure described by Zajicek (Zajicek, 1974). Deep-located lesions were aspirated under ultrasound control. One part of the aspirate was used to prepare smears, which were air-dried or methanol fixed and stained by May-Grünwald-Giemsa or Papanicolau technique, respectively. Additional air-dried smears were fixed in freshly prepared 4% buffered formalin and used to study proliferation. The other part of the aspirate was suspended in phosphate buffered saline (PBS). Cell viability by trypan blue exclusions and cell concentration in suspensions was assessed immediately after the FNA procedure. One half of the cell suspension was used for FCM study and from the other half cytospin preparations in a cytocentrifuge (Shandon, Cheshire, UK) for IC analysis (Tani, 1988) were made.

## 2.1. Immunocytochemistry

IC on cytospins was performed in 60% (253/424) of samples and during the first year of study in 70% (73/103) of samples. IC on cytospins was not performed in all cases because after the first year of study we found an excellent correlation between IC and FCM in low-grade B-cell NHLs (LG-NHL) and RH. Therefore, immunophenotyping was done subsequently only by FCM in most cases of RH and of recurrent LG-NHL.

A three-step alkaline phosphate anti-alkaline phosphatase method was employed (using alkalin phosphatase anti-alkaline phosphatase reagents from DAKO, Glostrup, Denmark). Single stainings were performed with the following monoclonal antibodies: anti-kappa (clone A8B5, dilution 1:10), CD10 (clone SS2/36, dilution 1:20), CD20 (clone L26, dilution 1:50) and Bcl-2 (clone 124, dilution 1:40) from DAKO. Anti-lambda (clone 1–155–2, dilution 1:50), CD3 (clone SK7, dilution 1:40) and CD5 (clone L1F12, dilution 1:40 from BD (San Jose, CA, USA).

# 3. Histopathology

Lymph node excision (n=91) or biopsies from extranodal tissues (n=33) were performed in 124 of 396 patients. Tissue specimens were fixed in formalin or B5 fixative and routinely stained for morphological examination only with hematoxylin and eosin, Gimesa, periodic acid-Schiff and Gordon-Sweet methods. Immunophenotyping was performed by FCM on lymph node cell suspensions or on by a standard immunoperoxidase method on paraffin sections.

# 4. Cytogentics of AML

Karyotypes were available in 43 patients and were classified into three groups essentially in accordance with Grimwade et al (Grimwade et al., 1998).

- two patients with t(8;21) and inv(16) formed a favorable group,
- five patients with 5q-, near-tetraploid and complex aberrant karyotypes (i.e. ≥3 clonal chromosome aberrations) were considered as an unfavorable group,
- 36 patients with trisomy 8, t(9;22), other aberrations and normal karyotypes belonged to an intermediate prognostic group.

# 5. Treatment and outcome

## 5.1. Young adult AML

Most patients with AML (n=42) received induction therapy with idarubicin (10 mg/m$^2$ infusion d 1–3), Ara-C (1000 mg/m$^2$ infusion twice daily d 1–4), and etoposide (100 mg/m$^2$ infusion d 1–5). CR patients were given 2 cycles of consolidation therapy consisting of idarubicin (10 mg/m$^2$ infusion d 1–2), Ara-C (1000 mg/m$^2$ infusion twice daily d 1–3), and etoposide (100mg/m$^2$ infusion d 1–4). The third cycle of consolidation consisted of Ara-C (1000 mg/m$^2$ infusion twice daily d 1–6). One patient with AUL was given ABCDV that consisted of Ara-C, betamethasone, cyclophosphamide, doxorubicin and vincristine and 2 patients were treated with mitoxantrone, etoposide and Ara-C (Björkholm et al., 1995). Allo-SCT in the first CR was performed in 16 patients. Auto-SCT as consolidation in the first CR was performed in 15 patients: CR patients with no sibling donor received total body irradiation and high-dose Ara-C followed by auto-SCT (Rohatiner et al., 1998; Smith et al., 2004). Thirty patients achieved mCR after one induction course, 12 after two and three patients after three courses.

MRD levels were determined at two time-points:

- (1) – at first mCR after induction treatment,
- (2) – at the end of post-remission chemotherapy or before allo-SCT or auto-SCT.

A relapse was diagnosed in 17/45 mCR patients (38%) at median 10 months after mCR (range 2–20 months). One patient had MDS. RFS for patients who had achieved CR was determined as the duration from the date of the first CR to censoring point, first relapse or death due to any cause. The median RFS time was 36 months (range 2–105 months). OS was measured from the date of diagnosis to death or the censoring point. Twenty patients (44%) had died and the median OS was 42 months (range 4–107 months). The cause of death was AML relapse in 13 patients, MDS in one patient, allo-SCT related complications in three patients and non-leukemia related factors in three patients.

### 5.2. Childhood ALL

Children with ALL were divided into two main groups based on the presence or absence of unfavorable features and stratified into five protocols according to NOPHO ALL-2000 (Table5; Figure 3). Detailed therapy protocols have been published elsewhere (Gustafsson et al., 2000; Schmiegelow and Gustafsson, 2005). CR was achieved in 75 children (98.7%). Two patients suffered systemic ALL relapse and one patients isolated central nervous system relapse. Three patients had died due to relapse or refractory disease. All patients without unfavorable features who were stratified into Standard Intensive therapy (SI) and Intermediate Intensive therapy (II) had good response to initial therapy and therefore were not up-graded into more intensive protocols.

Precursor B-ALL patients without unfavorable features who were stratified into Protocol SI (preB-ALL [SI]) and Protocol II (preB-ALL [II]) formed standard-risk (SR) group. DC subsets were evaluated in SR group at eight time-points: diagnosis, treatment days 15 (week 2), 29 (week 4), 50 (week 7), 106 (week 15), at the beginning of Maintenance (I) (week 130), at the end of treatment protocol and six months after the end of chemotherapy.

Precursor B-ALL patients with unfavorable features formed preB-ALL high-risk group (preB-ALL HR). Patients with preB-ALL HR and preT-ALL formed together high-risk group (HR group). DC subsets were evaluated in HR group at seven time-points: diagnosis, treatment days 15 (week 2), 29 (week 4), 50 (week 7), 106 (week 15), at the beginning of Delayed Intensification (week 28) and the end of treatment protocol.

MRD levels were determined at the same time points as DC levels.

## 6. Flow cytometry

***Sample preparations***

FNA aspirates were collected in tubes containing PBS and were processed within 2h. BM samples were collected in heparin anticoagulant tubes, directly diluted 1:1 (vol/vol) with 0.9% NaCl and maintained at room temperature (RT). All samples were processed within 24h. A "stain and then lyse/wash" technique was used: 100 μl of BM cell suspension with at least $1\times10^6$ nucleated cells were incubated for 10–15 min in RT, in the dark with saturating amounts of triple or quadruple combinations of moAb. After incubation with moAb, 2 ml of FACS lysing solution (BD, diluted 1/10 in distilled water) were added and samples were incubated for another 5 min at RT, in the dark. After erythrocytes lysis, the cells were centrifuged for 5 min with 500 g, washed in 2 ml PBS and suspended in 0.5 ml of PBS. In moAb combinations including cytoplasmic or nuclear antigens the staining for surface markers was performed as described above and was followed by a fixation and permeabilization of cell membrane with 800 μl Permeafix (OPM) from Ortho (Raritan, NY, USA), diluted 1:1 (vol/vol) with

distilled water for 30 min or with 100 µl Intrastain solution A from DAKO for 15 min. After OPM or Intrastain fixation the cells were centrifuged for 5 min, 500 g and washed with 2 ml PBS. A further incubation with moAb to cytoplasmic or nuclear antigens followed for 30 min in OPM prepared samples and 15 min in Intrastain-processed samples together with 100 µl of Intrastain solution B. Finally, the cells were washed in 2 ml PBS and suspended in 0.5 ml PBS. Both intracellular procedures gave equal results in detecting nuclear TdT or cytoplasmic CD3. In all cases, isotype-matched immunoglobulins with no reactivity against BM cells were used as negative controls.

### *Data acquisition and analysis*

Data acquisition was performed using FACScan or FACS-Calibur flow cytometer, equipped with Lysis II or Cell Quest software programs from BD (Franklin Lakes, NJ, USA). Light scatter characteristics and autofluoresence levels of normal peripheral blood lymphocytes were used as reference values for instrument settings. Instrument setup and calibration were performed with CaliFlow beads (Spherotech Onc., Libertyville, IL, USA).

On average 8 874 non-gated events (544–10 000) were acquired per tube in immunophenotyping of FNA material. Data analysis was performed with CellQuest (tubes 1–3) and Paint-a-Gate (BD, tube 4; Figure 4) software programs. Bcl-2 expression was evaluated by mean fluorescence intensity (MFI) for separately gated CD3+ T-cells, CD19+ B-cells and CD10+/CD19+ B-cells. Bcl-2 MFI for normal T-cells present in the samples remained the same throughout the study (MFI 93.6 ± 31.8, median 89), which confirmed stability of the measurements allowing comparison of Bcl-2 expression in various NHL categories.

FCM criteria for phenotypic classification of LG-NHL and RH are presented in Table 6. Only CLL, MCL and FL displayed characteristic immunophenotypes. FCM differentiation between immunocytoma/lymphoplasmocytic lymphoma (IC/LPL) and extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue type/nodal marginal zone lymphoma (MALT/NMZL) was not possible, but localization and clinical data allowed correct diagnosis in most cases. Scatter characteristics was one of the important features in diagnosis of HG-NHL. Monoclonality was defined as a kappa/lambda ratio (K/L) higher than six or lower than 0.3 (Dong et al., 2001).

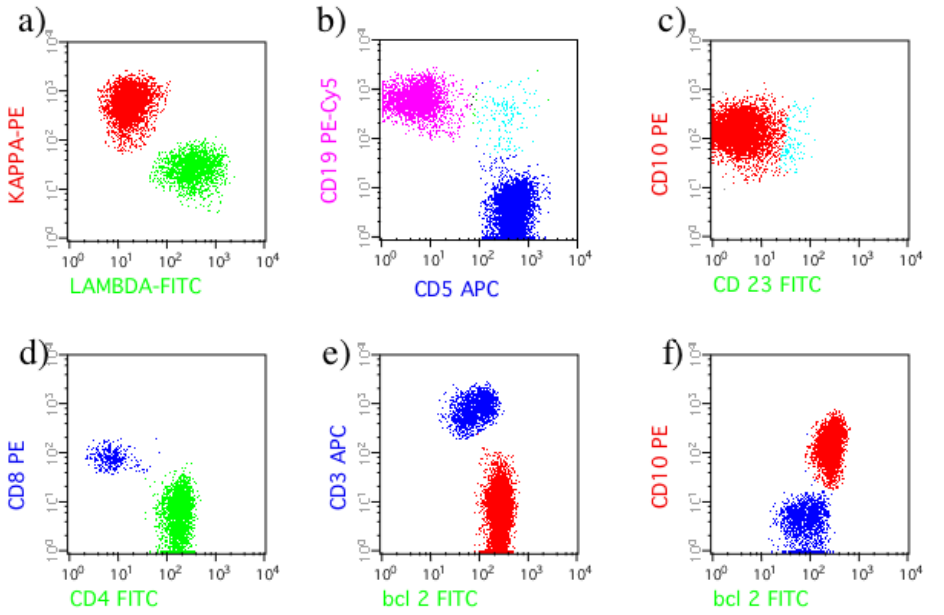


**Figure 4.** Illustration of FCM detection of antigen expression in lymphocyte subsets.

Expression of various markers was investigated in a "lymphocyte gate", set on a forward scatter (FSC) versus side scatter (SSC) plot or in B or T cells gated on SSC and CD19 or CD7 expression, respectively. a,b: The first tube was composed to investigate Ig light-chain restriction in separately gated CD19+ B-cells (a) and to determine a fraction of CD5+ B-cells (b). c: The second tube was designed to detect CD10 and/or CD23+ B-cells. d: The third tube allowed to determine CD4/CD8 ratio and to detect abnormal T-cell populations. e,f: The fourth tube was used to evaluate Bcl-2 expression in separately gated T cells, B cells and CD10+ B-cells. Plot a shows polyclonal expression of kappa and lambda in RH after CD19/SSC gating; kappa-positive B cells are shown in red, and lambda-positive B cells in green. Plot b shows lymphocyte subpopulations which were investigated in the lymphocyte gate set on FSC and SSC in the same sample; Normal CD19+/CD5- B-cells are shown in violet, normal CD5+/CD19- T-cells in blue and CD19/CD5 double positive cells in cyan. Plot c illustrates a Follicular lymphoma case in which malignant B-cells are CD10+/CD23- (red); CD10/CD23 double positive cells as cyan. Plot d shows the determination of CD4/CD8 ratio in reactive T cells after CD7/SSC gating; CD4+ T-cells are shown in green and CD8+ T-cells in blue. Plot e illustrates the Bcl-2 analysis in separately gated CD3+ T-cells (blue) and CD19+ B-cells (red) in Follicular lymphoma sample. Plot f shows that CD19+ B-cells in the same sample also express CD10 (red) and this population has a higher expression of Bcl-2 than do T-cells (blue). APC, allophycocyanin; Cy5, indodicarbocyanine; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

**Table 6.** FCM immunophenotypic criteria for classification of B-Cell NHL and RH (Jaffe, 2001).

| Diagnosis | CD19 | CD5 | CD23 | CD20 | CD10 | K/L |
|---|---|---|---|---|---|---|
| CLL | + | + | + | +(weak) | – | Clonal |
| FL | + | – | – | + | + | Clonal |
| IC/LPL | + | – | – | + | – | Clonal |
| MALT/NMZL | + | – | – | + | – | Clonal |
| MCL | + | + | – | + | – | Clonal |
| HG-NHL | + | – | –/+ | +/– | +/– | Clonal |
| RH | + | – | –/+ | +/– | –/+ | Polyclonal |

Abbreviations: CLL, chronic lymphocytic leukemia; FL, follicular lymphoma, HG-NHL, high-grade B-cell non-Hodgkin's lymphoma; IC/LPL, immunocytoma/lymphoplasmocytic lymphoma;K/L, kappa/lambda light-chain ratio; MALT/NMZL, extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue type/nodal marginal zone lymphoma; MCL, mantle cell lymphoma; RH, reactive hyperplasia.

At AML diagnosis a total of 15 x $10^3$ non-gated events were acquired. Based on immunophenotypes at diagnosis, phenotypic abnormalities were defined and used in follow-up samples. Applied three-color moAb (Table 3) combinations were tested in normal or reactive BM samples for sensitivity in detecting LAIPs in the current study or by other groups (Venditti et al., 2000; Feller et al., 2004; Macedo et al., 1995; Kern et al., 2003). At follow-up, at least 30 000 cells were analyzed in each tube. Live-gate analysis was used in five patient's follow-up samples. A total of 100 000–500 000 cells were analyzed in allowing sensitivity of at least 0.015%. Data-analysis was performed using Paint-a-Gate Pro (BD) software (Figure 5). In most cases CD34/SSC or CD117/SSC gates were applied. Detectable MRD was defined as a distinct cluster of 15–20 dots. Sensitivity levels were determined as:

- 0.1% if 30 000 events were acquired,
- 0.05% if 30 000 events were acquired in cases with highly aberrant LAIP as co-expression of CD34 and CD7, CD14, CD56 or CD65 and CD34+/CD15+/HLA-DR-,
- 0.015% if live-gate approach was used (Macedo et al., 1995).

