VILNIUS UNIVERSITY

GODA ELIZABETA VAITKEVIČIENĖ

WHITE BLOOD CELL COUNT AT DIAGNOSIS OF ACUTE LYMPHOBLASTIC LEUKEMIA AS A PROGNOSTIC FACTOR IN CHILDREN TREATED IN LITHUANIA AND THE NORDIC COUNTRIES

Doctoral dissertation

Biomedical sciences, medicine (06 B)

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VILNIAUS UNIVERSITETAS

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PRADINIO LEUKOCITŲ SKAIČIAUS PROGNOSTINĖ REIKŠMĖ GYDANT ŪMINE LIMFOBLASTINE LEUKEMIJA SERGANČIUS VAIKUS LIETUVOJE IR ŠIAURĖS ŠALYSE

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Štai mano paslaptis, - pasakė lapė. – Ji labai paprasta:
matyti galima tik širdimi. Svarbiausi dalykai akims nematomi.
- Svarbiausi dalykai akims nematomi, - pakartojo mažasis princas,
kad geriau įsimintų.
(*Antoine de Saint-Exupéry „Le Petit Prince“*)

Skiriu Paulei, Gustei, Mortai ir Andriui

Contents

ABBREVIATIONS

AIEOP - Italian Association of Pediatric Hematology and Oncology

ALL - acute lymphoblastic leukemia

AML - acute myeloid leukemia

BCP - B-cell precursor ALL

BFM - Berlin – Frankfurt – Münster group

CD - cluster of differentiation

CI - confidence interval

CLL - chronic lymphoid leukemia

CML - chronic myeloid leukemia

CNS - central nervous system

COH CH - Center for Oncology and Hematology, Children's Hospital, Affiliate of Vilnius University Hospital Santariskiu Klinikos

Cyp - cyclophosphamide

CTCAE - Common Terminology Criteria for Adverse Events

CS - corticosteroids

Doxo - doxorubicin

DCR1 - death in first complete remission

FAB - French – American – British classification system

FISH - fluorescence *in situ* hybridization

FU - follow-up

G6PD - glucose-6-phosphate dehydrogenase

Hb - hemoglobin

HD MTX - high dose methotrexate

HeH - high hyperiplod karyotype (modal chromosome count ≥50)

HR - high risk

ICU -intensive care unit

IF - induction failure

IR - intermediate risk

i.th. MTX - intrathecal methotrexate

LDH - lactate dehydrogenase

LPh - leukapheresis

6-MP - 6-mercaptopurine

MTX - methotrexate

MRD - minimal residual disease

NA - not available

NCI - National Cancer Institute

NOPHO - Nordic Society of Paediatric Oncology and Haematology

OR - odds ratio

PCR - polymerase chain reaction

pEFS - probability of event free survival

PEG-Asp - pegylated asparaginase

PLT - platelets

PRBC - packed red blood cells

r_s - coefficient of Spearman's rank correlation

pOS - probability of overall survival

SJCRH - St. Jude Children's Research Hospital

SMN - second malignant neoplasm

SR - standard risk

T-ALL - T-lineage ALL

6-TG - 6-thioguanine

TLS - tumor lysis syndrome

ULN - upper limit of normal

VCR - vincristine

WHO - World Health Organization

1. Introduction

1.1 Relevance of the study

Childhood acute lymphoblastic leukemia (ALL) comprises, depending on age, 20–30% of all pediatric malignancies and represents the largest group of childhood cancers (Gurney *et al*, 1995; Linabery & Ross, 2008). Five decades ago childhood ALL was a fatal disease, while nowadays based on risk-adapted antileukemic therapy and improved supportive care, treatment of nearly 90% of children are cured according to the best contemporary treatment protocols (Hunger *et al*, 2013).

Identification of clinical, biological and cytogenetic risk factors, and establishment of criteria for early response to therapy, which are currently used for treatment stratification in contemporary treatment protocols, became possible by international collaboration of large pediatric oncology groups, inclusion of patients into clinical trials, and systematic reporting on trial results (Tsuchida *et al*, 2000; Gustafsson *et al*, 2000a; Silverman *et al*, 2000; Harms & Janka-Schaub, 2000; Gaynon *et al*, 2000a; Schrappe *et al*, 2000b; Conter *et al*, 2000; Kamps *et al*, 2000; Maloney *et al*, 2000; Pui *et al*, 2000a; Pui *et al*, 2010; Gaynon *et al*, 2010; Krishnan *et al*, 2010; Escherich *et al*, 2010; Stary *et al*, 2010; Moricke *et al*, 2010; Schmiegelow *et al*, 2010a; Silverman *et al*, 2010; Conter *et al*, 2010a; Hudson *et al*, 2012).

International collaboration and inclusion of children into clinical trials have already shown significant improvement of health care (Pritchard-Jones, 2008; Schrappe *et al*, 2000a). Due to historical reasons and because of a population of only three million inhabitants, pediatric oncologists in Lithuania until recently have neither been able to take part in international collaboration nor to conduct their own clinical trials.

Childhood ALL in Lithuania has been treated since the early 1970s. However, data on population-based long-term results of children with ALL treated in Lithuania have not been described until 1995 (Savinas *et al*, 1995;

Rageliene *et al*, 1995) (articles in Lithuanian). The study of 206 children with ALL treated in Lithuania from 1986 to 1994 showed five-year survival rate of 60% which was almost 20% inferior compared to those published by the groups from Western countries (Reiter *et al*, 1994; Pui & Evans, 1998). Information on cure rates of childhood ALL in Lithuania for the two recent decades was lacking.