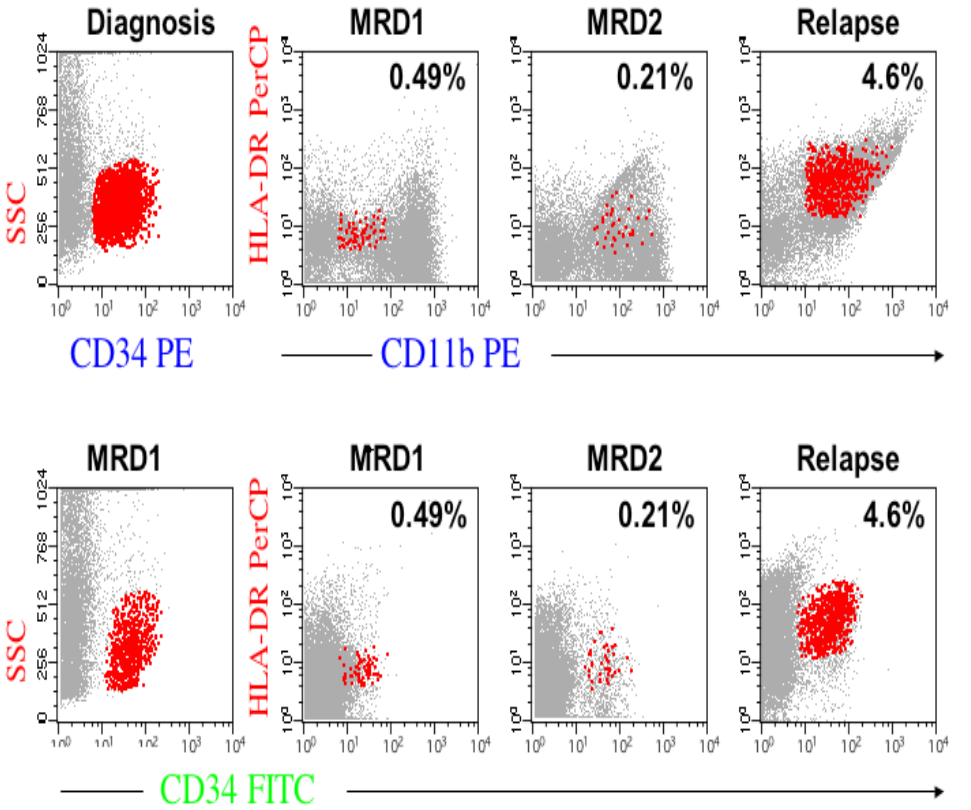


**Figure 5.** Illustration of sequential flow cytometry MRD analyses after induction (MRD1), at the end of post-remission treatment (MRD2) and before overt relapse in an AML patient who eventually relapsed.

The three-color moAb combination of CD34 FITC/CD11b PE/HLA-DR PerCP was used in the follow-up samples to detect MRD. The aberrant phenotype relied on over-expression of CD11b and lower than normal expression of HLA-DR in CD34+ cells. The positivity for CD11b and HLA-DR was similar at diagnosis and at incipient relapse (illustrated in the right upper and lower plots). The left upper plot shows CD34 positivity at diagnosis (CD34 PE/SSC plot) and left lower plot gating strategy during follow-up (CD34 FITC/SSC plot). The frequencies of CD34+/CD11b+/HLA-DRdim triple positive cells at indicated time-points expressed as percentages of total BM events are shown in upper and lower middle plots.

DC frequency was evaluated by Lineage cocktail 1 (lin)-FITC/CD123-PE/HLA-DR-PerCP/CD11c-APC (BD, San Jose, CA, USA), see Figure 6. Plasmacytoid DC immunophenotype was characterized as lin-/HLA-DR+/ CD123+ and myeloid DC as lin-/HLA-DR+/ CD11c+ (Figure 6). The lin marker included cocktail of CD3, CD14, CD16, CD19, CD20 and CD56. On

average, 150 000 non-gated events were acquired. A DC subset was detected when a distinct cluster of at least 10 dots was found. Data-analysis was performed using Paint-a-Gate Pro (BD) software (Figure 6).

MRD was analyzed by four-color flow cytometry as described in previous publication (Björklund et al., 2003). MRD was defined as a presence of a distinct cluster of cells with LAIP in BM.

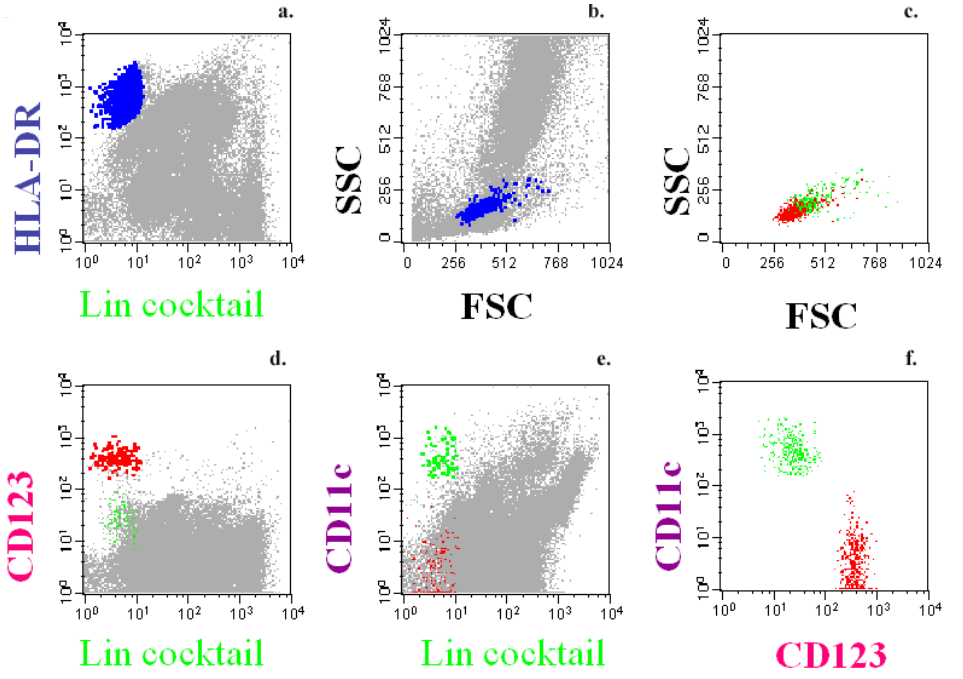


**Figure 6.** Detection of dendritic cell (DC) subsets.

Illustration of DC subset analysis in bone marrow sample from a hospital control group patient. 100 µl of sample was incubated with following antibodies: 20 µl of the Lineage cocktail 1 FITC (cat nr. 340546, contains CD3, CD14, CD16, CD19, CD20 and CD56), 10µl CD123 PE (cat nr. 340545), 10µl anti-HLA-DR PerCP (cat nr. 347402) and 5 µl CD11c APC (cat nr. 3337402). All antibodies were obtained from Becton & Dickinson Biosciences (BD), Franklin Lakes, NJ, USA.

Upper left plot (a) shows the gating strategy of Lin cocktail negative and HLA-DR strongly positive cells. Middle upper plot (b) shows localization of DCs on forward scatter (FSC)/ side scatter (SSC) plot. Plasmacytoid DCs (pDCs) were identified by high levels of CD123 and were negative for CD11c {red dots, lower left (d) and right (f) plot}. Myeloid DCs (mDCs) expressed high levels of CD11c and low CD123 {green dots, lower middle (e) and right

(f) plot}. Plasmacytoid DCs were slightly smaller than mDCs {red and green dots, respectively, upper right plot (c)}. DC subsets were quantified as percentage of total BM events. Analysis was performed by Paint-A-Gate software (BD).

Abbreviations: FITC, flourescein isothiocyanate; PE, R-phycoerythrin; PerCP, peridinin chlorophyll protein; APC, allophycocyanin.

The diagnostic flow cytometry panel is listed in Table 7.

## 7. Statistical analysis

Descriptive statistics, analysis of variance and Dunnett's T3 post hoc tests were used in SPSS 9.0 (SPSS, Inc., Chicago, IL, USA) to analyze differences in frequencies of B and T cells, PI and Bcl-2 expression across NHL categories .

Dichotomous variables were compared between different groups using the $\chi^2$ test and continuous variables by Student's t test. Spearman rank correlation ($r$) was used to investigate correlations between continuous variables. RFS and OS were estimated according to the Kaplan-Meier method and differences between groups were analyzed using the log-rank test. The Cox proportional hazard method was used for multivariate analysis.

Non-parametric Mann-Whitney test was used for comparison of DC subset levels at different time-points. Spearman rank correlation ($r$) was used to investigate correlations between continuous variables and Wilcoxon's rank sum test for comparison of DC levels in different patient groups. P-values $<0.05$ were considered significant. All reported P-values are two-sided. All calculations were performed using SPSS 12.0 (SPSS, Chicago, IL, USA) software.

**Table 7.** Panel of Monoclonal antibodies.

| Triple combination | FITC | PE | PERCP/PECy5/TRI | APC |
|---|---|---|---|---|
| Standard panel of MoAb for immunophenotyping of NHL | | | | |
| 1. lambda/kappa/CD19/CD5 | DAKO-lambda (code F0435) | DAKO-kappa (code F0436) | IM-CD19 clone: J4.119 | BD-CD5 clone: L17/F12 |
| 2. CD23/CD10/CD20/CD19 | DAKO-CD23 clone: MHM 6 | DAKO-CD10 clone: SS2/36 | BD-CD20 clone: SK1 | BD-CD19 clone: SJ25C1 |
| 3. CD4/CD7/CD8/CD3 | DAKO-CD4 clone: MT310 | IM-CD7 clone: 8H8.1 | BD-CD8 clone: L27 | BD-CD3 clone: SK7 |
| 4. Bcl-2/CD10/CD19/CD3 | DAKO-Bcl-2 clone: 124 | DAKO-CD10 clone: SS2/36 | IM-CD19 clone: J4.119 | BD-CD3 clone: SK7 |
| Standard panel of MoAb for immunophenotyping of AML at diagnosis | | | | |
| 1. Simultest control | BD-control γ1/γ2 clone: X40,X39 | BD-control γ1/γ2 clone:X40,X39 | BD-G1/G2 clone: mouseIgG1/IgG2 | |
| 2. CD61/GPA/CD45 | DAKO-CD61 clone: Y2/51 | IM-GPA clone: 11E48-7-6 | BD-CD45 clone: 2D1 | |
| 3. CD19/CD34/CD45 | BD-CD19 clone: 4G7 | IM-CD34 clone: class III 581 IgG1 | BD-CD45 clone: 2D1 | |
| 4. CD10/CD19/CD13 | BD-CD10 clone:W8E8 | BD-CD19 clone: 4G7 | CALTAG-CD13 clone:MHCD1306 | |
| 5. CD15/CD33/CD20 | DAKO-CD15 clone: C3D-1 | DAKO-CD33 clone: WM-54 | BD-CD20 clone: L27 | |
| 6. CD7/CD5/CD3 | BD-CD7 clone: 4H9 | BD-CD5 clone: L17F12 | BD-CD3 clone: SK7 | |

| | | | | |
|---|---|---|---|---|
| 7. CD65/CD2/HLADR | GmbH-CD65<br>clone: VIM2 | DAKO-CD2<br>clone: S5.2 | BD-HLADR<br>clone: L243 | |
| 8.CD15/CD34/HLADR | DAKO-CD15<br>clone: C3D-1 | IM-CD34<br>clone: class III<br>581 IgG1 | BD-HLADR<br>clone: L243 | |
| 9. CD15/CD117/CD14 | DAKO-CD15<br>clone: C3D-1 | IM-CD117<br>clone: 95C3 | CALTAG-CD14<br>clone: TüK4 | |
| 10. CD2/CD56/CD33 | DAKO-CD2<br>clone: MT910 | BD-CD56<br>clone: MY31 | CALTAG-CD33<br>clone: TüK1 | |
| 11. CD7/CD13/CD19 | BD-CD7<br>clone: 4H9 | DAKO-CD13<br>clone: WM-47 | CALTAG-CD19<br>clone: MHCD1906 | |
| 12. CD65/CD11b/CD4 | GmbH-CD65<br>clone: VIM2 | DAKO-CD11b<br>clone: 2LPM19C | BD-CD4<br>clone: SK3 | |
| 13. CD4/CD8/CD3 | DAKO-CD4<br>clone: MT310 | BD-CD8<br>clone: SK1 | BD-CD3<br>clone: SK7 | |
| 14.lambda/kappa/CD19 | DAKO-lambda<br>polyclonal rabbit<br>$F(ab)_2$ fragment<br>of affinity | DAKO-kappa<br>polyclonal rabbit<br>$F(ab)_2$ fragment<br>of affinity | CALTAG-CD19<br>clone: MHCD1906 | |
| 15. CD22/CD5/CD20 | BD-CD22<br>clone: S-HCL-1 | BD-CD5<br>clone: L17F12 | BD-CD20<br>clone: L27 | |
| 16. CD34/CD38/CD19 | BD-CD34<br>clone: antiHPCA-2 | BD-CD38<br>clone: HB7 | CALTAG-CD19<br>clone: MHCD1906 | |
| 17. CD15/CD33/CD34 | DAKO-CD15<br>clone: C3D-1 | DAKO-CD33<br>clone: WM-54 | IM-CD34<br>clone: classIII<br>581 IgG 1mouse | |

| | | | | |
|---|---|---|---|---|
| 18. TDT/CD34/cytCD3 | DAKO-TdT clone: HT-6 | IM-CD34<br>clone: class III<br>581 IgG1 | BD-CD3<br>clone: SK7 | |
| Standard panel of MoAb for immunophenotyping of DCs subsets | | | | |
| 1. Lin-cocktail/CD123/HLA-DR/CD11c | BD-CD3,CD14,<br>CD16,CD19, CD20, CD56<br>cat nr. 340546 | BD-123<br>cat nr. 340545 | BD-HLADR<br>cat nr. 347402 | BD-CD11c<br>cat nr. 3337402 |

APC: allophycocyanin; FITC: fluorescein isothiocyanate; PE: R-phycoerythrin; PERCP: peridinin chlorophyll protein; PE-Cy5: tandem conjugate system which combines R-phycoerythrin and a cyanine dye; TRI: TRI-COLOR, PE-Cy5 tandem conjugate from CALTAG; BD: Becton and Dickinson (San José, CA, USA); DAKO: DakoCytomation (Glostrup, Denmark); IM, Immunotech: (Marseille, France); CALTAG: Caltag Laboratories (San Francisco, CA, USA); GmbH: An-der-Grub Biosearch (Vienna, Austria); TdT: terminal deoxynucleotidyl transferase; cyt: cytoplasmic.

# RESULTS

## 1. Diagnostic value of FCM in NHL diagnosis in FNA aspirates (Paper I)

### 1.1. Accuracy of FCM in diagnosis of NHL

#### *1.1.1. Low-grade NHL*

Our FCM panel was very efficient in detecting LG-NHL. The LG-NHL categories (FL, CLL, IC/LPL, MALT/NMZL, MCL, B-NHL-UN) were correctly diagnosed in 95% (177 of 186) of cases (Table 8).

**Table 8.** Accuracy of primary and recurrent lymphoma diagnoses

| Cytopathologic diagnosis | Cases (n) | FCM concordance[a] | Histopathologic confirmation |
|---|---|---|---|
| Primary | | | |
| FL | 47 | 45/47 (96%) | 28/47 (60%) |
| CLL | 26 | 26/26 (100%) | 10/26 (38%) |
| IC/LPL | 8 | 8/8 (100%) | 2/8 (25%) |
| MALT/NMZL | 15 | 15/15 (100%) | 2/15 (13%) |
| MCL | 6 | 4/6 (67%) | 4/6 (67%) |
| HG-NHL | 29 | 22/29 (76%) | 10/29 (34%) |
| T-NHL | 7 | 3/7 (43%) | 5/7 (71%) |
| B-NHL-UN | 4 | 0/4 | 0/4 (0%) |
| Total | 142 | 123/142 (87%) | 61/142 (43%) |
| Recurrent | | | |
| FL | 33 | 33/33 (100%) | |
| CLL | 32 | 32/32 (100%) | |
| IC/LPL | 2 | 2/2 (100%) | |
| MALT/NMZL | 8 | 7/8 (88%) | |
| MCL | 5 | 5/5 (100%) | |
| HG-NHL | 7 | 6/7 (86%) | |
| T-NHL | 10 | 6/10 (60%) | |
| Total | 97 | 91/97 (94%) | |

Abbreviations: B-NHL-UN, B-cell non-Hodgkin's lymphoma, unspecified; CLL, chronic lymphocytic leukemia; FCM, flow cytometry; FL, follicular lymphoma; HG-NHL, high-grade B-cell non-Hodgkin's lymphoma; IC/LPL, immunocytoma/lymphoplasmocytic lymphoma; MALT/NMZL, extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue type/nodal marginal zone lymphoma; MCL, mantle cell lymphoma; T-NHL, T-cell non-Hodgkin's lymphoma.

[a] Cases in which the FCM results was in concordance with the final diagnosis. In case of FL, CLL and MCL characteristic pathologic immunophenotype was detected by FCM. In case of IC/LPL, MALT/NMZL and HG-NHL pathologic monoclonal B-cell population was detected. In T-NHL an aberrant phenotype within the T-cell population was found.