Collaboration with the Nordic Society of Paediatric Oncology and Haematology (NOPHO) in the form of participation in training courses or meetings started in the late 1990s and played an important role in improving qualifications of pediatric oncologists in Lithuania. The next step towards improvement of cure results of childhood ALL in Lithuania was to join the prospective NOPHO ALL treatment protocol, which is one of the leading international ALL treatment protocols. However, before being accepted as full member of the study group, several requirements had to be fulfilled. First, the long-term childhood ALL treatment results in Lithuania had to be evaluated. For that reason retrospective clinical and laboratory data had to be collected and analyzed in detail in order to establish the long-term cure rates and prognostic factors impacting survival rates of children with ALL in Lithuania. Second, contemporary diagnostic methods, and contemporary strategies for the risk-group stratification and monitoring of minimal residual disease (MRD) consistent with the requirements of the NOPHO ALL-2008 treatment protocol had to be implemented. Third, prospective registration of the patients to the ALL-2008 Register had to be started.

Fulfilment of all the above-listed conditions in order to join the NOPHO ALL protocol and consequently to offer the most contemporary treatment for children with ALL in Lithuania was the object of this doctoral dissertation.

Historically, white blood cell count in peripheral blood (WBC) at diagnosis of ALL was considered to be one of the strongest independent risk factors and was used for the risk-group stratification in Lithuania and other

countries (Moricke *et al*, 2008; Hann *et al*, 2000; Riehm *et al*, 1990; Moghrabi *et al*, 2007; Hunger *et al*, 2012; Oudot *et al*, 2008). As novel risk factors were identified such as cytogenetic aberrations of leukemic cells or early response to therapy at the MRD level, WBC at diagnosis has tended to lose its significance.

Thus, WBC at diagnosis of ALL is no longer used as a stratifying factor by the international Berlin–Frankfurt–Münster (BFM)-study group. In contrast, the groups in the United States as well as in the Nordic countries still use WBC at diagnosis for treatment stratification. To explore to which extent WBC is still a prognostic factor and may be used for treatment stratification after including into analysis the other well known risk factors such as cytogenetic aberrations and MRD, data on 2363 Nordic children with ALL were analyzed, and the results are described in this dissertation. The results of the study will be used for the development of a new NOPHO ALL treatment protocol.

A small subset of patients comprising 5-8% of all childhood ALL present with very high WBC ($\geq$200 x 10^9/L) at the time of diagnosis (Lund *et al*, 2011; Porcu *et al*, 2000; Porcu *et al*, 2002). Such tumor burden itself is associated with high risk of early morbidity and mortality since it may cause multi-organ dysfunction both by hyperviscosity and leukostasis in microcirculation or by severe metabolic derangements, which occur when rapid and extensive cell lysis of massive amount of blasts is induced by antileukemic therapy (Lowe *et al*, 2005a; Lund *et al*, 2011; Zonfrillo, 2009). Large clinical studies analyzing the rates of early morbidity and mortality as well as clear guidelines for initial management of such patients are lacking. Despite the small number of patients at risk there is a need for contemporary guidelines to address this life-threatening complication. To analyze hyperleukocytosis caused complications, the impact of different strategies of initial treatment on early morbidity and survival, and to develop guidelines for the management of children with ALL and WBC $\geq$200 x 10^9/L was the goal pursued in the third part of this doctoral dissertation.

1.2 Scientific novelty of the study

For the first time in Lithuania the detailed epidemiological, clinical, laboratory and treatment data for children with ALL were analyzed to determine cure rates and to detect risk factors having an impact on survival.

Lithuanian children with cancer were for the first time enrolled into an international treatment protocol (NOPHO ALL-2008), and started to be prospectively registered into the multicenter NOPHO ALL-2008 Register.

New laboratory markers were introduced into routine practice for diagnostic work-up and monitoring of treatment effect. The flow cytometry lab in Lithuania had to standardize their diagnostic procedures and participate in the NOPHO cross-validation program.

An access to the register data of 2363 Nordic children with ALL enabled exploration of biological background of WBC at diagnosis of ALL, and detection of a subgroup of patients with WBC $\geq$100 x 10^9/L with a significantly poorer outcome after adjusting for other prognostic features such as immunophenotype, karyotype, extramedullary disease at diagnosis and the early response to therapy measured at the level of MRD.

The study of 221 children with ALL and hyperleukocytosis (WBC $\geq$200 x 10^9/L) treated in 1992-2011 in Lithuania or Nordic countries (Denmark, Finland, Iceland, Norway or Sweden) was the largest and the first international population-based study that explored clinical presentation and an impact of different initial treatment strategies on early morbidity and cure rates for those patients.

1.3 Practical application

Clinical, laboratory and treatment data of children with ALL from the recent two decades in Lithuania were collected from patient paper forms into a common database. The results of the analysis have served and will serve in the future as a basis for decision making for further improvement of treatment strategy and thus increase of cure rates for children with ALL in Lithuania.

Being participants in the multi-center prospective protocol, Lithuanian pediatric oncologists/hematologists were provided with the information on the interim results of the protocol and had a possibility to participate in the discussions on complicated cases of both Lithuanian and Nordic patients. This led to a steady increase of understanding of the biology of childhood ALL, which in turn may have improved clinical decisions.

Joining the international protocol, analyses of retrospective data, and starting of prospective registration of data showed its benefits and was a learning phase to serve as an example for collaboration with the other international pediatric cancer study groups.

The findings on prognostic impact of WBC for high risk patients will be useful in the process of the development of a new NOPHO ALL-2015 protocol.

Based on the results of hyperleukocytosis study presented in this thesis, Nordic/Baltic guidelines for the initial management of patients with high risk for tumor lysis syndrome (TLS) (WBC $\geq$100 x 10^9/L at diagnosis) were developed, approved by the NOPHO Leukemia and Lymphoma Committee and the Board, and will soon be practically applied.

1.4 Aims

1.4.1 Primary aim

To improve survival rates of childhood ALL in Lithuania by joining an international treatment protocol.

1.4.2 Secondary aim

To explore biological background and prognostic impact of high WBC in childhood ALL on early events and long-term survival after adjustment for other well-known prognostic factors.