In four cases lymphoma subclassification was not possible (B-NHL-UN) and was therefore defined as FCM discordant.

In total, histopathologic examination was available in 46% (86 of 186) of LG-NHL cases. Cytologic diagnosis was concordant with histopathology in all cases. Eight samples were diagnosed by FCM as unspecified LG-NHL. In two of these cases histopathology confirmed CD10- FL based on morphologic observations and Bcl-6 expression. Another two samples appeared to be MCL. On review of FCM plots of both cases, we found a low level of CD5 expression that was not correctly evaluated during initial analysis. Cyclin D1+ MCL was confirmed in one case by lymph node histopathology and in the second case by BM examination. LG-NHL subclassification was not possible in four patients. Cytologic findings suggested diagnosis of FL because of a predominance of small to medium-sized centrocyte-like cells. However, theses lymphomas were CD10- and in three studied cases FISH analysis did not show t(14;18), t(11;14) or t(11;18). However, FISH analysis showed extra copies of chromosomes 11, 14 and 18 in one case and trisomy 18 in one case. In one patient genetic studies were not available due to lack of material. In these four patients biopsy for histologic examination was not performed because patients were older than 76 years and the biopsy was considered uninformative regarding treatment decisions. In one sample of recurrent MALT/NMZL, FCM did not show a monoclonal B-cell population. This sample was obtained from soft tissue and had a very low cell count; on average only 1500 events were registered per tube.

### *1.1.2. High-grade NHL*

HG-NHL was diagnosed in 36 FNA samples from 35 patients (31 cases of DLBCL, two of Burkitt-like lymphoma, two of TCRBCL and one of anaplastic B-cell lymphoma). Anaplastic and both TCRBCL diagnoses were confirmed by tissue biopsy. Immunoglobulin (Ig) heavy-chain gene rearrangement analysis was done in one case of TCRBCL, which showed clonal Ig rearrangement.

In 78% (28 of 36 samples) FCM suggested a diagnosis of lymphoma. From these 28 samples Ig light-chain restriction was detected in 22 samples of DLBCL and two of Burkitt-like NHL. In four samples malignant B-cells did not express surface Ig (sIg). In eight samples FCM was not able to diagnose lymphoma: one case of anaplastic B-cell lymphoma, two cases of TCRBCL and five cases of DLBCL. In these samples FCM analysis showed a dominant reactive T-cell population with very few B cells.

### *1.1.3. T-NHL*

T-NHL was found in 17 patients. T-NHL diagnosis was confirmed in 13 cases by tissue histopathology. T-cell receptor gene rearrangement analysis was done in four primary and three recurrent cases. In 53% of T-NHL cases (three of seven primary lymphomas and six of 10 relapsed lymphomas) FCM showed aberrant findings within T-cell population. These aberrant findings were a high

CD4/CD8 ratio (11–23) in three samples, CD4+CD7– population in three samples, CD4/CD8 double negative in one sample or double positive population in one sample. T-cell precursor leukemia/lymphoma diagnosis was confirmed by an additional study that showed expression of terminal TdT. In one sample FNA and cytology showed reactive changes in an enlarged lymph node. However, in this case CD4/CD8 ratio was 17.7 and recurrence of T-NHL was confirmed by T-cell receptor gene rearrangement analysis.

## 1.2. Accuracy of diagnosis in RH

FCM was concordant with the diagnosis of RH in 97% of 172 samples. In four aspirates with final diagnosis of RH both FCM and IC suggested a possibility of lymphoma. In the first case FCM showed a K/L ratio of 10.6 and cytology showed a mixed-cell population of small lymphocytes and relatively large blast-like cells. Clonality analysis by IC was not representative due to background staining. In the second case a subpopulation of monoclonal lambda CD10+ B cells with high Bcl-2 expression was found. Therefore, a possible partial engagement of FL was reported by FCM. In these two patients a subsequent lymph node biopsy could not confirm a diagnosis of lymphoma and Ig heavy-chain rearrangement analysis showed a polyclonal pattern. In another patient FCM revealed a subpopulation of B cells expressing dim kappa, dim CD5 and high levels of Bcl-2, indicating thus suspicious lymphoma. The IC findings were discordant to FCM by showing dominant lambda-positive B-cells (K/L 0.6). Lymph node excision biopsy was not performed in the latter two patients, but FISH analysis showed normal chromosomes 11, 14 and 18. Importantly, none of these patients developed lymphoma during two-year clinical follow-up. In the fifth discrepant sample FCM showed dim kappa positivity in the CD19+ B-cell population, but FNA cytology and IC were concordant with infectious mononucleosis, which was later confirmed by laboratory tests.

## 1.3. Diagnostic parameters obtained from FCM panel

### *1.3.1. B-cell and T-cell counts*

Frequencies of B cells, T cells and CD4/CD8 ratio determined by FCM in different NHL categories are given in Table 9.

The B-cell count was significantly higher in LG-NHL as compared to RH:

- $P < 0.001$ for FL, CLL, IC/LPL, MALT/NMZL
- $P = 0.002$ for MCL.

**Table 9.** CD19+, CD7+ cells in the lymphocyte gate, and CD4/CD8 ratio in NHL and RH

| Cytopathologic diagnosis | %CD19+ | %CD7+ | CD4/CD8 |
|---|---|---|---|
| FL | 64.8 ± 18.2 (80) | 26.1 ± 14.9 (80) | 3.9 ± 2.7 (80) |
| CLL | 79.5 ± 13.3 (58) | 16.6 ± 12.5 (58) | 3.6 ± 2.1 (58) |
| IC/LPL | 79.1 ± 13.6 (10) | 17.8 ± 11.5 (10) | 3.7 ± 2.4 (10) |
| MALT/NMZL | 69.7 ± 19.8 (23) | 19.6 ± 15.1 (23) | 3.6 ± 2.2 (23) |
| MCL | 82.2 ± 20.5 (11) | 12.9 ± 15.0 (11) | 1.8 ± 1.6 (11) |
| HG-NHL | 50.4 ± 25.3 (35) | 25.7 ± 19.5 (35) | 2.0 ± 2.1 (35) |
| T-NHL | 27.6 ± 21.7 (17) | 61.6 ± 22.3 (17) | 7.8 ± 7.2 (15) |
| RH | 45.6 ± 17.6 (172) | 44.9 ± 17.1 (172) | 4.5 ± 2.9 (172) |

Values are mean ± standard deviation (number of cases).
Abbreviations: CLL, chronic lymphocytic leukemia, FL, follicular lymphoma; HG-NHL, high-grade B-cell non-Hodgkin's lymphoma; IC/LPL, immunocytoma/lymphoplasmocytic lymphoma; MALT/NMZL, extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue type/nodal marginal zone lymphoma; MCL, mantle cell lymphoma; RH, reactive hyperplasia; T-NHL, T-cell non-Hodgkin's lymphoma.

No difference in B-cell counts was found between RH and HG-NHL ($P = 0.998$). T-cell counts showed a significant difference between RH and all B-cell NHL categories ($P < 0.001$). Among B-cell lymphomas B-cell count was lowest in HG-NHL, with a significant difference between HG-NHL and categories of LG-NHL except for FL ($P = 0.085$):

- $P < 0.001$ for CLL, $P = 0.001$ for IC/LPL
- $P = 0.041$ for MALT/NMZL
- $P = 0.007$ for MCL.

We also found a significant difference in B-cell count and T-cell count between CLL and FL ($P < 0.001$ for both cell subsets). Results of statistical analysis performed in 124 histopathologically confirmed cases did not differ from overall results.

### *1.3.2 K/L ratio*

K/L was assessed within a gated CD19+ B-cell population. On average, K/L in RH was 1.7 (median 1.6, range 0.4–4.7). Of 222 B-cell NHL samples 128 (58%) expressed monoclonal kappa and 77 (35%) monoclonal lambda. We did not find sIg expression in eight cases (3%) (four HG-NHL, three FL and one CLL). Two of these cases were also negative for both light chains by IC. IC showed cytoplasmic kappa expression in one sample and cytoplasmic lambda in four samples. In one sample IC staining was not representative due to technical reasons.

In nine (4%) lymphoma samples (one of anaplastic B-cell lymphoma, two of TCRBCL, five of DLBCL and one MALT/NMZL) FCM showed polyclonal B-cells. Two of these samples were monoclonal for kappa and one for lambda by IC. In five samples clonality could not be demonstrated by IC due to background staining. K/L staining by IC was not performed in one case of recurrent MALT/NMZL because of characteristic cytologic findings.

### *1.3.3. CD4/CD8 ratio*

The CD4/CD8 ratio was highest in RH (4.5 ± 2.9) and lowest in MCL (1.8 ± 1.6, P = 0.002 between RH and MCL) and HG-NHL (2.0 ± 2.1, P < 0.001 between RH and HG-NHL), Table 9. Among different lymphoma categories, CD4/CD8 ratio was significantly lower in MCL and HG-NHL than in FL (P = 0.013 and P = 0.001, respectively) and lower in HG-NHL than in CLL (P= 0.008). When only histopathologically confirmed cases were included in statistical analysis, the results remained the same.

### *1.3.4. Bcl-2 expression*

Expression of Bcl-2 was examined in 72 cases of FL, 52 cases of CLL, eight cases of IC/LPL, 18 cases of MALT/NMZL, 10 cases of MCL, 29 cases of HG-NHL, and 137 cases of RH (Figure 7).

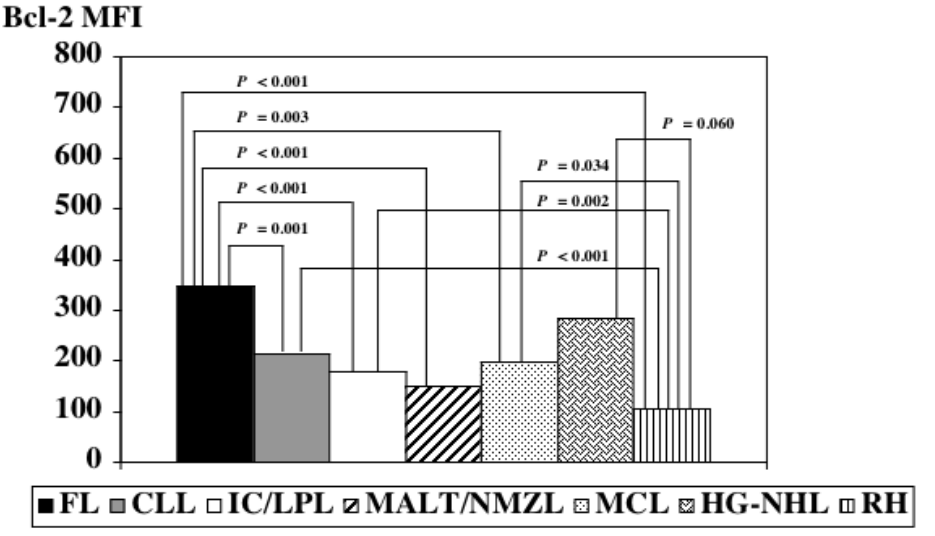


**Figure 7.** MFI of Bcl-2 fluorescein isothiocyanae (FITC) monoclonal antibody in B cells in B-cell NHL and RH.

There was no significant difference between FL and HG-NHL and between RH and MALT/NMZL.

We found similar levels of Bcl-2 expression in T cells in B-cell NHL (MFI 94.7 ± 44.7) and RH (MFI 94.8 ± 28.3). In contrast, malignant B cells had significantly higher mean Bcl-2 MFI values (266.3 ± 207.6) than did reactive CD10- B cells (105.3 ± 31.6). Furthermore, normal germinal center CD10+ B-cells in RH expressed considerably lower levels of Bcl-2 (MFI 54.4 ± 30.9) than did T cells and CD10- B cells. The mean MFI value for Bcl-2 in malignant B cells was highest in FL (347.0 ± 248.6), Figure 7.

Statistical analysis of Bcl-2 expression showed that B cells in RH samples expressed significantly lower levels of Bcl-2 than did B cells in lymphomas (Figure 7).

In HG-NHL we noted a heterogeneous Bcl-2 expression. Ten of 29 HG-NHL samples were considered "low" in Bcl-2 because the level of Bcl-2 expression was lower than or equal to that of reactive T cells. In nine samples of CD10+ HG-NHL very high levels of Bcl-2 were found (MFI 440.6 ± 268.1).

We also analyzed corrected MFI values for Bcl-2 defined as the ratio of Bcl-2 MFI in malignant B cells to that in T cells from the same sample. Analysis confirmed the results obtained with MFI alone. The highest corrected MFI value for Bcl-2 was found in

- FL (3.69 ± 2.62) and the lowest value was found in RH (1.14 ± 0.27).

We found no substantial overlap between Bcl-2 MFI in B cells of RH and B-cell NHL. Only in nine cases results obtained in reactive B cells exceeded the first quartile of corrected MFI value for Bcl-2 of malignant B-cells. The first, second, and third quartile values of corrected Bcl-2 MFI in B cells were

- 0.97; 1.10 and1.27 in RH and
- 1.55; 2.34 and 3.40 in B-cell NHL.

Statistical analysis of Bcl-2 expression in histopathologically confirmed cases of B-cell NHL and RH showed similar results.

The addition of a moAb combination that included Bcl-2 was especially helpful in lymphoma samples with presence of rests of normal germinal centers. In these samples the results of K/L determination could be misleading, but a population of cells with high Bcl-2/CD10 expression could allow the correct diagnosis. Further, in other cases determination of K/L could be difficult due to weak or negative expression of sIg. We found 49 such cases (30 of FL, 10 of HG-NHL, five of MALT/NMZL, three of CLL and one of MCL). Of those, Bcl-2 expression was analyzed in 39 cases. In 35 of these, high expression of Bcl-2 supported a lymphoma diagnosis. In addition, Bcl-2 analysis helped to establish a diagnosis in one case of CLL, one of HG-NHL, and three of sIg-negative FL.

Of particular interest was one case of primary DLBCL in which K/L staining by FCM did not show clonality even in large cell subpopulation (B cells were

sIg negative). These large cells demonstrated a CD10+/CD19+ immunophenotype with very high levels of Bcl-2. By IC the malignant large cells expressed cytoplasmic lambda and had a proliferation rate of 50%.

# 2. Prognostic relevance of MRD detected by FCM in young adult AML (Paper II)

## 2.1. Frequency of aberrant phenotype in AML

AML blasts from 93.5% of all patients (58/62) and 94% of those in patients in morphological CR (50/53) expressed LAIP at diagnosis. Aberrant phenotypes were divided into four groups (San Miguel et al., 1999; Terstappen et al., 1992):

- Cross-lineage infidelity (n=32)
- Asynchronous antigen expression (n=48)
- Antigen over-expression (n=9)
- Lack of antigen expression (n=5).

Blasts from 28 of 50 patients (56%) expressed highly aberrant phenotypes (mainly cross-lineage infidelity) and blasts from 22 patients (44%) had aberrant phenotypes, based mostly on maturation asynchrony (Table 10). At diagnosis 13, 20, seven and five patients expressed one, two, three and four LAIP, respectively.

**Table 10.** Aberrant phenotypes applied in MRD studies

| Aberrant phenotype | No (%) |
|---|---|
| Cross-lineage infidelity | 32 (34) |
| CD2 | 5 |
| CD7 | 14 |
| CD19 | 5 |
| CD56 | 8 |
| Antigen over-expression | 9 (10) |
| CD34++ | 9 |
| Antigen under-expression | 5 (5) |
| CD33- | 5 |
| Asynchronous antigen expression | 48 (51) |
| CD34+/CD38-/My+ | 1 |
| TdT+/My+ | 6 |
| CD34+/CD11b+ | 11 |
| (CD34+,CD117+)/HLA-DR-/My+ | 5 |
| (CD34+,CD117+)/CD4+/My+ | 5 |
| (CD34+,CD117+)/CD15++/My+ | 15 |
| (CD34+,CD117+)/CD14+ or CD65+/My+ | 5 |

Total 94 aberrant phenotypes were detected in 45 patients.
My: myeloid marker

## 2.2. Prediction of survival by MRD

MRD was analyzed at the first time-point in 43 patients in morphological remission and at the second time-point in 31 patients. MRD was evaluated at both time-points in 30 patients. MRD was detectable in 32 (74%) patients at the first time-point and in 17 (55%) at the second time-point. The mean MRD levels were 0.23% (median 0.13%, range 0.01–2.48%) after the induction and 0.23% (median 0.06%; range 0.04–2.0%) after the end of chemotherapy or before transplantation. By Cox analysis, detectable MRD after the induction did not predict either RFS or OS, but there was a trend for longer RFS in patients with no detectable MRD at the second time-point ($P = 0.061$).

Prolonged RFS and OS was found in patients subjected to SCT as compared to the remainder (median RFS 53.5 m vs 8 m, $P < 0.001$ and median OS 54,5 m vs 14,3 m $P = 0.001$, respectively). However, by log-rank test MRD1 and MRD2 positive young adult AML patients had significantly shorter five-year RFS than patients with no detectable MRD {51% versus 90% for time-point (1) ($P = 0.044$) and 42% versus 85% for time-point (2) ($P = 0.039$), respectively}. OS was not significantly different between MRD1 and/or MRD2 positive and negative patients. The percentage of blasts in BM at diagnosis, absolute WBC count, Hb levels, platelet count, number of cycles to achieve mCR, and cytogenetic risk group were not significant predictors of RFS and OS.

## 2.3. Prognostic significance of SCT in relation to MRD status

### *2.3.1. Allo-SCT in relation to MRD*

Since SCT was the only significant prognostic factor in the studied group of AML patients we decided to investigate how this therapy influenced survival of MRD positive and MRD negative patients. For this analysis, the patients were divided into 4 categories:

- no detectable MRD and allo-SCT;
- detectable MRD and allo-SCT;
- no detectable MRD and no allo-SCT;
- detectable MRD and no allo-SCT.

These groups had significantly different RFS based on both MRD1 and MRD2 (Figure 8 i and ii) and showed a trend for difference in OS ($P = 0.069$) based on MRD2. MRD1 and/or MRD2 positive patients, who did not undergo allo-SCT, had the worst outcome (five-year RFS 24% and 20%, respectively; and OS 34% and 35%). MRD positive patients that were subjected to allo-SCT had similar cumulative RFS (Figure 8 i and ii) when compared to patients who were MRD1 and/or MRD2 negative. Similar OS was also observed in both these groups (67% versus 70% for the first time-point and 75% for the second time-point, respectively).

### *2.3.2. Auto-SCT in relation to MRD*

During 1994–1997 eligible AML patients were included in an extended British-Italian-Swedish phase II trial of auto-SCT as consolidation treatment in first mCR. The treatment results have been promising. Since auto-SCT improved RFS and OS, we analyzed the potential impact of auto-SCT on RFS and OS in the MRD positive young adult AML patients in more detail. Indeed, MRD1 and/or MRD2 positive patients subjected to auto-SCT had significantly better RFS (Figure 8 iii and iv) than mCR patients who received only conventional post-induction chemotherapy. However, the comparison of RFS (Figure 8 iii and iv) showed that MRD positive patients, who underwent allo-SCT had a significantly better prognosis as compared to patients who received auto-SCT or conventional chemotherapy. The five-year OS was also significantly better in allo-SCT patient group than in auto-SCT or conventional chemotherapy patient groups {67%, 46 % and 31% for the first time-point (P = 0.021) and 75%, 53% and 25% for time-point two (P = 0.046), respectively}.

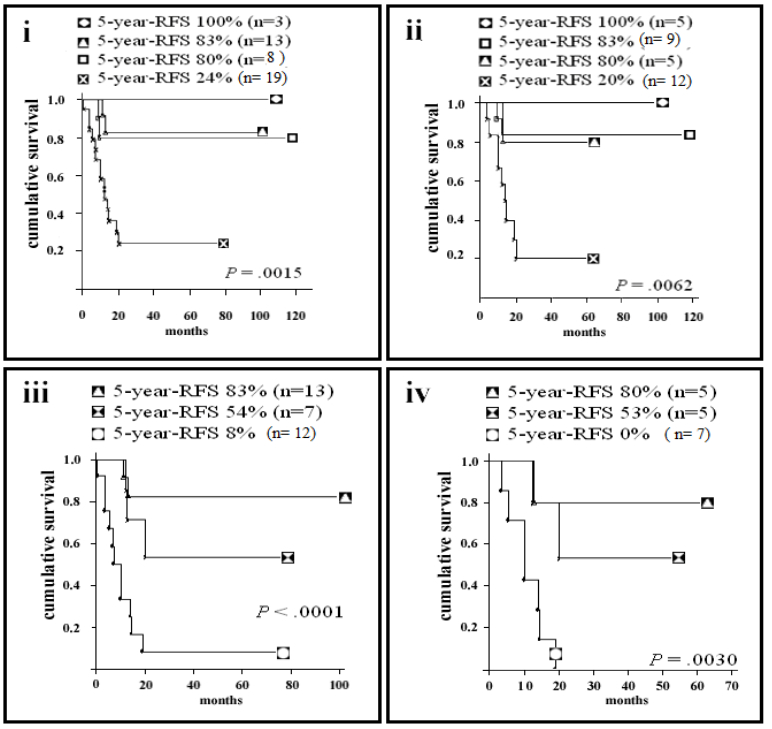


**Figure 8.** Relapse-free survival (RFS) of younger adult AML patients divided according to MRD status and type of post-remission treatment.

MRD status was studied after induction treatment (i and iii) and at the end of post-remission chemotherapy or before stem cell transplantation (ii and iv).

i and ii: AML patients 19–60 years old were divided in four groups: no detectable MRD (MRD-) and allo-SCT (); detectable MRD (MRD+) and allo-SCT (); MRD- and no allo-SCT (); and MRD+ and no allo-SCT (). MRD+ patients who received allo-SCT had similar RFS to MRD- patients who did not receive allo-SCT.

iii and iv: Only MRD+ patients were considered. The MRD+/no allo-SCT patient group was divided into two subgroups: auto-SCT () and only conventional chemotherapy (). MRD+ patients subjected to allo-SCT () had significantly longer RFS after the induction treatment (iii) or before stem cell transplantation (iv) than patients who received auto-SCT () or only conventional chemotherapy ().

# 3. DC levels in BM evaluated by FCM in childhood ALL (Paper III)

## 3.1. DC levels in children with ALL at diagnosis

DC levels in BM at diagnosis were evaluated in seven children with preT-ALL and 62 with preB-ALL (Table 11). We found a profound deficiency of both DC subset levels in most patients. Only two patients (one preB-ALL (SI), one preT-ALL) had DC subset levels within the reference range. In the preB-ALL HR patients, we found a complete absence of both DC subsets. In the SR preB-ALL (II) group only one of 28 patients (4%) had very low levels of DCs (both pDC and mDC 0.01%). In the SR preB-ALL (SI) group, low levels of pDCs (mean 0.10%) were found in 30% and mDCs (mean 0.06%) in 22% of patients. Therefore, the levels of pDC and mDC in preB-ALL (SI) group were significantly higher than in B-ALL HR group ($P < 0.001$ for pDC and $P = 0.012$ for mDC). The levels of pDC were also significantly higher in the SR preB-ALL (SI) than in the SR preB-ALL (II) group ($P = 0.007$) and the levels of mDC showed the trend ($P = 0.053$) for higher numbers in the SR preB-ALL (SI) group.

In the preT-ALL group, three patients (43%) had detectable pDCs (mean 0.11%) and two of patients (29%) had mDCs (0.04%; 0.28%). The levels of pDCs and mDCs were significantly higher in preT-ALL patients as compared to preB-ALL group ($P = 0.044$ and $P = 0.041$, respectively).

**Table 11.** The level of DC subsets in bone marrow of children without leukemia and in childhood ALL at diagnosis {Median (range)}.

| Patient group | Hospital control group | | preT-ALL | | preB-ALL HR | | preB-ALL (II) | | preB-ALL (SI) | |
|---|---|---|---|---|---|---|---|---|---|---|
| DC subtype | pDC | mDC | pDC | mDC | pDC | mDC | pDC | mDC | pDC | mDC |
| Patient no | n = 9 | | n = 7 | | n = 11 | | n = 28 | | n = 23 | |
| Median (range) | 0.28 (0.14–0.53) | 0.15 (0.10–0.28) | 0 (0–0. 26) | 0 (0–0.28) | 0 | 0 | 0 (0–0.01) | 0 (0–0.01) | 0 (0–0.40) | 0 (0–0.20) |
| Mean ± ST | 0.31±0.11 | 0.16±0.06 | 0.05±0.10 | 0.05±0.10 | 0 | 0 | 0.01 | 0.01 | 0.03±0.08 | 0.01±0.04 |
| No of pts. with DCs | 9 (100%) | 9 (100%) | 3 (42.9%) | 2 (28.6%) | 0 | 0 | 1 (3.6%) | 1 (3.6%) | 7 (30.4%) | 5 (21.7%) |

* DC levels are given as % of total bone marrow cells Abbreviations: pDC – plasmacytoid dendritic cell; mDC – myeloid dendritic cell; preB-ALL HR- high-risk precursor B ALL patients; preB-ALL (II) – precursor B ALL patients stratified to therapy Protocol II; preB-ALL (SI) – precursor B ALL patients stratified to therapy Protocol SI; preT-ALL – precursor T lymphoblastic leukemia

## 3.2. Correlation of DC subset levels with other haematological values at diagnosis

The median neutrophil counts at diagnosis were 10.9 x $10^9$/L (range 0.6 –16.4 × $10^9$/L) for preT-ALL and 0.45 × $10^9$/L, (range 0.0–5.2 × $10^9$/L) for preB-ALL patients. We found a positive correlation between blood neutrophil counts and levels of both DC subsets in BM ($P = 0.005$; $r = 0.331$ and $P < 0.001$; $r = 0.441$ for pDC and mDC levels, respectively). pDC levels, but not mDC levels, also correlated with platelet counts ($P = 0.010$). There was no correlation between DC levels and Hb values.

## 3.3. Regeneration of DCs during treatment

The DC levels in the BM increased in parallel with the total BM cellularity, which was on average 10% at day 15 (week 2), 30% at day 29 (week 4), 50–70% at day 50 (week 7) and 70–90% onwards. There was no significant correlation between pDC and mDC levels in the BM with peripheral WBC count. Detailed data on pDC regeneration is presented in Table 12 and Figure 9 and an example of follow-up of one representative patient is demonstrated in Figure 10.

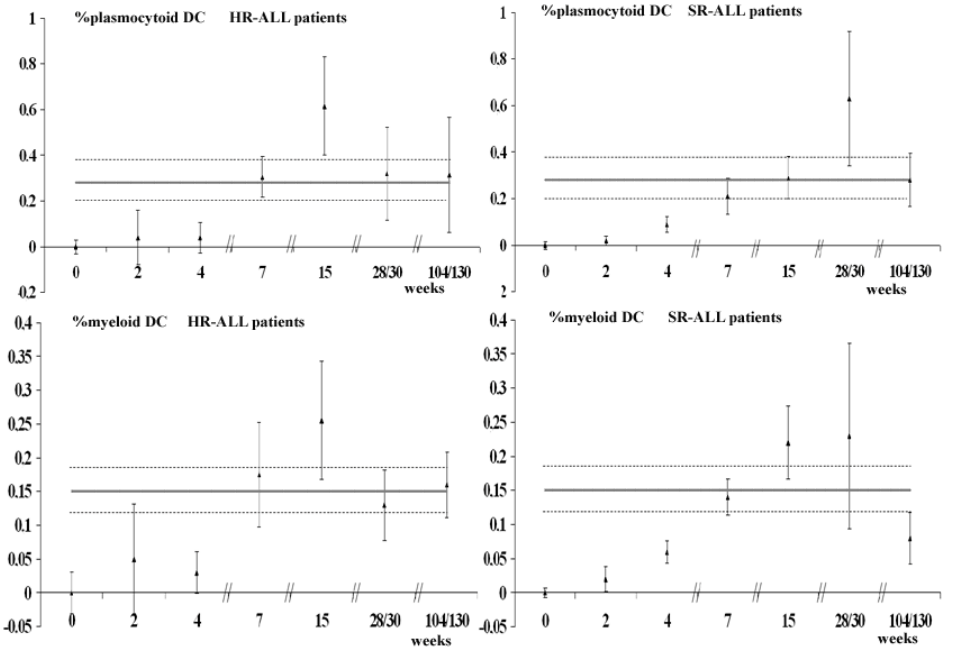


**Figure 9.** Regeneration of dendritic cell (DC) subsets in bone marrow.

**Table 12.** Regeneration of DC subsets in bone marrow of children treated by NOPHO ALL-2000 protocol {Median (range)}.

| | preT-ALL | | preB-ALL HR | | preB-ALL (II) | | preB-ALL (SI) | |
|---|---|---|---|---|---|---|---|---|
| Time-point in treatment | pDC | mDC | pDC | mDC | pDC | mDC | pDC | mDC |
| d 15 (week 2) | n = 5 | | n = 8 | | n = 26 | | n = 21 | |
| | 0.04 (0.02–0.24) | 0.04 (0.03–0.12) | 0.06 (0.02–0.77) | 0.055 (0.02–0.57) | 0.015 (0–0.35) | 0.01 (0–0.17) | 0.03 (0–0.22) | 0.05 (0–0.30) |
| d 29 (week 4) | n = 6 | | n = 9 | | n = 28 | | n = 24 | |
| | 0.035 (0–0.18) | 0.035 (0.01–0.14) | 0.08 (0–0.53) | 0.03 (0–0.25) | 0.075 (0–0.47) | 0.045 (0–0.20) | 0.10 (0–0.61) | 0.075 (0–0.29) |
| d 50 (week 7) | n = 7 | | n = 11 | | n = 28 | | n = 23 | |
| | 0.33 (0.09–0.57) | 0.15 (0.06–0.29) | 0.27 (0.11–0.77) | 0.18 (0.02–0.77) | 0.215 (0.03–0.91) | 0.135 (0.01–0.33) | 0.19 (0.03–0.72) | 0.15 (0.02–0.39) |
| d 106 (week 15) | n = 7 | | n = 11 | | n = 25 | | n = 20 | |
| | 0.65 (0.41–1.38) | 0.29 (0.17–0.67) | 0.52 (0.20–1.84) | 0.23 (0.05–0.65) | 0.29 (0.07–0.73) | 0.21 (0.06–0.36) | 0.31 (0.14–1.17) | 0.26 (0.09–1.12) |
| Week 28/30 | n = 3 | | n = 6 | | n = 13 | | | |
| | 0.41 (0.16–0.70) | 0.13 (0.13–0.14) | 0.235 (0.07–0.86) | 0.08 (0.01–0.28) | 0.63 (0.1–1.87) | 0.23 (0.05–1.03) | nd | nd |
| End of treatment | n = 2 | | n = 6 | | n = 5 | | n = 7 | |
| | 0.15; 0.28 | 0.19; 0.15 | 0.425 (0.04–0.95) | 0.15 (0.02–0.26) | 0.17 (0.06–0.46) | 0.06 (0.01–0.20) | 0.395 (0.16–0.46) | 0.095 (0.05–0.20) |
| | | | | | n = 2 | | n = 6 | |
| 6m post treatment | nd | nd | nd | nd | 0.09; 0.04 | 0.13; 0.35 | 0.325 (0.18–0.46) | 0.115 (0.07–0.17) |

* DC levels are given as % of total bone marrow cells Abbreviations: pDC – plasmocytoid dendritic cell; mDC – myeloid dendritic cell; preB-ALL HR-high-risk precursor B ALL patients; preB-ALL (II) – precursor B ALL patients stratified to therapy Protocol II; preB-ALL (SI) – precursor B ALL patients stratified to therapy Protocol SI; preT-ALL – precursor T acute lymphoblastic leukemia

Median levels of plasmocytoid DCs (pDC) (a,b) and myeloid DCs (mDC) (c,d) in bone marrow at indicated time-points during treatment according to NOPHO ALL-2000 protocol (see Figure 3) are given. The median hospital control values are indicated at each plot (——) with 95% confidence intervals (dotted lines). Data for high-risk (HR) and standard-risk (SR) patients are shown as separate curves. Error bars represent 95% confidence interval. In HR group 18, 13, 15, 18, 18, nine and eight patients were analyzed at diagnosis, week 2, 4, 7, 15, 28, and 104, respectively. In SR group 51, 47, 52, 53, 45, 13 and 12 patients were analyzed at diagnosis, week 2, 4, 7, 15, 30 and 130, respectively. End of treatment was at 104 weeks in the HR group and 130 weeks in the SR group.