1.5 Objectives

1. To analyze the data on epidemiology and cure rates for children with ALL diagnosed in 1992-2012 in Lithuania.
2. To explore the association of the biology of leukemic cells, clinical presentation, and treatment response to WBC in childhood ALL among Lithuanian and Nordic patients.
3. To study biological characteristics and clinical presentation of hyperleukocytosis (WBC $\geq$ 200.0x10^9/L) among the children 0–14.9 years of age at diagnosis of ALL.
4. To explore the impact of different initial treatment strategies on early morbidity and cure rates among childhood ALL patients with hyperleukocytosis (WBC $\geq$ 200.0x10^9/L).
5. To develop Nordic/Baltic guidelines for initial management of hyperleukocytosis (defined as WBC$\geq$100 x 10^9/L) in childhood ALL.

2. Literature review

2.1 Background

Leukemia is a cancer of bone marrow and peripheral blood characterized by dysregulated clonal expansion of immature lymphoid or myeloid progenitor cells that are blocked at a particular stage of differentiation (Figure 1).

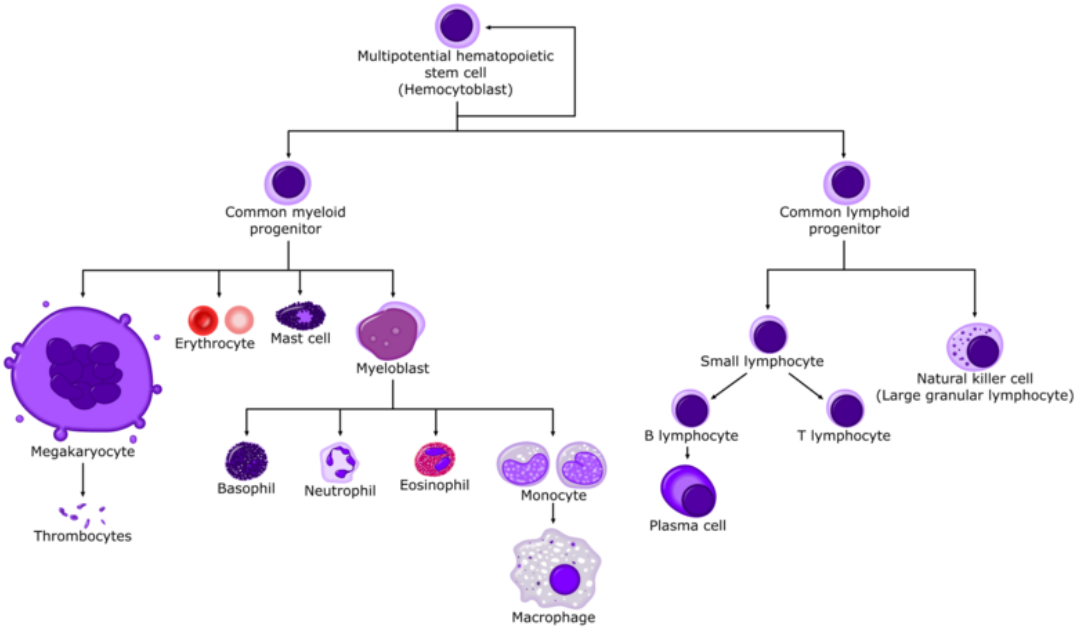

Figure 1. Schematic picture of normal haematopoiesis. Source: Image: Hematopoiesis_ (human) _diagram.png by A. Rad

Leukemia is a heterogeneous group of diseases which differ in etiology, pathogenesis, natural history and prognosis. Leukemia is broadly divided into (a) acute leukemia, which, if untreated, usually causes death in weeks or months and (b) chronic leukemia, which, if untreated, causes death within months or years. Both acute and chronic types of leukemia according to the line of origin are classified into (a) lymphoid or (b) myeloid leukemias. Thus, four main types of leukemia exist: acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), chronic lymphoid leukemia (CLL) and chronic myeloid leukemia (CML). The studies presented in this dissertation are focused on ALL in children.

2.2 Historical perspective

Generally, the ancient Greeks are credited with being the first to recognize cancer in the 4^{th} – 5^{th} century B.C. The modern knowledge about leukemia started in Europe as late as the 19^{th} century. In 1844, the French doctor Alfred Donné was the first to describe a patient with "white cells dominating in his blood" calling it an unknown disease (Diamantis *et al*, 2009). In 1845 the German pathologist Rudolf Virchow for the first time used the name "leukhemia" (from greek *leukos* – white, *heima* – blood) that referred to the abundance of white blood cells in the body. Five years later doctor Henry Fuller reported a case of a 9-year-old girl that died of "leucocythaemia", and this was considered to be the first record of childhood leukemia (Piller, 2001).

Half a century later, Paul Ehrlich developed new methods of staining slides and for the first time differentiated between types of blood cells thus classifying leukemia into a myeloid group ('granulated cells') and a lymphoid group ('cells without granules') (Piller, 2001). In the mid-1970's, a group of seven French, American and British hematologists proposed the French–American–British (FAB) cell classification system determined by the morphological and cytochemical features of lymphoid blasts, which distinguished three ALL subtypes: L1 (predominance of small blasts), L2 (larger and more heterogeneous blasts) and L3 (large and homogeneous blasts, mostly mature B-lymphocytes) (Bennett *et al*, 1976). The FAB classification system was used for leukemic cell classification for almost two decades; however, it showed poor correlation with immunological and genetic findings and therefore has not proven to have an independent prognostic significance in the risk stratification and clinical management of ALL.

A new classification was proposed by the International Workshop and Conference held in Paris, in 1982 (Bernard & Boumsell, 1984; 1984). Classification was based on the combination of glycoprotein molecules, present

on the surface or in the cytoplasm of the cells, so called clusters of differentiation (CD). Flow cytometry techniques now use monoclonal antibodies for detection of combinations of these antigens, which are specific for B- or T-lineage lymphocytes at different stages of their differentiation. Recently the EuroFlow Consortium has been initiated to standardize antibody panels and flow cytometric immunophenotype procedures in Europe (van Dongen *et al*, 2012).