### *3.3.1. Regeneration of pDC subset*

At day 15 (week 2), all HR patients had already detectable pDCs in BM samples and no increase of pDC levels between days 15 (week 2) and day 29 (week 4) was found. At day 15 (week 2), pDC were detected in 67% and 58% of patients from preB-ALL (SI) and (II) groups, respectively, and at day 29 (week 4), in 96% and 89% of patients from preB-ALL (SI) and (II) groups, respectively. The levels of pDC increased significantly between days 15 (week 2) and 29 (week 4) in both SI and II groups ($P < 0.001$). However, at day 29 (week 4) the levels of pDC in both SR and HR groups remained significantly lower than reference levels ($P < 0.001$, respectively).

At day 50 (week 7) pDCs were detected in all analyzed BM samples and the levels reached the HC group levels in both SR and HR patients (Figure 9). The peak of pDC regeneration was found at day 106 (week 15) in HR patients and at the beginning of Maintenance-I (week 30) in the SR group (Table 12; Figure 9). These two time-points were comparable, since in both groups BM was sampled two weeks after the administration of chemotherapy. At these time-points, the levels of pDCs in patients were significantly higher than those of HC group ($P = 0.008$ and $P = 0.032$ for HR and SR patients, respectively). After the peak in the pDC regeneration, we observed a decrease to levels within reference range (Table 12; Figure 9). pDC levels remained stable at the end of treatment and at six months after the end of chemotherapy.

### *3.3.2. Regeneration of mDC subset*

The mDC regeneration followed similar pattern to the pDC regeneration in both HR and SR patients (Table 12; Figure 9). At day 15 (week 2), mDCs were detected in BM samples from 71% of preB-ALL (SI) patients, 58% of preB-ALL (II) patients and in all HR patients. At day 29 (week 4), mDCs were detected in 96% of preB-ALL (SI) patients and 89% of preB-ALL (II) patients. However, the mDC levels both in SR and HR groups remained below the HC levels ($P < 0.001$ and $P = 0.002$, respectively).

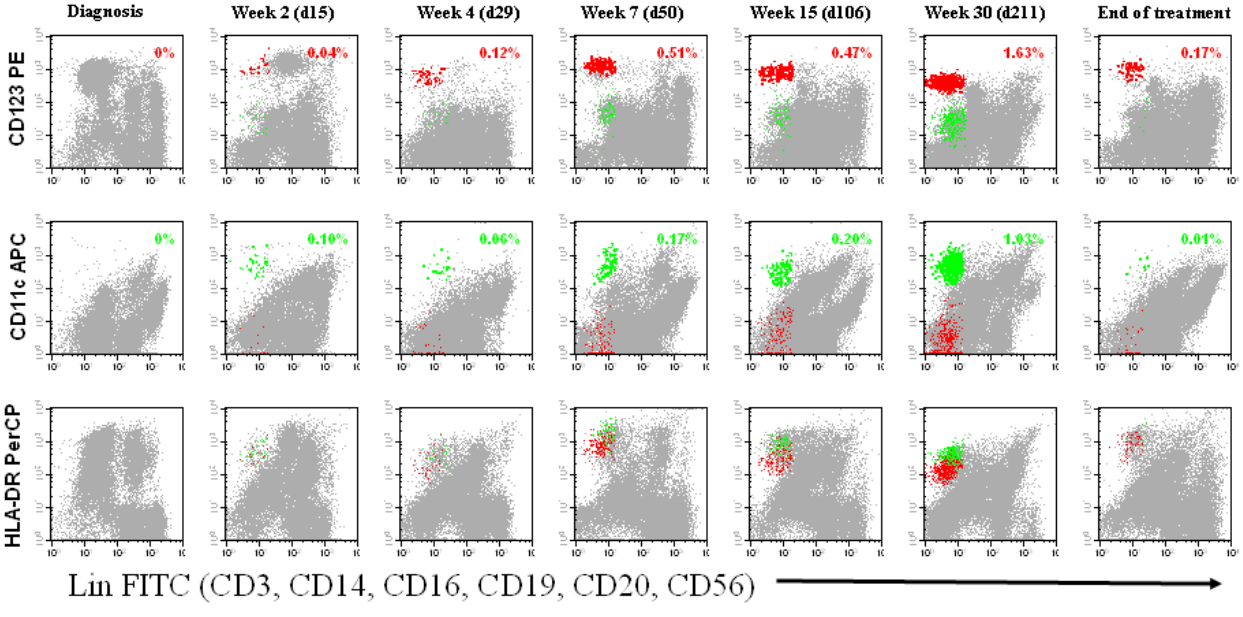


**Figure 10.** Flow cytometry detection of dendritic cell (DC) subsets at acute lymphoblastic leukemia (ALL) diagnosis and DC regeneration during ALL treatment in bone marrow.

Illustration of DC subset analysis in a BM sample from a representative SR group patient treated according to NOPHO ALL-2000 intermediate intensity treatment protocol (see Figure 3) is presented in Figure 10.

DC levels were evaluated from single four-color Lineage (Lin) cocktail (CD3, CD14, CD16, CD19, CD20 and CD56)/CD123/HLA-DR/CD11c flow cytometry panel (Figure 6) Upper row shows the level of CD123+/Lin- plasmacytoid DCs (pDCs) at indicated time points (red dots). The middle row shows the level of CD11c+/Lin- myeloid DCs (mDCs) at indicated time points (green dots). DC subsets were quantified as percentage of total BM events (red color indicates the level of pDCs and green color the level of mDCs). The lower row illustrates that pDCs and mDCs express high levels of HLA-DR, mDCs show higher HLA-DR expression than pDC subset. Analysis was performed by Paint-A-Gate software (BD).

The mDC levels reached the reference range at day 50 (week 7) in all groups of patients (Figure 9). The peak of mDC regeneration was detected in HR patients at day 106 (week 15) and in SR patients at the beginning of Maintenance-I (week 30). At these time-points, the levels of mDC were significantly higher than the control levels ($P = 0.024$ for HR group and $P = 0.041$ for SR group). At the end of treatment the mDC levels in HR patients did not differ from the HC levels. However, in SR group, mDC levels decreased further becoming significantly lower than reference values ($P = 0.032$) and remained lower in the follow-up samples taken six months after the end of chemotherapy ($P = 0.028$).

## 3.4. DC levels in relation to MRD

We were also interested if the levels of DC subsets were related to the presence of MRD in BM samples of patients under treatment. For that analysis we divided patients in the MRD-positive and MRD-negative categories and compared the DC levels in BM samples obtained at day 15 (week 2), 29 (week 4) and 50 (week 7) from these two groups. No significant differences were found in BM DC levels between MRD-positive and MRD-negative patients at day 15 and 29. At day 50, MRD-positive patients (N=13) had significantly higher pDC levels in BM than MRD-negative group (N=55) (median 0.36%, range 0.11–0.91% vs. median 0.20%, range 0.03–0.77%; $P = 0.021$), Figure 11.

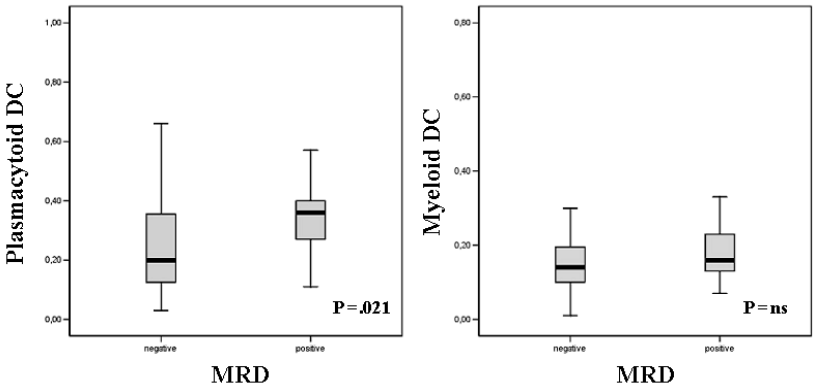


**Figure 11.** Dendritic cell (DC) subset levels in relation to minimal residual disease (MRD) levels at the end of Induction treatment according to NOPHO 2000 protocol.

Patients who were MRD positive at the end of Induction treatment (day 50/week 7) had significantly higher pDC levels than patients who were MRD negative at this time-point. The levels of myeloid DC did not differ between MRD positive and negative patients groups. DC subsets were quantified as percentage of total BM events.

## 3.5. DC levels in refractory/very slow responding ALL patients

Three of studied patients were still not in mCR at day 50 (week 7). In one preT-ALL patient with refractory disease we could detect DC subsets at the very low levels (<0.1%). Another slow-responding preT-ALL patient, who reached morphological CR at day 58, had normal DC subset levels in BM at day 75, when the MRD level in BM was 0.8%. When this patient subsequently relapsed, both DC subsets were detectable (pDC 0.1% and mDC 0.03%), but disappeared from BM when the disease progressed to refractory stage, Figure 12. The third patient with HR preB-ALL had a very slow response to treatment achieving mCR at day 64 with BM MRD level 1.1%. At this time-point, both DC subsets appeared in BM at 0.01% level.

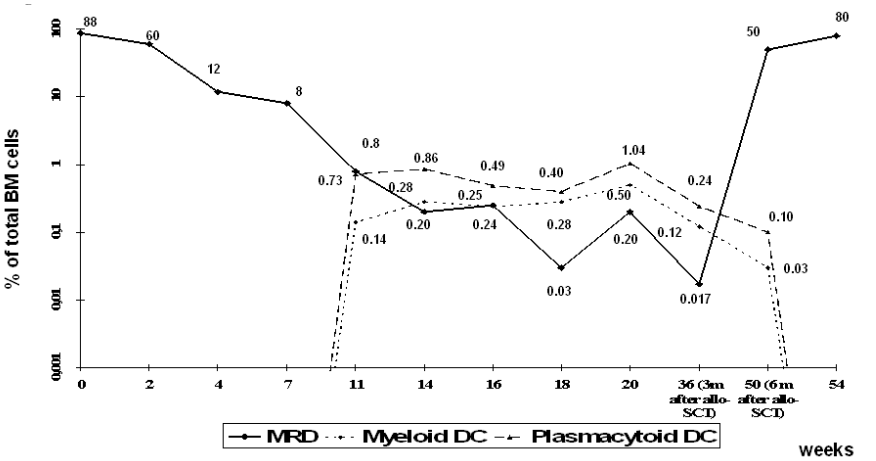


**Figure 12.** Dendritic cell levels in relation to levels of leukemic cells in a patient with precursor T-cell acute lymphoblastic leukemia (preT-ALL) resistant to treatment.

preT-ALL cell levels were measured as percentages of cells positive for TdT FITC/CD7 PE/cytoplasmicCD3 PerCP /CD34 APC. Levels of pDC and mDC were determined as shown in Figure 6. Exact levels at various time points are given.

# DISCUSSION

Immunophenotyping with multiparameter FCM is a rapid and sensitive method to detect small cell populations in a variety of cellular background. FCM is especially useful to detect lymphoid abnormal cell populations, but FCM is increasingly used in detecting small populations of abnormal blasts in acute leukemias and detecting different cell populations in the immune system. The thesis includes three studies where FCM was used a principal method in detecting cell populations of interest. The first study was focused for accuracy of lymphoma diagnosis in FNA material. The second study was aimed to investigate the power of FCM to detect MRD in young adult AML patients and especially to evaluate the prognostic significance of MRD in relation to transplantation. The third study investigated DCs, the central cells in immune system which orchestrate the whole immune response in childhood ALL.

## 1. The usefulness of FCM in lymphoma diagnosis in FNA

The WHO classification of haematopoietic and lymphoid tumors classifies lymphoid malignancies into distinct biologic entities based on morphology, immunophenotype, genetics and clinical features (Jaffe, 2001). The relative importance of each of these methods varies among different lymphomas, but generally biopsy for histologic examination and demonstration of clonality is required. The diagnosis of NHL on cytologic material only has been controversial (Hanson, 1994). However, when FNA is combined with immunophenotyping by IC, the accuracy of NHL detection exceeds 90%. However, immunophenotyping can also be accomplished with FCM. Both techniques have advantages and limitations. The main advantage of IC over FCM is the requirement of a small number of malignant lymphoma cells and the preservation of cellular morphology. Intensity of staining, staining pattern and background can be assessed easily. Moreover, fragile neoplastic cells which could be destroyed during FCM preparation and analysis could be successfully assessed in cytospins. The disadvantage of IC is that the technique is time-consuming, routinely only one antigen can be assessed per cell and scoring is semi-quantitative. FCM is a rapid and sensitive method, which allows to assess several antigens on one cell simultaneously. Current techniques allow detection of intracytoplasmic antigens, closing thus the gap between FCM and IC. Furthermore, FCM provides quantitative results and can detect small abnormal cell populations in reactive background. These FCM features significantly improve the diagnostic sensitivity and therefore are particularly useful in lymphoma diagnostics. However, the main disadvantage of FCM is unawareness of cytomorphology.

In our study we demonstrated that a simple four tube four-color FCM panel allows quick and precise classification of NHL and detection of RH in FNA material. The inclusion of Bcl-2 into FCM was useful because malignant B cells in most samples expressed a higher levels of Bcl-2 than did normal B cells and T cells. In our study FCM and IC were equally effective in distinguishing NHL from RH and also in the diagnosis of primary and recurrent lymphomas.

We found in literature search two studies that directly compared FCM with IC on FNA aspirates in large series of cases (Robins et al., 1994; Simsir et al., 1999). These studies demonstrated that FCM and IC can be used interchangeably and the overall concordance of the FCM and IC results was similar to that in our study. FCM has been used an aid to FNA diagnostics in several other studies, showing the rate of accuracy more than 90% (Dunphy and Ramos, 1997; Meda et al., 2000; Nicol et al., 2000; Dong et al., 2001).

In our study FCM panel suggested lymphoma in five samples finally diagnosed as RH. In four of these samples also IC suggested lymphoma. However, similar cases have been reported in literature. Our results are in agreement with the study of Meda et al (Meda et al., 2000) who reported four cases of RH that were suggestive of lymphoma in FNA. Another study by Levy et al (Levy et al., 1983) described 12 patients with RH, in whom sIg studies showed monoclonal Ig staining pattern.

FCM was especially useful in detecting low-grade B-cell lymphomas, 95% of cases were diagnosed and classified accurately. The only problematic category was CD10- FL. Interfollicular lymphoma cells may lack CD10 (Dogan et al., 1998) and in these cases FCM is not able to diagnose lymphoma subtype correctly.

The diagnostic accuracy in the detection of HG-NHL was much lower than that of LG-NHL. However, our results are similar to the study of Verstovsek et al (Verstovsek et al., 2002) who reported 27% false negative FCM results in HG-NHL. Preparation of FCM may lead to preferential loss of large cells due to increased death. We noted that in HG-NHL, acquired cell counts were approximately 1000 events lower than average: 7831 vs 8874, respectively. In addition, B-cell counts were lowest in HG-NHL.

Diagnosis of T-cell NHL by FCM is complicated because there is no sensitive marker for clonality. We could detect a pathologic immunophenotype in T-NHL in 53% of cases. This result is similar to report by Meda et al (Meda et al., 2000), but the frequency of a pathologic phenotype was somewhat lower than obtained by Yao et al (Yao et al., 2001) who found aberrant expression of T-cell markers in 67% (14 of 21) of peripheral T-NHL samples.