About thirty years ago it was recognized that chromosomal changes were an independent prognostic indicator in childhood ALL (Secker-Walker *et al*, 1978). Discovery and characterization of genetic abnormalities have increased the understanding of the biology of the disease and provided important prognostic and predictive markers which have improved the patients' outcome. A wide range of chromosomal and genomic abnormalities have been reported as being associated with the patient outcome, but only a subset are currently used to risk stratify patients (Moorman, 2012).

2.3 Etiology

Causes of ALL are still largely unknown. Higher risk of ALL development is associated with several congenital disorders such as Down's syndrome, neurofibromatosis, Fanconi's anemia, ataxia telangiectasia (Hasle *et al*, 2000; German, 1997; Morrell *et al*, 1986; Wang *et al*, 2011). Association of ALL development with prenatal exposure to X-rays or chemotherapeutic agents, maternal smoking and exposure to chemical agents during pregnancy, or to child's birth weight has also been investigated (Brauner *et al*, 2010; Hjalgrim *et al*, 2004; Turner *et al*, 2010b; Chang *et al*, 2006; Chokkalingam & Buffler, 2008; Milne *et al*, 2013). However, all these causes together may explain less than 10% of the cases (Wiemels, 2012).

Studies of Guthrie cards found leukemia associated mutations including translocation t(12;21)/*TEL-AML1* on neonatal blood spots already at birth (Wiemels *et al*, 1999; McHale *et al*, 2003; Greaves & Alexander, 1993). The commonly held belief of 1% of newborns harboring the functional t(12;21)-transcripts detectable at levels of 10^{-3}-10^{-4} and almost exclusively supported by the study of H. Mori *et al*. (Mori *et al*, 2002) had recently been challenged by the study of U. Lausten-Thomsen *et al*. (Lausten-Thomsen *et al*, 2011) which indicated far lower levels. Based on the fact that only about 1% of the neonates with 'preleukemic' clone later develop an overt ALL, M. Greaves elucidated the 'two-hits' model indicating the need of a second genetic 'hit' later in life to induce an overt leukemia (Greaves, 2006). The 'second hit' is believed to be provoked by the immune system, and that led to the development of two related hypotheses: (i) L. J. Kinlen's 'population mixing' hypothesis noting that leukemia incidence was increased when children and families were moved and mixed to different countries (Kinlen, 1995), and (ii) M. Greaves hypothesis on the developing of a higher risk of leukemia for children who received lower levels of immune stimulation during childhood (Schmiegelow *et al*, 2008; Greaves, 2006; Rudant *et al*, 2010).

However, it is likely that childhood ALL may result from interaction of both genetic and environmental factors (Metayer *et al*, 2013).

2.4 Epidemiology

ALL is the most common pediatric malignancy, comprising 25% of cancers occurring under 15 years of age and 19% among those under 20, which translates to the incidence of 3–4 cases per 100 000 children per year (male : female prevalence of roughly 1.3 : 1) in Europe and the United States (Kaatsch, 2010; Coebergh *et al*, 2006; Ries LAG *et al*, 2013). ALL affects both children and adults with an incidence peak prevalence in young children aged 2 to 7 years (Figure 2) (Forestier & Schmiegelow, 2006; Hjalgrim *et al*, 2003).

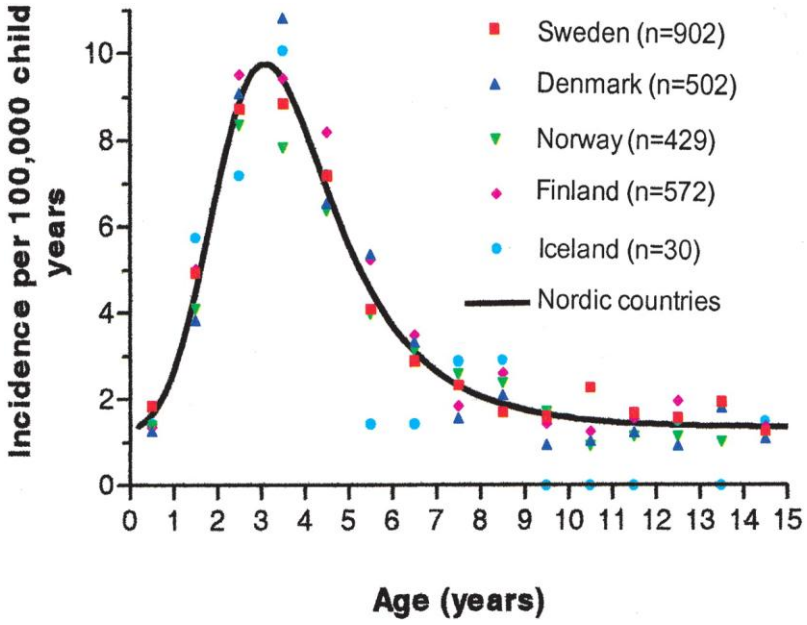

Figure 2. Age-specific incidence rates of B-precursor acute lymphoblastic leukemia in the Nordic countries from January 1, 1986, through December 31, 2001 (Hjalgrim *et al*, 2003)

Increasing incidence rates for ALL were repeatedly reported by a number of developed countries in Europe with an average increase of close to 1% per year in the last two decades (Kaatsch *et al*, 2006; Kaatsch & Mergenthaler, 2008). The same trend was reported in the United States (Ries LAG *et al*, 2013; Linabery & Ross, 2008). The exact reason for such changes is not clear. Factors challenging the immune system, such as infectious agents or atopic diseases are considered to play a role in the development of leukemia in the countries that have a higher socioeconomic status (Maule *et al*, 2006; Dahl *et al*, 2009; Greaves, 2006; Bunin, 2004; Schmiegelow *et al*, 2008). However, such increase may also reflect the improvement in completeness of registration of childhood cancer or advanced diagnostic procedures. The Nordic countries which have one of the most complete registries in the world report a stable incidences of acute lymphoblastic leukemia overall also within specific ALL immunophenotypes over the last two decades (Hjalgrim *et al*, 2003).