We also studied the usefulness of FCM analysis of Bcl-2 expression in differential diagnosis of malignant lymphoma. This has been previously investigated in only two studies of lymph node biopsies (Cornfield et al., 2000; Cook et al., 2003). Cornfield et al reported that simple dual staining with monoclonal antibodies to Bcl-2 and CD20 is useful in differentiating neoplastic from benign germinal center cells, i.e. FL from RH. Cook et al (Cook et al., 2003) evaluated

a three-color FCM panel using antibodies against CD10, CD20 and Bcl-2. Our four-color FCM panel had an advantage allowing simultaneous evaluation of Bcl-2 expression in B and T cells and, if present, in CD10+ B cells. Using this approach non-malignant T cells present in the sample served as internal control for the comparison of levels of Bcl-2 expression. Similarly to previous reports we found that the presence of CD10+ B cells with high Bcl-2 expression was highly predictive for FL (Cornfield et al., 2000; Cook et al., 2003). In contrast, CD10+ B cells in RH expressed much lower levels of Bcl-2 than did T cells and CD10- B cells. We also noted high Bcl-2 expression in most cases of LG-NHL, which was not investigated in detail previously (Cook et al., 2003). Cornfield et al (Cornfield et al., 2000) surprisingly found that T cells express higher levels of Bcl-2 in FL than in RH. In contrast, we found Bcl-2 expression in T cells very similar between RH and B-cell NHL. Analysis of Bcl-2 expression was most useful in FL cases with partial lymph node involvement with nests of reactive germinal centers that made evaluation of light-chain restriction difficult. In HG-NHL Bcl-2 expression was less informative because malignant B cells may downregulate Bcl-2 (Lai et al., 1998). Our results are similar with the report of Menendez et al (Menendez et al., 2004) who also found that malignant B cells in BM and peripheral blood samples from patients with mature B-cell neoplasms, except BL, express consistently higher levels of Bcl-2 than their normal counterparts.

The major weakness of the study is that histopathologic confirmation was available in only 124 of 424 (29%) of cases. Biopsy was mainly performed in diagnostically difficult cases or when histopathology was needed for making treatment decisions. However, cytologic diagnosis was concordant with histopathology in all lymphoma cases. Unfortunately, biopsy is not always readily available, for example when enlarged lymph nodes are present only in abdomen or thorax. If in such cases patients are too fragile for operation, FNA remains an important option for lymphoma diagnosis. In addition FNA could be helpful selecting the most representative malignant lymph node for biopsy if multiple sites are involved from suspect lymphoma.

Based on the study results, we recommend first to perform a stain of an FNA smear. If small to medium-size lymphatic cells predominate, indicating low-grade lymphoma, FCM should be the method of choice for immuno-phenotyping. However, when large cell predominate, IC should be preferable due to a high false-negative rate of FCM. It has to be noted that Hodgkin's lymphoma and some NHLs like TCRBCL and anaplastic large cell lymphoma cannot be reliably detected by FCM. For the best diagnostic accuracy a strong communication should exist between cytopathologists and FCM laboratories.

# 2. The usefulness of FCM in detecting MRD in young adult AML

Studies of childhood and adult AML have shown that the levels of MRD detected either early after induction or later after consolidation are powerful prognostic factors. We focused on the prognostic significance of MRD in relation to SCT in young adult AML patients 19–60 years old. Allo-SCT was performed in 36% (16/45) of patients and 33% (15/45) underwent auto-SCT. Fourteen patients (31%) were treated with conventional chemotherapy only. In our study MRD levels detected either after induction or at the end of post-remission chemotherapy or before SCT were not predictive for RFS or OS, but there was a trend for a longer RFS in patients with no detectable MRD at the end of chemotherapy or before SCT. Statistically, SCT was the only significant prognostic factor. Patients who underwent SCT had significantly longer OS and RFS than had patients who received only chemotherapy. However, patients with MRD detected after the induction treatment and/or at the end of chemotherapy had significantly lower five-year RFS than patients without detectable MRD. Patients without detectable MRD had also a slightly but not significantly longer OS.

Since prognosis of AML was predicted only by SCT, we investigated how SCT influenced survival of MRD-positive and MRD-negative patients. The worst outcome was observed in patients, who were MRD-positive either after induction or at the end of treatment and did not undergo SCT. MRD-positive patients, who underwent allo-SCT had similar cumulative RFS and OS to MRD-negative patients, who did not receive allo-SCT. Finally, MRD-positive patients who underwent auto-SCT had worse outcome as compared to MRD-positive patients who underwent allo-SCT. However, autografted MRD-positive patients had significantly longer RFS and OS than patients treated by chemotherapy only. Our data indicates that the prognostic significance of MRD differs depending on the applied post-remission therapy. Although our study included a small number of patients, the results strongly indicate that patients who remain MRD-positive after the induction treatment and/or at the end of chemotherapy have worse prognosis and benefit from allo-SCT or intensified post-remission therapy with auto-SCT.

We used FCM as a method to detect MRD and therefore several aspects of this technique need to be discussed in detail.

### *Applicability and reliability of the FCM method*

In our study we could detect LAIPs in 94% of patients at AML diagnosis. Our results are in line with the previous studies by San Miguel et al (San Miguel et al., 2001) and Coustan-Smith et al (Coustan-Smith et al., 2003), who reported the frequency of LAIPs at diagnosis in 75% and 85% of patients, respectively. Kern et al (Kern et al., 2004), by using more antibody combinations and

numerous fluorochromes, demonstrated that at least one LAIP could be detected in every studied patient and MRD could be followed by FCM in all AML patients. The limitation of this approach was a lower sensitivity due to increased use of less aberrant LAIPs that may be present in very low frequencies in normal or reactive BM.

The phenotype changes in AML are the major concern when discussing the reliability of FCM detection of MRD, since this phenomenon may lead to false-negative results. Macedo et al (Macedo et al., 1996) and Baer et al (Baer et al., 2001) have reported that immunophenotypic shifts occur in 11% of LAIPs. In addtion, Voskova et al (Voskova et al., 2004) reported, that in 24% of patients, none of the original LAIPs were present in more than 1% of BM cells at relapse. Nevertheless, by using several LAIPs, relapse could be detected in virtually all patients (Voskova et al., 2004).

Phenotypic changes occurred frequently also in our study. Major phenotypic changes (i.e. loss or gain of an antigen) were found in AML blasts from 9 of 15 patients (60%), in whom FCM analysis was performed at relapse. The following antigens were lost at relapse (each in one case): CD2, CD56 and TdT. At relapse AML blasts acquired: HLA-DR in two cases and CD4, CD33, CD34 and CD38 each in one case.

In six of the nine patients, the prognostic evaluation of MRD was not affected by the loss or gain of individual antigens since other studied LAIPs remained aberrant. In the remaining three patients (33%) the change of antigen expression occurred in the most aberrant LAIP used for MRD follow-up. However, in two of these three patients MRD was still detectable, since a small population with the original LAIP persisted during treatment and at relapse. Phenotype changes rendered MRD not detectable in only one patient. In this patient the CD34+ blast population constituted only 0.2% of total blasts at diagnosis. Therefore, a CD34 negative population was selected for MRD studies, but the major AML blast population at relapse was CD34 positive. Thus, phenotype changes resulted in false-negative MRD measurements in 1 out of 15 patients (7%) analyzed at relapse.

We also noted minor changes in antigen expression levels in 33% of relapse patients. This indicates a potential interpretation hazard if strictly predefined dot-plot gates are used for MRD evaluation.

A study by Voskova et al (Voskova et al., 2004) showed that LAIP changes in AML at relapse may be associated with a biologically different disease, especially when there are also changes in cytomorphology and molecular genetics. In our study, one phenotype change occurred in a patient with AML-M0 at diagnosis that at relapse displayed a phenotype consistent with preT-ALL. MRD monitoring may be a challenging task is such patients.

### *Sensitivity of the method and clinically relevant MRD levels*

A maximum sensitivity of $10^{-4}$–$10^{-5}$ could be achieved using live-gate acquisition approach together with highly aberrant LAIPs (Coustan-Smith et al., 2003; San Miguel et al., 2001). However, this high level of sensitivity is difficult to achieve with less aberrant LAIPs due to very small populations with similar phenotypes that could be present in normal regenerating BM. Therefore, data from published studies, as well as our study indicate that MRD at cut-off level around 0.1% defines the patient group with the highest risk of relapse. However, in a study by San Miguel et al (San Miguel et al., 2001) approximately 30% of patients had MRD level between 0.01–0.1% with a three-year cumulative relapse rate of 14%, whereas patients with MRD levels between 0.1–1% had the relapse rate of 45%. Therefore, MRD levels between 0.01–0.1% may also define patients with an increased relapse risk. In our study, relapse occurred in four out of six patients who had MRD levels between 0.01–0.09% after induction treatment and in two patients with similar levels at the end of post-remission chemotherapy.

### *The most relevant time-point for MRD follow-up*

Two studies in adult AML (Feller et al., 2004; San Miguel et al., 2001) and two studies in childhood AML (Coustan-Smith et al., 2003; Sievers et al., 2003) indicate that early MRD detection i.e. after induction chemotherapy, when the first mCR is achieved, is highly significant for prognosis. Two other adult AML studies (Kern et al., 2004; Venditti et al., 2000) indicated that the late MRD detection, i.e. after post-remission treatment could be more relevant for outcome. In our study eight patients had no detectable MRD at both above-mentioned time-points and none of them relapsed. However, two out of six patients who had no detectable MRD at the end of chemotherapy treatment but were MRD-positive after induction treatment, suffered relapse.

### *MRD follow-up after the end of treatment*

Only few reports are available on the significance of long-term MRD follow-up in AML patients. Results from studies by Feller et al (Feller et al., 2004) and Venditti et al (Venditti et al., 2003) indicate that in order to predict relapses MRD should be followed at least every three months.

We followed MRD in 11 patients after the completion of therapy (23 BM samples collected at different time-points). Auto-SCT was performed in 10 of these patients. MRD was followed at least twice in five patients and once in six patients. Of eight patients with no detectable MRD, only two patients relapsed within seven months after the last follow-up. In contrast, relapse was detected in all five patients in whom MRD levels were over 0.1% during 1–2 months.

Finally, two patients, who showed decreasing MRD levels from 0.13% or 0.07% to MRD-negativity, did not relapse.

***Relationship of MRD levels with other known prognostic features***

All studies discussed above, have clearly shown that the level of MRD is more powerful independent prognostic factor than other known pre-treatment prognostic factors such as age, WBC count and cytogenetic risk group. In addition, results from a study by San Miguel et al (San Miguel et al., 2001) demonstrated that patients with favorable cytogenetics had significantly lower MRD levels as compared to patients with intermediate or unfavorable karyotypes. Patients who achieved mCR after the first chemotherapy cycle or had WBC counts less than 50 $x10^9$/l also had significantly lower MRD levels as compared to patients who achieved mCR after the second chemotherapy cycle or had WBC counts greater than 50 $x10^9$/l. Kern et al (Kern et al., 2004) have also reported that MRD levels have prognostic impact even within cyto-genetically defined risk groups.

In our study we did not find significant correlation between MRD levels and age, cytogenetic risk-group and WBC count. However, all analyzed five patients with unfavorable cytogenetics had detectable MRD after induction treatment and two of them relapsed. Two of these five patients subjected to allo-SCT and one patient subjected to auto-SCT did not relapse. Two patients had favorable cytogenetics and one of them, who remained MRD-positive at the end of post-remission chemotherapy, suffered relapse. We performed analysis of the significance of MRD in the intermediate cytogenetic risk-group. Patients with no detectable MRD after induction had significantly longer cumulative five-year RFS than patients with detectable MRD (90% versus 49%, P = 0.041). OS was also prolonged in patients with no detectable MRD, but this difference did not reach to statistical significance.

Based on the results of the study we recommend FCM based monitoring in every AML patient in order to refine and individualize post-remission therapy according to treatment response at the submicroscopic level. Since our study included a relatively limited number of patients, large prospective studies are needed, where MRD is thoroughly followed and used for therapy decisions in uniformly treated patient groups. These studies would further improve possibilities of treatment stratification in individual AML patient.

## 3. DC levels in BM evaluated by FCM in childhood ALL

DCs originate from CD34+ BM precursor cells and play a pivotal role in the development of immune responses and immune tolerance. Very little is known about DC subset levels and their functional status in ALL. Two studies have

investigated DC subset levels in peripheral blood from patients with ALL at diagnosis (Maecker et al., 2006; Mami et al., 2004). Both authors reported severely reduced levels and impairment function of both mDCs and pDCs in patients with B-ALL. However, DC subsets were quantitatively and functionally comparable with healthy control group and in patients with preT-ALL. To our best knowledge, there is no published data on DC subset levels in BM obtained at diagnosis of ALL and during ALL treatment. Our study is the first to demonstrate a severe deficiency of DCs in BM of childhood ALL at diagnosis. Our findings are in concordance with previous studies, which demonstrated a severe reduction of DC subsets in blood of ALL patients at diagnosis and higher levels in preT-ALL as compared to preB-ALL patients (Maecker et al., 2006; Mami et al., 2004). The latter finding can be in part explained by correlation of peripheral blood neutrophil counts at diagnosis with DC subset levels in BM and higher neutrophil counts in patients with preT-ALL as compared to patients with preB-ALL. However, on the contrary to findings in peripheral blood (Maecker et al., 2006; Mami et al., 2004), we did not find normal levels of pDC and mDC subsets in BM in patients with preT-ALL. This discrepancy could be explained by different source of DCs or different antibody panels and method of analysis. We performed the DC subsets analysis in whole BM and not after Ficoll-densitiy centrifugation. Using four-color analysis we evaluated both subsets in the same staining tube. One of the drawbacks of our study is that we could not evaluate the absolute numbers of DCs. The cell concentration in aspirates could not be assessed since BM samples for flow cytometry analysis were diluted in heparinized saline to prevent clotting. BM cellularity was evaluated only on BM biopsy sections.

In preB-ALL patients DC levels were related to the extent of the disease. Patients in the SR group had significantly more pDCs and mDCs in BM than patients in higher risk groups. These differences may be related to more pronounced BM suppression in higher risk groups, especially in patients with HR preB-ALL. The relationship between DC levels and BM suppression is also supported by the finding that DC levels correlated with platelet and neutrophil counts at diagnosis.

The levels of both DC subsets in BM reached to reference values at the end of Induction treatment block (day 50/week 7). Up to day 50 (week 7), treatment of patients in both SR and HR groups was essentially identical. The regeneration peak of the relative levels of both pDC and mDC subsets occurred at the beginning of Consolidation-1 (day 106/week 15) in HR patients and at the beginning of Maintenance-1 in SR preB-ALL (II) patient group (week 30). At this time points, the relative levels of both pDC and mDC subsets were significantly higher than control values. These two time-points were comparable, since in both patient groups BM were sampled two weeks after the end of previous chemotherapy cycles, which were essentially identical.

At the end of therapy, the level of pDC subset in BM was comparable to reference values in both SR ad HR patient groups. However, the level of mDC

was significantly reduced in SR group but not in HR group of patients. Notably, the SR group was under treatment 6 months longer than HR group. Therefore, the prolonged treatment may have more severe effect on mDC subset by comparison to pDC subset. Another explanation is that the suppression of DCs could also be related to low WBC counts during Maintenance therapy when WBC count is targeted to 1.5–3.5 x$10^9$/L. However, at the end of therapy DC subsets levels did not correlate with neutrophil and platelet counts. This may indicate that at the end of treatment decreased DC levels are not caused only by BM suppression. Importantly, mDC subset levels remained significantly lower than control values at six months after the end of treatment protocol when BM biopsies usually showed normal cellularity.

DCs provide essential link between innate and adaptive immunity. Immature DCs are present in peripheral tissues where they are posed to capture antigens and after antigen capture, antigen-loaded tissular dendritic cells migrate through afferent lymphatics into draining lymph nodes where they present processed antigens to T-cells. It has been demonstrated that the mDC is the predominant subtype in the peripheral tissues such as human deciduas (Gardner and Moffett, 2003), in human lung (Masten et al., 2006; Demedts et al., 2007) and in human renal tissue under normal and pathological conditions (Woltman et al., 2007). Furthermore, mDC subtype predominates also in human peripheral blood (Vakkila et al., 2004; Maraskovsky et al., 2000). However, in primary and secondary lymphoid tissues like in human thymus (Bendriss-Vermare et al., 2001), human tonsils (Summers et al., 2001) and in human BM (Szabolcs et al., 2003) pDC subtype predomiantes. Further, pDCs have been shown to be the major DC subset innately producing cytokines in human lymph nodes (Cox et al., 2005). Finally, both mDC and pDC orchestrate the migration of immune effectors like T and B cells, launching and shaping adaptive immune response. In accordance with above-mentioned published data, we found that pDC is the predominant subtype in BM. This finding was true both for non-leukemic (HC group) patients and for BM from ALL patients in CR.

It has be noted, that in addition to pDC and mDC, the third subtype of DC is characterized in humans. These cells are lineage marker negative, express high levels of HL-DR and co-express CD16 and low levels of CD14. Almeida et al have found that the overall frequency of those cells in normal adult peripheral blood is 0.72% ± 0.35% of all nucleated cells, which is higher than remaining lin-/CD16-/HLA-DR+ DCs (Almeida et al., 2001). The frequency of these CD16+ DCs in BM was 0.19% ± 0.15% which was lower than in PB (Almeida et al., 2001). The role of these DCs in immune response is not fully established, but Kanaya et al have shown that these DCs exhibit weak immunostimulatory activity and may play a role in the induction of chronic inflammation (Kanaya et al., 2004). In our study we could not evaluate this DC subset since our lineage marker combination included CD16. The level of CD16+/HLA-DR+/CD14-/low DCs at ALL diagnosis and during treatment has to be determined in the future studies.

The defects in mDC maturation in cancer patients attract great attention (Gabrilovich, 2004). It has been found that patients with breast and head and neck cancer had significantly reduced mDC, but not pDC levels (Hoffmann et al., 2002; Della Bella et al., 2003). Furthermore, this reduced mDC levels were restored after cancer removal (Hoffmann et al., 2002; Della Bella et al., 2003). We also observed no regeneration of DC cells in three patients who did not achieve mCR, indicating suppression of DC regeneration by remaining leukemia. It has been reported that in cancer patients DCs exhibit several functional abnormalities, such as decreased expression of co-stimulatory molecules (Nestle et al., 1997; Troy et al., 1998) insufficient T-cell stimulatory capacity and accumulation of immature DCs (Nestle et al., 1997; Gabrilovich, 1997). The accumulation of immature DCs may be especially important, since they induce T-cell tolerance (Chaux et al., 1997; Enk et al., 1997; Bonifaz et al., 2002).

The major impact of decreased mDC levels on immune functions is emphasized by a concept that mDCs are central cells in T-cell priming that transfer signals from the innate immunity to adaptive immune system (Dubsky et al., 2005). It is established that children treated for ALL may have profound impairment of the immune system (Brodtman et al., 2005; Nilsson et al., 2002; Smith et al., 1995).

It could be clinically relevant to establish if there a relationship between DC levels at diagnosis or at early time-point during treatment and clinical outcome. We observed that MRD-positive patients had significantly higher pDC levels in the BM than the MRD-negative group at the end of Induction treatment. Follow-up time is too short to determine the prognostic significance of this finding. However, this observation is in agreement with a recent study reporting negative prognostic impact of high pDC counts at diagnosis in childhood cancer patients (Vakkila et al., 2004). It has been suggested that pDC may induce tolerance of the immune system against cancer cells (Wei et al., 2005). Further, hepatic lymph nodes of patients with hepatocellular carcinoma have significantly reduced numbers of mDCs and elevated levels of pDCs in the T-cell areas when compared to lymph nodes from patients with viral hepatitis without involvement of cancer (Tang et al., 2006). Additionally, it has been shown that pDCs accumulate in the ovarian carcinoma microenvironment where they are able to induce suppressive CD8+ regulatory T-cells (Wei et al., 2005).

We acknowledge that the major drawback of our study is that we did not study the function of DCs due to shortage of BM cells. Studies in future should aim on investigating DC function *ex vivo* at different time-points during and after ALL therapy.

Based on the results of the study we propose that reduced levels of DC subsets may be one of the important factors in the impairment of the immune system in children after treatment for ALL. Future studies should focus on the functional status of DC subsets in ALL patients after treatment. The relevance of higher pDC counts in MRD positive patients should also be investigated.

# CONCLUSIONS

Multiparametric FCM is useful to detect the correct NHL subtype and for NHL differentiation from reactive hyperplasia.

- FCM allows precise lymphoma classification from FNA material, a panel of four tubes of four-color antibody combinations is reliable of NHL diagnosis of 90% of samples.
- Staining for Bcl-2 is helpful for differentiation between RH and NHL, especially in samples with partial lymph node involvement.
- FCM technique is more efficient in detecting LG-NHL than HG-NHL due to the fragility and preferential loss of large lymphoma cells.
- FCM technique is more reliable in detecting B-NHL than T-NHL due to the lack of sensitive marker for clonality.

MRD analysis by flow-cytometry may be used for refining the selection of therapeutic strategies and improving clinical outcome in individual patients.

- MRD assessment by FCM is applicable over 90% of patients with AML and is reliable. Due to possibility of phenotype change in AML blasts, for efficient MRD monitoring two or more LAIPs should be followed.
- The prognostic significance of MRD levels in AML patients depends on the type of post-remission therapy.
- Young AML patients who have detectable MRD at the first CR and/or at the end of post-remission chemotherapy may benefit from SCT.
- AML patients in whom MRD is not detectable after induction treatment and who remain MRD-negative at the end of post-remission chemotherapy have long-term RFS and OS similar to allografted patients.

Children with ALL have a severe deficiency of DCs in BM at diagnosis, which is more pronounced in preB-ALL than in preT-ALL.

- In preB-ALL patients DC levels were related to the extent of the disease: children without unfavorable features had significantly more pDCs and mDCs than patients with unfavorable prognostic features.
- DCs subset levels regenerate to reference levels by the end of induction treatment according to NOPHO ALL-2000 protocol at day 50/week 7.
- The regeneration of mDC subset is more severely affected by ALL chemotherapy than the pDC subset in SR group, decreasing to significantly lower than control levels at the end of chemotherapy and remaining significantly reduced at six months after the end of ALL treatment.
- In patients with treatment-resistant ALL, both pDC and mDC regeneration is suppressed, possibly by remaining leukemia burden.

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# SUMMARY IN ESTONIAN

## Läbivoolu tsütomeetria kasutamine pahaloomuliste vereloomehaiguste korral

Läbivoolu tsütomeetria on kaasaegne laseril põhinev meetod rakkude uurimiseks, mille abil on võimalik iseloomustada rakkude koostist – rakkude sees olevaid valke-, ning markereid, mis asuvad rakkude pinnal. Samuti on võimalik määrata rakkude suurust ja granulaarsust. Kuigi läbivoolu tsütomeetriat on eelkõige kasutatud immunoloogias lümfotsüütide arvu määramiseks ja nende iseloomustamiseks, on tehnoloogia arenedes meetodit hakatud kasutama ka pahaloomuliste vereloomehaiguste korral. Hematoloogiliste patsientide luuüdi ja lümfisõlmede punktaadid on ideaalsed substraadid, kus saab kasutada uurimiseks läbivoolu tsütomeetrilist meetodit. Läbivoolu tsütomeetria võimaldab leida suurest rakkude hulgast spetsiifilised rakud, mis eristuvad teatud kindla tunnuse järgi, nagu näiteks iseloomulik rakupinna marker. Uuemate monoklonaalsete antikehade abil on võimalik uurida täpsemalt erinevaid vereloomest pärinevaid rakke ning nende muutusi ravi käigus. Viimastel aastatel kasutatakse läbivoolu tsütomeetriat järjest sagedamini vereloomehaiguste diagnoosimisel, ravitaktika planeerimisel, ravitulemuste ja ravimõjude hindamisel.

### *Uurimistöö eesmärgid*

Uurimistöö peamiseks eesmärgiks oli hinnata läbivoolu tsütomeetria kui meetodi rakendatavust pahaloomuliste hematoloogiliste haiguste korral. Täpsemateks töö eesmärkideks olid:

1) hinnata läbivoolu tsütomeetria rakendatavust lümfoomide diagnostikas peennõelbiopsial saadud materjalist.
2) Hinnata läbivoolu tsütomeetria abil minimaalse residuaalse haiguse olemasolu ja selle prognostilist väärtust noortel ägeda müeloidse leukeemiaga täiskasvanutel.
3) Uurida läbivoolu tsütomeetria abil dendriitiliste rakkude arvu ägeda lümfoblastse leukeemiaga lastel haiguse diagnoosimise hetkel ja rakkude taastumist keemiaravi ajal.

### *Materjalid ja meetodid*

Uurimisgrupis oli 526 patsienti, 256 meest/poissi ja 270 naist/tüdrukut vanuses 2 kuud kuni 94 aastat, keda uuriti ja raviti Karolinska Haiglas ja Astrid Lindgreni nimelises Lastehaiglas Stockholmis Rootsis ajavahemikul 1994–2005.

Lümfoomide diagnostilises uuringus peennõelbiopsial saadud materjalist osales kokku 396 patsienti, ägeda müeloidse leukeemia ning minimaalse

residuaalse haiguse hindamise uuringus oli 45 patsienti ja dendriitiliste rakkude arvu määramise uuringus 85 last.

Lümfoomide diagnostiline uuring tehti 396 patsiendil, kokku 424 peennõelbiopsiat. Kahekümne kuuel patsiendil teostati biopsia kaks korda ja ühel patsiendil kolm korda. Kõikidel nendel juhtudel võeti peennõelbiopsia erinevatest kohtadest ja/või erinevatel ajahetkedel. Lümfisõlmi punkteeriti 352 korral ja teisi kudesid 72 korral. Peennõelbiopsial saadud rakke ja luuüdi rakke uuriti läbivoolu tsütomeetriga. Uurimistöös kasutati FACSCalibur ja FACScan läbivoolu tsütomeetreid, mis olid varustatud 'Lysis II' või 'Cell Quest' programmidega. Rakkude hulga täpsemaks määramiseks kasutati 'Paint-A-Gate' arvutiprogrammi. Nii tsütomeetria aparaadid kui andmetöötlusprogrammid olid Becton Dickinson korporatsioonilt.

Minimaalset residuaalset haigust jälgiti kokku 45 patsiendil, kellel keemiaraviga saavutati täielik morfoloogiline remissioon. Patsientide jälgimise aeg oli keskmiselt viis aastat. Minimaalset residuaalset haigust uuriti kahel korral: pärast induktsioonravi lõppu ja kas keemiaravi lõppedes või enne autoloogset või allogeenset siirdamist.

Dendriitilisi rakke uuriti 76 ägeda lümfoblastse leukeemiaga lapsel ja üheksal leukeemia kahtlusega lapsel, kelle luuüdi leid osutus normaalseks. Need üheksa last moodustasid kontrollgrupi dendriitiliste rakkude arvu hindamisel. Ägeda lümfoblastse leukeemiaga lastel uuriti dendriitilisi rakke haiguse diagnoosimisel, ravi ajal viiel korral, ravi lõppedes ja kuus kuud pärast ravi lõppu. Luuüdi dendriitiliste rakkude uurimiseks võeti ajal, mil toimus ka luuüdi rutiinne kliiniline uurimine vastavalt raviprotokollile.

Lümfoomide diagnostiline paneel koosnes neljast neljavärviliset markerite kombinatsioonist: (1) lambda/kappa/CD19/CD5, (2) CD23/CD10/CD20/CD19, (3) CD4/CD7/CD8/CD3 ja (4) Bcl-2/CD10/CD19/CD3.

Ägeda müeloidse leukeemia diagnostiline läbivoolu tsütomeetriline paneel sisaldas: CD2, membraani CD3, tsütoplasmiline CD3, CD4, CD5, CD7, CD8, CD10, CD11b, CD13, CD14, CD15, CD19, CD20, CD22, CD33, CD34, CD38, CD45, CD56, CD65, CD117, anti-müeloperoksüdaas, anti-TdT ja HLA-DR. Leukeemia diagnoosimisel koostati igale patsiendile individuaalne leukeemiat iseloomustav immuunfenotüüp – kolmevärviline rakumarkerite kombinatsioon, mida jälgiti ravi käigus. Minimaalset residuaalset haigust analüüsiti 'Paint-A-Gate' programmi abil.

Dendriitilisi rakke uuriti neljavärvilise paneeli abil, mis koosnes (1) Linkokteil markerist, mis sisaldas endas rakuliini kuuluvuse näitajaid CD3, CD14, CD16, CD19, CD20 ja CD56; (2) CD123; (3) HLA-DR ja (4) CD11c. Dendriitiliste rakkude iseloomulikus tunnuseks on HLA-DR tugev positiivsus ja liinikuuluvuse markerite puudumine rakupinnal. Plasmatsütoidsed dendriitilised rakud on tugevalt CD123 positiivsed, kuid CD11c negatiivsed. Müeloidsed dendriitilised rakud on seevastu CD11c positiivsed ja CD123 on nende pinnal nõrk.

Fluorokroomideks antud uurimistöös olid fluorestsiin isothiotsüanaat, fükoerüthriin, peridiniin klorofüll proteiin või tandem konjugaat R-fükoerüthriin-indodikarbitsüaniin ja allofükotsüaniin.

## *Tulemused*

Peennõelbiopsial saadud materjalist saadi immuunfenotüpiseerimisel läbivoolu tsütomeetria abil õige diagnoos madalmaliigse B-rakulise mitte-Hodgkini lümfoomi korral 95%-l juhtudel, kõrgmaliigse B-rakulise mitte-Hodgkini lümfoomi korral 78%-l juhtudel ja T-rakulise lümfoomi korral 53%-l juhtudel.

Reaktiivne lümfoidne hüperplaasia diagnoositi peennõelbiopsia materjalist õigesti 97%-l juhtudest. Seega aitab läbivoolu tsütomeetria usaldusväärselt eristada healoomulist reaktiivset põletikulist protsessi pahaloomulistest lümfoomidest.

Kõige olulisem roll oli läbivoolu tsütomeetrial madalmaliigsete B-rakuliste lümfoomide diagnoosimisel. Kõrgmaliigsete B-rakuliste lümfoomide diagnostikas on läbivoolu tsütomeetria usaldusväärsus väiksem, sest suured rakud võivad biopsia materjali töötlemise käigus hukkuda. Samuti on T-rakuliste lümfoomide diagnoosimine ainult rakkude immuunfenotüpiseerimist rakendades problemaatiline, sest paljudel juhtudel puudub iseloomulik immuunfenotüüp ja puudub ka rakkude klonaalsuse marker. Kui B-rakulistel lümfoomidel puudub klonaalsuse marker, siis võib lümfoomi kinnitamisel olla abistavaks uuringuks Bcl-2 kvantitatiivne määramine lümfoidsetel rakkudel.

Meie uuring näitas, et maliigsetes B rakkudes on oluliselt rohkem Bcl-2 valku kui reaktiivsetes B või T rakkudes. Kõige suurem oli Bcl-2 valgu sisaldus follikulaarse lümfoomi rakkudel. Keskmine Bcl-2 eksperssiooni tase, mis oli korrigeeritud sama proovi T rakkude suhtes, oli follikulaarse lümfoomi B rakkudel 3,69, kuid reaktiivsetel B rakkudel ainult 1,14. Bcl-2 määramine aitas kaasa õigele diagnoosile 49 juhul, kui ei leitud selget lümfotsüütide kappa või lambda monoklonaalsust või kui lümfoomi rakkudel puudus pinna immunoglobuliin.

Minimaalset residuaalset haigust hinnati 43 patsiendil pärast induktsioonravi lõppu ja 31 patsiendil keemiaravi järgselt või enne vereloome tüvirakkude siirdamist. Mõlemal juhul uuriti minimaalset residuaalset haigust 30 patsiendil. Allogeenne siirdamine teostati 16 patsiendil ja autoloogne 15 patsiendil. Pahaloomuliste blastide hulk ehk minimaalne residuaalne haigus oli keskmiselt 0,23% kogu luuüdi rakkude arvust nii pärast induktsioonravi lõppu kui keemiaravi järgselt või enne siirdamist. Statistiline analüüs näitas üllatuslikult, et ägeda müeloidse leukeemiaga patsientidel ei ole minimaalse residuaalse haiguse olemasolul prognostilist väärtust. Samas aga määras patsientide prognoosi tüvirakkude siirdamine. Seetõttu uurisime ka minimaalse residuaalse haiguse seost allogeensesse ja autoloogsesse tüvirakkude siirdamisse. Selleks analüüsiks jaotasime patsiendid esmalt nelja rühma: 1. Patsiendid minimaalse residuaalse haigusega, kellele tehti allogeenne siirdamine; 2. Patsiendid, kellel

puudus minimaalne residuaalne haigus, kellele tehti allogeenne siidamise. 3. Patsiendid, kellel oli minimaalne residuaalne haigus, kellele ei tehtud allogeenset siirdamist ja 4. Patsiendid, kellel puudus minimaalne residuaalne haigus ja kellele allogeenset siirdamist ei tehtud. Selgus, et halvim prognoos oli patsientidel, kellel oli leitav minimaalne residuaalne haigus, aga kellele allogeenset siirdamist ei tehtud. Selles patsientide grupis oli viie aasta jooksul haiguse retsidiivideta ainult 20–24% haigetest ja elulemus 34–35%. Üllatuslikult näitasid töö tulemused, et minimaalse residuaalse haiguseta ja siirdamiseta patsientide elulemus oli samasugune nendega, kellel oli minimaalne residuaalne haigus ja kellele tehti allogeenne siirdamine. Üldine viie aasta elulemus oli mõlemas patsientide grupis 75%. Nendel patsientidel, kellel leiti minimaalne residuaalne haigus induktsioonravi lõppedes, oli viie aasta üldiseks elulemuseks 67%, juhul kui teostati allogeenne siirdamine; 46% kui teostati autoloogne siirdamine ja 31% kui siirdamist ei teostatud (P = 0,021). Kui minimaalne residuaalne haigus leiti keemiaravi lõppedes ja vereloome tüvirakkude siirdamist ei järgnenud, oli viie aasta elulemuseks vaid 25%. Patsientidel, kellel tehti allogeenne siirdamine, oli viie aasta elulemuseks 75%. Kui teostati autoloogne siirdamine, oli elus viie aasta möödudes 53% haigetest (P = 0,046).