2.5 Childhood ALL in Lithuania

In-depth studies on epidemiology, survival rates and risk factors for children with ALL in Lithuania are scarce. The first scientific study on childhood ALL in Lithuania was performed by L. Rageliene in 1988 who in her dissertation explored the activity of insulin growth factor inhibitors in the blood of children with ALL (Rageliene L, 1988). Epidemiology and treatment results of 208 children with ALL treated in Lithuania during the period from 1986 to 1994 were analyzed and described in 1995 (Rageliene *et al*, 1995; Savinas *et al*, 1995) (articles in Lithuanian). The incidence of childhood ALL in Lithuania was 3.4-3.6 per 100 000 children per year which was in consistence with the data in other European countries (Lanzkowsky P, 2011). The 60% five-year overall survival for children with ALL was also reported. Another survey on epidemiology of childhood cancer in Lithuania in 1988-2002 detected no evident changes in the incidence rates of leukemias among children during the period (Zukauskaite R *et al*, 2005). V. Rutkauskaite and L. Rageliene analyzed causes of death for children treated for leukemia in 2001-2005. Septic complications during chemotherapy induced myelosuppression revealed to be the most common cause of death (Rutkauskaite V & Rageliene L, 2007). Few literature reviews on central nervous system (CNS) leukemia (Karalkeviciene E *et al*, 2001) and the use of colony stimulating factors for supportive care at the time of chemotherapy of childhood ALL (Gudleviciene Z & Rageliene L, 1999) were published. However systematic population-based long-term results of childhood ALL in Lithuania were missing.

Data report to international cancer registries on childhood ALL as well as on other childhood cancers in Lithuania is also insufficient. A disappointing aspect of poor representation of data from Eastern Europe countries was also noted in the recent report of the European Cancer Registry (EUROCARE-4) (Gatta *et al*, 2009).

There was an obvious need to collect and analyze in-depth data on childhood ALL in Lithuania. This was the object of Study I represented in this dissertation. Retrospective data from journals of the patients were thoroughly collected and analyzed starting from the year 1992 since earlier records were scarce and incomplete.

2.6 Biological characteristics

Modern laboratory techniques enabled detection of leukemic cell characteristics both by the level of differentiation and by recurrent genetic abnormalities. Many of them have been reported as being associated with patient outcome, however not all of them are used as prognostic factors for treatment stratification (Mullighan & Willman, 2011; Moorman, 2012; Pui *et al*, 2011).

Childhood ALL originates from the precursors of T- or B-lineage lymphocytes in bone marrow (Figure 1) (Bethesda (MD): National Cancer Institute (US) 2002-, 2013). B-lineage ALL is further divided to early pre-B cell, pre-B cell, and B-cell ALL. Leukemia of the majority of young children (2 to 10 years old) is of B-cell precursor phenotype, or "common acute lymphoblastic leukemia", expressing CD10+, CD19+ and CD79a+, and is associated with a favourable prognosis. In contrast, immunophenotype of infant ALL is often immature early B-cell precursors, and is characterized by a lack of CD10 expression and co-expression of myeloid-associated antigens. ALL in infancy is associated with a high WBC at presentation, hepatosplenomegaly, CNS involvement, presence of 11q23/*MLL*$^{-}$ gene rearrangement and with a poor prognosis (Reaman *et al*, 1985; Chessells *et al*, 1994; Hilden *et al*, 2006; Tauchi *et al*, 2008).

Recent studies have identified a novel very high-risk immature T-cell subtype termed "early T-cell precursor" ALL. These cells are characterized by immunological markers and a gene expression profile reminiscent of very early

arrest in T-cell differentiation that retain the ability to differentiate into both T-cell and myeloid, but not B-cell lineages. Whole genome sequencing frequently showed mutations involved in the genes regulating hematopoietic development (*GATA3, ETV6, RUNX1, IKZF1*), *RAS* signalling and histone modifications sharing characteristics with acute myeloid leukemia (Coustan-Smith *et al*, 2009; Zhang *et al*, 2012; Neumann *et al*, 2012).

Although all chromosomal translocations have been observed at virtually all age groups, there is a strong correlation between the age and the frequency of each translocation with the tendency of higher risk aberrations generally increasing with age (Figure 3) (Moorman, 2012; Toft *et al*, 2013; Chiaretti *et al*, 2013).

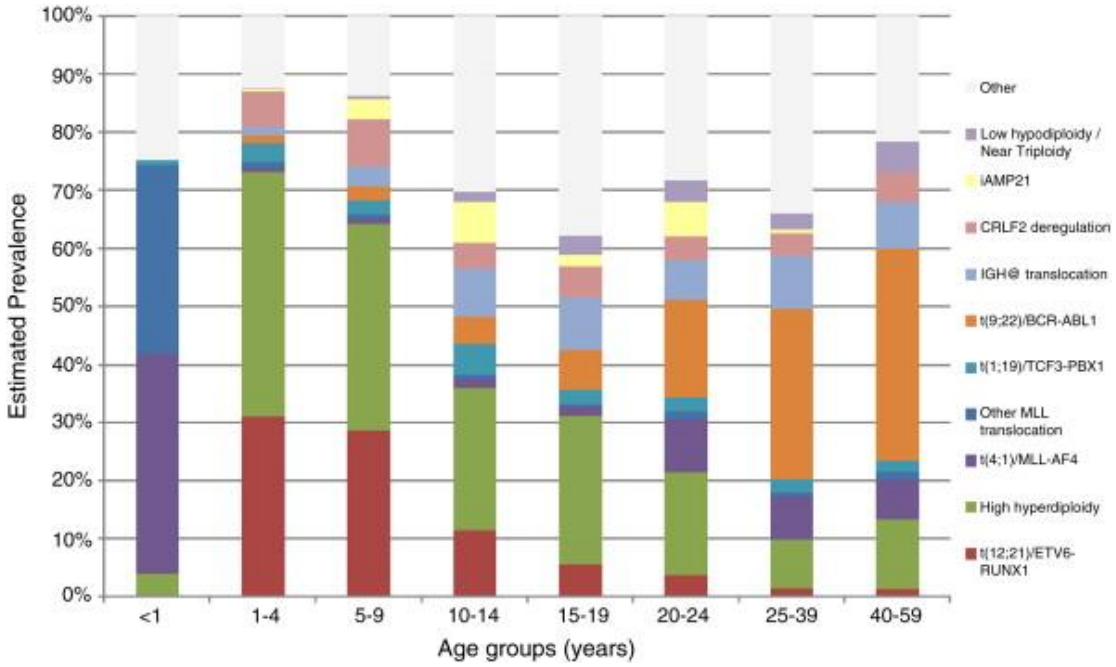

Figure 3. Estimated age-specific frequency of selected chromosomal abnormalities in ALL. *(Moorman, 2012).*

Hyperdiploidy with gain of at least 5 chromosomes and translocation t(12;21)(p13;q22) resulting in the fusion gene *ETV6-RUNX1* are characterized by a prominent incidence peak between 1 and 7 years of age together encompassing more than 70% of all BCP ALL cases with an aberrant karyotype

in this age group, and are associated with young age, low WBC, CD10+ immunophenotype and a favourable outcome (Forestier & Schmiegelow, 2006; Romana *et al*, 1995; Shurtleff *et al*, 1995). In contrast, hypodiploidy with less than 44 chromosomes or ALL with 11q23/*MLL*- gene rearrangement is associated with a poor prognosis (Nachman *et al*, 2007; Harrison *et al*, 2004a; Cerveira *et al*, 2012; Muntean & Hess, 2012). Translocation t(9;22)/*[BCR-ABL]* which has increasing incidence with increasing age, is associated with higher WBC, and previously has been considered to have a poor prognosis, until it became to be the first genetic subtype that benefitted from targeted therapy with imatinib, a selective inhibitor of the *BCR-ABL1* tyrosine kinase (Schultz *et al*, 2009).

Acute leukemia genomes commonly harbor submicroscopic genetic alterations that disrupt genes with key roles in leukocyte development or activate oncogenes, and are important events in leukemogenesis (Mullighan, 2011; Roberts & Mullighan, 2011). These include *IKZF1*, *TCF3*, *EBF1*, *PAX5*, *JAK 1/2* and *CRLF2* aberrations. They encode transcriptional regulators of early lymphoid maturation and commitment to B-cell lineage, and are associated with poor prognosis (Mullighan *et al*, 2007; Mullighan *et al*, 2008; Kuiper *et al*, 2007; Mullighan *et al*, 2009; Russell *et al*, 2009).

There has been intensive effort in recent years to identify novel genetic alterations, and some of them are now well established and are used in the official WHO Classification of ALL (Turner *et al*, 2010a). However, the prognostic impact of many of these novel mutations has yet to be established in conjunction with traditional risk factors including WBC.

2.7 White blood cell count as a prognostic risk factor

The poorer prognosis for infants and adolescents as well as its association with increasing WBC had been confirmed in many studies (Crist *et al*, 1986; Pui & Crist, 1994; Sather, 1986), and were accordingly used by the United States

National Cancer Institute (NCI) in 1993 as criteria to define more uniform application of prognostic factors in risk classification system for comparison of the results from different clinical trials (Smith *et al*, 1996). BCP patients, 1 to 9 years of age, and with WBC <50 x 10^9/L were assigned to the standard-risk (SR) category, and the rest of the patients (T-ALL, age ≥10 years, and WBC ≥50 x 10^9/L) were assigned to the high risk (HR) category (Smith *et al*, 1996). WBC at diagnosis had been considered as one of the strongest independent predictors of induction failure, resistant disease, and risk of relapse in childhood ALL and had accordingly been used for treatment stratification by all major study groups (Gaynon *et al*, 2000b; Eden *et al*, 2000; Schrappe *et al*, 2000c; Pui *et al*, 2000b; Schultz *et al*, 2007a; Gustafsson *et al*, 2000b).

However, no optimal WBC cut-point for risk group categorization had been observed. The French group in the data of three consecutive FRALLE protocols found an exponential distribution of WBC with the risk of relapse or toxic death in the highest quintile being 1.9 times higher than that in the lowest quintile with a continuous nature of WBC as a predictor of prognosis (Donadieu *et al*, 2000).

As treatment has been intensified, several previously prognostic factors such as sex, race, even immunophenotype or initial CNS involvement have lost their independent prognostic significance (Conter *et al*, 2010b), and new prognostic factors have emerged, including prognostic karyotypes (Sinnett *et al*, 2006; Forestier *et al*, 2000; Bhojwani & Pui, 2008) and early assessment of MRD (Nyvold *et al*, 2002; Ratei *et al*, 2009; Irving *et al*, 2009; Coustan-Smith *et al*, 2002; Flohr *et al*, 2008).

WBC at diagnosis of ALL is no more used as a stratifying factor by several international study groups including BFM or Italian Association of Pediatric Hematology and Oncology (AIEOP) study groups. However, the groups in the United States such as St. Jude Children's Research Hospital (SJ CRH), Dana–Farber Cancer Institute (DFCI) or Children's Oncology Group

(COG) still use the NCI criteria. Furthermore, the NOPHO group in its recent NOPHO ALL-2008 protocol stratifies ALL patients of B-cell precursor origin having WBC <100 x 10^9/L at diagnosis to the lower risk and those having WBC ≥100 x 10^9/L to the higher treatment risk arms. Nevertheless, published studies on long-term treatment results by the large study groups still reveal WBC as a risk factor for an event (Schultz *et al*, 2007b; Rives *et al*, 2012; Maurer *et al*, 1988a; Escherich *et al*, 2010; Bowman *et al*, 2011).