Dendriitiliste rakkude uurimisel selgus, et ägeda lümfoblastse leukeemiaga lastel on haiguse diagnoosimisel dendriitilisi rakke vähe. Kontrollgrupis oli plasmatsütoidsete dendriitiliste rakkude keskmiseks hulgaks 0,28% (0,14–0,53%) ja müeloidsete dendriitiliste rakkude hulgaks 0,15% (0,10-0,28%) kogu luuüdi rakkude arvust. Kõrgriski B-rakulise ägeda leukeemia haigetel dendriitlised rakud diagnoosimisel puudusid. Standardriski grupis leiti vähesel hulgal plasmatsütoidseid dendriitilisi rakke 30%-l haigetest (keskmiselt 0,03%) ja müeloidseid dendriitlisi rakke 22%-l haigetest (keskmiselt 0,015%). Keskriski B-rakulise ägeda leukeemia grupis leiti nii plasmatsütoidseid kui müeloidseid dendriitilisi rakke 0,01% kogu luuüdi rakkude arvust ainult ühel 28 patsiendist. Seevastu T-rakulise ägeda leukeemia grupis leiti plasmatsütoidseid dendriitilisi rakke 43%-l haigetest (keskmiselt 0,05%) ja müeloidseid dendriitilisi rakke 29%-l haigetest (keskmiselt 0,05%). Diagnoosimisel oli T-rakulise ägeda lümfoblastse leukeemiaga lastel nii plasmatsütoidseid kui müeloidseid dendriitilisi rakke statistiliselt rohkem võrreldes B-rakulise vormiga, vastavalt P = 0,044 ja P = 0,041.

Induktsioonravi järgselt olid kõikidel uuritud lastel dendriitilised rakud luuüdis leitavad ja nende arv normaliseerus induktsioonravi järgselt 50. ravi-päevaks (7. ravinädalal). Keskmine plasmatsütoidsete dendriitiliste rakkude arv oli induktsioonravi lõppedes T-rakulise ägeda leukeemia grupis 0,33%, kõrgriski B-rakulise ägeda leukeemia grupis 0,27%, keskriski grupis 0,22% ja standardriski grupis 0,31%. Keskmine müeloidsete dendriitiliste rakkude arv oli T-rakulise ägeda leukeemia grupis 0,15%, kõrgriski B-rakulise ägeda leukeemia grupis 0,18%, keskriski grupis 0,14% ja standardriski grupis 0,15%. Ravi lõppedes jäi plasmatsütoidsete dendriitiliste rakkude arv kontrollväärtuste

piiridesse. Ka kuus kuud peale ravi tehtud kontrollproovides leiti kõikides leukeemia riskigruppides plasmatsütoidseid dendriitilisi rakke normaalsel hulgal. Seevastu müeloidsete dendriitiliste rakkude arv langes B-rakulise ägeda leukeemia standardriski patsientidel uuesti alla kontrollväärtuse. Kõrgriksi patsientidel, kuhu kokku kuulusid T-rakulise ägeda leukeemiaga ja kõrgriski B-rakulise ägeda leukeemiaga lapsed, jäid müeloidsed dendriitlised rakud kontrollväärtuste piiridesse. Leitud erinevus võis olla põhjustatud ravi kestvusest. Ägeda lümfoblastse leukeemia ravi kestis standardriski grupis pool aastat kauem – kokku 3 aastat. Kõrgriski grupi ravi kestvuseks oli kaks ja pool aastat. Standardriski grupis ei taastunud müeloidsete dendriitiliste rakkude arv ka pool aastat pärast ravi lõppu. Kolmel lapsel ei saavutanud 50. ravipäevaks morfoloogilist remissiooni. Nendel keemiaravile refraktaarsetel lastel ei täheldatud ka dendriitiliste rakkude regeneratsiooni. Ainult ühel lapsel leidsime nii plasmatsütoidseid kui müeloidseid dendriitilisi rakke 50. ravipäeval 0,01% kogu luuüdi rakkude arvust, teisel kahel lapsel dendriitilisi rakke luuüdis ei leitud.

## *Uurimistööst tulenevad järeldused*

Uurimistöö näitas, et läbivoolu tsütomeetrial on oluline roll mitte-Hodgkini lümfoomide õigel diagnoosimisel, lümfoomi täpsema alavormi määramisel ja lümfoomide eristamisel reaktiivsest lümfoidsest hüperplaasiast.

Läbivoolu tsütomeetria abil on võimalik peennõelbiopsia materjalist täpne lümfoomide diagnostika 90%-l juhtudest. Bcl-2 lisamine diagnostilisse paneeli võimaldab paremini eristada mitte-Hodgkini lümfoomi rakke reaktiivsetest lümfoidsetest rakkudest, mis tavaliselt paljunevad infektsiooni korral. Bcl-2 määramine on abiks eriti nendel juhtudel, kui lümfisõlm on haaratud lümfoomist vaid osaliselt, sest siis säilub osaliselt ka lümfisõlme normaalne koeline ja rakuline ülesehitus. Kõige olulisem roll on läbivoolu tsütomeetrial madalmaliigsete lümfoomide diagnoosimisel. Vea võimalus on suurem kõrgmaliigsete lümfoomide korral, sest suured pahaloomulised rakud on haprad ja hävivad proovi töötlemise käigus. Uurimistöö põhjal võib soovitada osa peennõelbiopsia materjalist koheselt kanda uuringuklaasile rakkude vaatamiseks. Kui domineerivateks rakkudeks on väikesed või keskmist suurust lümfotsüüdid, siis on soovitav määrata lümfotsüütide immuunfenotüüp läbivoolu tsütomeetrial. Kui aga domineerivad suured rakud, on õigem rakkude immuunfenotüpiseerimine teostada immuuntsütokeemiliste meetoditega.

Läbivoolu tsütomeetria võimaldab jälgida minimaalset residuaalset haigust 90%-l ägeda müeloidse leukeemia patsientidest. Usaldusväärse tulemuse saamiseks on vaja jälgida vähemalt kahte või enamat leukeemiale-iseloomulikku immuunfenotüüpi. Minimaalse residuaalse haiguse hindamine aitab valida kuni 60.a. vanustele patsientidele individuaalse parima induktsioonravi järgse raviviisi. Juhul, kui morfoloogilise remissiooni saavutamisel ja/või ravi lõppedes on leitavad pahaloomulised müeloidsed blastid ehk minimaalne residuaalne haigus, saavutatakse parim ravitulemus allogeense vereloome tüvirakkude

siirdamisega. Seega kokkuvõttes võimaldab minimaalse residuaalse haiguse määramine parandada patsientide prognoosi, kuid minimaalse residuaalse haiguse korral sõltub prognoos siirdamisest.

Uurides dendriitilisi rakke lastel ägeda lümfoblastse leukeemiaga, leidsime, et diagnoosimisel on nende hulk luuüdis märkimisväärselt langenud. Dendriitiliste rakkude puudulikkus on enam väljendunud B-rakulise ägeda lümfoblastse leukeemia kui T-rakulise ägeda leukeemia korral. B-rakulise ägeda leukeemia korral sõltub aga dendriitiliste rakkude arv haiguse raskusest. Nendel lastel, kellel haigus oli vähem väljendunud, oli suhteliselt rohkem nii plasmatsütoidseid kui müeloidseid dendriitilisi rakke. Ravi käigus taastus dendriitiliste rakkude arv normväärtuste piiridesse induktsioonravi lõpuks, seitsme nädala möödudes alates ravi algusest. Kuid nendel patsientidel, kellel keemiaravi oli efektitu, ei toimunud nii plasmatsütoidsete kui müeloidsete dendriitiliste rakkude taastumist, tõenäoliselt leukeemia pärssiva mõju tõttu luuüdi dendriitilistele rakkudele. Ravi Põhjamaade laste ägeda lümfoblastse leukeemia raviprotokolli NOPHO ALL-2000 järgi mõjutab oluliselt rohkem müeloidseid dendriitilisi rakke standardriski grupi lastel. Kui ravi lõppedes plasmatsütoidsed dendriitilised rakud jäid normväärtuste piiridesse, siis müeloidsete dendriitiliste rakkude arv langes allapoole normväärtusi. Uurimistöö näitas, et müeloidsete dendriitiliste rakkude arv ei taastu ka kuus kuud peale ravi lõppemist. Pikaaegne ägeda lümfoblastse leukeemia keemiaravi kahjustab müeloidseid dendriitilisi rakke ja see kahjustus võib olla üheks immuunsuse languse põhjuseks lastel, kes on tervistunud ägedast lümfoidsest leukeemiast.

Kokkuvõttes näitas uurimistöö, et läbivoolu tsütomeetria on oluline meetod pahaloomuliste vereloomehaiguste diagnoosimisel, ravitulemuste ja ravimõjude hindamisel.

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All patients participated in this study

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My colleagues in Tartu **Ain Kaare**, **Mari Punab**, **Marju Hein**, **Jane Lehtmaa** and **Sirje Mikkel** for your support.

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**Andreas**, my brother, for helping me always.

My father **Peeter.** I am so grateful for your support. I have always admired you!

**Siim** and **Elise**, my prince and princess. My son and daughter, there is nothing greater than your smile! I am proud of you.

My mother **Aili**, *in memoriam.*

# PUBLICATIONS

# CURRICULUM VITAE


## Edward Laane

Date and place of birth 23.06.1972, Tallinn, Estonia
Citizenship: Estonian
Address: Department of Haematology and Oncology, Tartu University
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### *Education*

| | |
|---|---|
| 1990 | Tallinn Secondary School No.3, gold medal |
| 1996 | Tartu University, Faculty of Medicine, *cum laude* |
| 1996–1997 | Tartu University, internship |
| 1997–2001 | Tartu University, residency in haematology |
| 2001–2011 | Tartu University, Department of Haematology and Oncology, post-graduate student |
| 2002–2006 | Karolinska Institutet, oncology and experimental oncology, PhD |

### *Professional employment*

| | |
|---|---|
| 2001–2001 | Tallinn Central Hospital, physician |
| 2006–2007 | North Estonia Medical Centre Foundation, haematologist |
| 2008–2010 | North Estonia Medical Centre Foundation, head of haematology department |
| 2008– | Tartu University, Department of Haematology and Oncology, assistant, 0.5p |
| 2010– | North Estonia Medical Centre Foundation, head of haematology centre |

### *Scientific work*

Main fields of research:

- Investigation of cell death mechanisms on lymphoid cells, especially the mechanism of glucocorticoid-induced cell death in lymphoid cells;
- Multiparameter flow cytometry, especially investigating minimal residual disease in acute myeloid leukemia, lymphoma diagnostics and dendritic cells in leukemias.

Altogether 8 CC articles.

Membership in scientific organizations:

- Estonian Society of Hematology, president (2010–)
- The Middle-Swedish Leukemia Group, member
- Nordic Myeloma Study Group, investigator
- Nordic Chronic Myeloid Leukemia Study Group, member
- HOVON – the Haemato-Oncology Foundation for Adults in the Netherlands, member
- European Hematology Association, member
- I am the linker of the European Hematology Association H-Net project which is aimed for harmonization of Haematology Curriculum in Europe.

I have been organizer of European Hematology Association and European School of Hematology Tutorials on lymphoid and myeloid malignancies (2009; 2011).

## *Special Courses*

- Karolinska Hospital, Hematopathology 2000
- Karolinska Hospital, Hematology Centre 2001
- Postgraduate Athens Leukemia and Lymphoma Course 2002
- European Hematology Association Congresses 2006–2011
- American Society of Hematology Annual Meetings 2008–2010

## *Awards*

The 1'st place Diploma, Days of the Faculty of Medicine, Tartu University, Scientific Conference 2005

The 1'st place Diploma, Days of the Faculty of Medicine, Tartu University, Scientific Conference 2008

# ELULOOKIRJELDUS

## Edward Laane

Sünniaeg ja -koht 23.06.1972, Tallinn
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Aadress: Hematoloogia-onkoloogia õppetool, Tartu Ülikool
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### *Haridus*

| | |
|---|---|
| 1990 | Tallinna 3. Keskkool, kuldmedal |
| 1996 | Tartu Ülikool, arstiteaduskond, ravi eriala, *cum laude* |
| 1996–1997 | Tartu Ülikool, internatuur |
| 1997–2001 | Tartu Ülikool, residentuur, hematoloogia eriala |
| 2001–2011 | Tartu Ülikool, Hematoloogia-onkoloogia õppetool, doktorant |
| 2002–2006 | Karolinska Instituut, onkoloogia ja eksperimentaalne onkoloogia, PhD |

### *Erialane teenistuskäik*

2001–2001 Tallinna Keskhaigla hematoloogiaosakond, üldarst
2006–2007 SA Põhja-Eesti Regionaalhaigla, hematoloog
2008–2010 SA Põhja-Eesti Regionaalhaigla, hematoloogiaosakonna juhataja
2008– Tartu Ülikool, Hematoloogia-onkoloogia õppetool , assistent, 0.5k
2010– SA Põhja-Eesti Regionaalhaigla hematoloogiakeskuse juhataja

### *Teadustegevus*

Peamised uurimisvaldkonnad:
- Rakusurma mehhanismide uurimine lümfoidsetel rakkudel, eelkõige glükokortikoidide lüütilisest toimemehhanismist lümfoidsetele rakkudele
- Läbivoolu tsütomeetria hematoloogias, eelkõige minimaalse residuaalse haiguse uurimine ägeda müeloidse leukeemia patsientidel, lümfoomide diagnostika ja dentriitiliste rakkude uurimine.

Kokku 8 artiklit CC ajakirjanduses.

Teaduslike organisatsioonide liikmelisus:
- Eesti Hematoloogide Seltsi president (2010–)
- Kesk-Rootsi Leukeemia Grupi liige
- Põhjamaade Müeloomi Uurimisgrupi uurija

– Põhjamaade Kroonilise Müeloidse Leukeemia Uurimisgrupi liige
– Hollandi Täiskasvanute Hematoloogia-Onkoloogia Sihtasutuse liige
– Euroopa Hematoloogia Assotsiatiooni liige

Olen esindanud Eesti Hematoloogide Seltsi Euroopa Hematoloogia Assotsiooni H-Net projektis, mille eesmärgiks on harmoniseerida hematoloogide väljaõpet Euroopas. Euroopa Hematoloogide Assotsiatsiooni ja Euroopa Hematoloogide Kooli koolituste korraldaja lümfoidsetest ja müeloidsetest haigustest (2009; 2011).

## *Erialane enesetäiendus*

– Karolinska Haigla, Hematopatoloogia 2000
– Karolinska Haigla, Hematoloogiakeskus 2001
– Ateena Lümfoomide ja Leukeemiate täienduskoolitus 2002
– Euroopa Hematoloogia Assotsiatsiooni Aastakongress 2006–2011
– Ameerika Hematoloogide Seltsi Aastakongress 2008–2010

## *Auhinnad*

Teadustöö preemia, I koha Diplom, Tartu Ülikooli Arstiteaduskonna Teaduskonverents 2005

Teadustöö preemia, I koha Diplom, Tartu Ülikooli Arstiteaduskonna Teaduskonverents 2008

# DISSERTATIONES MEDICINAE UNIVERSITATIS TARTUENSIS

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