High WBC in infant ALL is generally associated with *MLL* gene rearrangements. Furthermore, within infants, the multivariate analysis on recently conducted large-scale clinical studies has revealed a rearranged *MLL* gene, younger age (<3 or 6 months), and very high white blood cell count (≥300,000/μL), to be independently associated with poor outcome (Hilden *et al*, 2006; Pieters *et al*, 2007).

Interestingly, higher WBC count (NCI high risk) has been associated with poorer prognosis as compared with NCI standard risk even within cytogenetically homogeneous subsets as Ph-positive ALL (Arico *et al*, 2000; Schrappe *et al*, 1998). Furthermore, NCI risk group revealed to be the only risk factor in a large study performed by the group from the United Kingdom of ALL patients with translocation t(12;21), which generally is associated with a very good outcome (Enshaei *et al*, 2013). This suggests of some yet unknown factors having an impact both on WBC and prognosis.

2.8 Clinical presentation

ALL is a disease of bone marrow and peripheral blood. The symptoms may be slowly progressive over weeks to months, or they may be acute and explosive. Peripheral blood picture may vary widely from pancytopenia to very high WBC (≥200 x 10^9/L) which may cause life-threatening complications due to increased hyperviscosity or severe metabolic derangements caused by the intensive lysis of leukemic cells. Thus, the clinical presentation of ALL is

dominated by the degree of bone marrow failure with anemia, thrombocytopenia or infectious complications, or by the involvement of extramedullary sites by leukemia causing lymphadenopathy or hepatosplenomegaly and/or by hyperleukocytosis caused complications.

High WBC at presentation, peripheral lymphadenopathy, mediastinal masses and CNS involvement are much more common in children with T-ALL than in children with BCP (Pui *et al*, 1990a; Greaves, 1981). Different patterns of clinical presentation for BCP and T-lineage ALL may reflect the different ways of multistep process of cell-differentiation and proliferation as well as migration from bone marrow to peripheral lymphoid organs during the normal development of B-lineage and T-lineage cells. B-cell precursors undergo multiple cycles of differentiation and proliferation in bone marrow, and migrate to peripheral compartments at the stage of development to naïve B-cells only (Ghia *et al*, 1996; van Zelm *et al*, 2005; Hystad *et al*, 2007). In contrast, the commitment to T-lineage takes place in the thymus microenvironment where the progenitors undergo differentiation and maturation to mature T-lymphocytes (Graux *et al*, 2006).

In addition, ALL subsets having certain cytogenetic aberrations have a characteristic pattern of presentation such as lower WBC and preschool age for translocation t(12;21)(p13;q22) or hyperdiploid karyotype, higher WBC and increasing incidence with increasing age for translocation t(9;22)/*[BCR-ABL]*, or age <1.0 year, high WBC, hepatosplenomegaly and CNS involvement for 11q23/*MLL*$^{-}$ rearrangement (described also in chapter 2.5).

2.8.1 Central nervous system and other extramedullary compartments

Approximately 2 to 3% of children with ALL have identified blasts in their spinal fluid (Pui & Howard, 2008). Furthermore, many international childhood ALL treatment groups report the involvement of CNS in up to 30 – 40% of all relapses (Pui & Howard, 2008; Pui & Thiel, 2009). CNS is supposed

to be a pharmacological ‘sanctuary’ as most antileukemic agents fail to achieve therapeutic levels in cerebrospinal fluid.

The mechanisms of leukemic cells to egress into CNS are not completely understood. Leukemic cells can reach CNS in case of CNS hemorrhage if the blood contains circulating blasts, for example in case of very high WBC at presentation, or may be introduced iatrogenically at the time of the diagnostic lumbar puncture, especially if the puncture is traumatic (Burger *et al*, 2003; Gajjar *et al*, 2000; Te Loo *et al*, 2006).

However, most likely leukemic cells in most of the cases invade CNS and other extramedullary compartments by active, rather than passive way. The UK study group in a murine model of CNS disease identified an expression of adhesion molecules on the surface of leukemic and endothelial cells. The group suggested a complex model of interaction between leukemic and host endothelial cells with the expression of molecules important for cell migration, adhesion and invasion. Only specific subpopulations of pre-B lymphoblasts may be capable of invading CNS, and also of surviving in cytotoxic surrounding. Similar processes may be responsive to the invasion and survival of leukemic blasts from bone marrow to peripheral blood and other extramedullary organs. Identifying and targeting the process of adhesion and invasion might prevent leukemic cells from spreading and surviving in the selected niches providing the cells a protective environment (Holland *et al*, 2011).

Recurrence of the disease in CNS may reflect the same underlying biological mechanisms as in cases of high WBC indicating to cell aggressiveness and ability to survive in extramedullary surrounding. Children with high WBC at presentation and with T-ALL have CNS involvement more often compared with those with lower WBC count and BCP ALL (Marwaha *et al*, 2010a). Moreover, many large clinical trials reported high WBC at presentation to be a risk factor for CNS involvement at the time of relapse. The joint study of BFM and Italian AIEOP groups showed that children and

adolescents with T-cell ALL and a presenting WBC ≥100 have a very high risk for CNS relapse and requires a very intensive CNS-directed treatment which if inadequate might increase the risk for not only CNS relapse, but also for hematological relapse (Conter *et al*, 1997).

2.8.2 Leukostasis and hyperviskosity

Patients with very high WBC (≥100.0 x 10^9/L) at diagnosis of ALL constitute 5-8% of all childhood ALL patients and are known to have significantly lower survival rates (Moricke *et al*, 2008; Lund *et al*, 2011). These patients represent a challenging therapeutic problem because of a very high risk of early mortality if not recognized and treated promptly and appropriately. Leukostasis can affect any organ however some organ-specificity is seen since intraparenchymal brain hemorrhage and respiratory failure account for the majority of early deaths (Fritz *et al*, 1959b; van Buchem *et al*, 1987). Mechanical cytoreduction procedures (leukapheresis or exchange transfusions) are often considered for the initial management with hyperleukocytosis. Leukapheresis was introduced more than 20 years ago (McCarthy *et al*, 1997), however no randomized, prospective studies on the efficacy of leukapheresis had been published, and its beneficial role is still controversial. So far there has been no benefit proved in terms on survival rates among the relatively large cohorts of pediatric or adult patients both for ALL or for AML (Haase *et al*, 2009a; Porcu *et al*, 2002; Porcu *et al*, 1997a).

Pathophysiology of hyperleukocytosis is not completely understood. Symptoms of hyperleukocytosis are thought to be caused primarily by the sludging of circulating leukemic blasts in microvasculature and an increase in whole blood viscosity. *In vitro* studies have demonstrated an increased volume of cell fraction in the blood (leukocrit) and increased stiffness of leukemic cells to possibly contributing to the development of leukostasis and its related clinical symptoms. Several years ago Lichtman showed that the viscosity of suspensions

of leukemic cells was dependent logarithmically on a cytocrit. He suggested that high counts of leukemic cells may interrupt the capillary blood flow and tissue oxygen delivery thereby causing clinical manifestation of leukemia (Lichtman, 1973; Lichtman & Rowe, 1982). Fractions of stiff leukemic cells in leukemic patients with symptoms consistent with CNS and pulmonary leukostasis were later detected by other groups which suggested that increased cell stiffness may be an additional independent leukostasis risk factor (Lam *et al*, 2008).

Recent studies show and it is now generally believed that adhesion reactions promoted by the interaction between leukemic blasts and endothelial cells may also play an essential role. Upregulated expression of several adhesion molecules (CD54, CD26E, CD62P, CD106) following interaction with leukemic blasts was found by A. Stucki et al. in the endothelial cells of AML patients with leukostasis (Stucki *et al*, 2001). Such findings may be in favor of systemic antileukemic therapy rather than mechanical reduction of leukemic blasts in peripheral circulation to be the method of choice for treatment of life-threatening intraparenchymal leukemic infiltrates.

2.8.3 Tumor lysis syndrome

Another potentially life-threatening disease-related oncologic emergency caused by the high WBC arises from a rapid and massive lysis of chemosensitive malignant cells and the concomitant release of intracellular contents into a bloodstream that in turn causes metabolic abnormalitites including hyperuricaemia, hyperphosphatemia, hyperkalemia and hypocalcemia. Clinical manifestations of tumor lysis syndrome (TLS) typically occur 12-72 hours after treatment initiation and include renal failure, seizures or cardiac arrhythmias, which may cause death if not recognized and treated appropriately (Hochberg & Cairo, 2008; Mughal *et al*, 2010; Maurer *et al*, 1988a; Cohen *et al*, 1980). Hyperuricemia together with hyperphosphatemia are the most frequently recognized manifestations of TLS. Hyperuricemia results from rapid release and

catabolism of intracellular nucleic acids. Purines are catabolized by xanthine oxidase to hypoxanthine, then xanthine, and finally to uric acid which is the final product in humans (Figure 4).

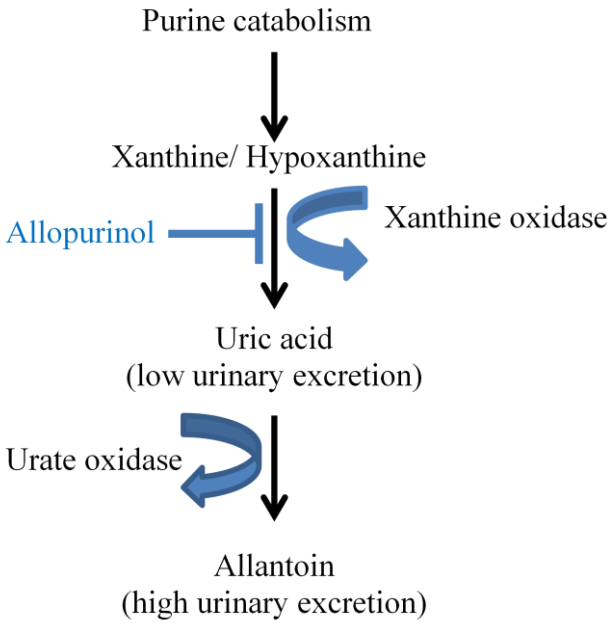

Figure 4. Purine catabolism pathway. *(Hochberg & Cairo, 2008)*

Phosphates are released into circulation from leukemic blasts which may contain up to four-fold higher amount of phosphates than normal cells. Increased concentrations of uric acid or phosphates can overwhelm the body's homeostatic mechanisms and result in deposition of uric acid or calcium phosphates crystals in the renal tubules causing acute renal damage (Locatelli & Rossi, 2005; Rieselbach *et al*, 1964; Nagai *et al*, 2011; Hochberg & Cairo, 2008; Canet *et al*, 2013).

Uric acid is rapidly degraded to allantoin by an enzyme urate oxidase which is produced in all mammals except humans and particular primates (Merriman & Dalbeth, 2011; Oda *et al*, 2002). Allantoin is five to ten times more water-soluble than uric acid, and is therefore easily excreted by the kidney (Figure 3). Urate oxidase has been recently synthesized and its recombinant form rasburicase is now available in the clinics (Coiffier *et al*, 2003; Bertrand *et al*, 2008). When uric acid is broken down to allantoin, a byproduct hydrogen peroxide is generated. It causes oxidative stress, and may cause hemolysis and

methemoglobinemia in patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency. Thus, patients with known G6PD deficiency are regarded as a contraindication for urate oxidase therapy.

Studies with rats ascertained another potential mechanism of renal damage caused by hyperuricemia independently of crystal formation. Hyperuricemia induces impairment in autoregulatory response of afferent arterioles, resulting in glomerular hypertension. Lumen obliteration induced by vascular wall thickening produces severe renal hypoperfusion. Resulting ischemia is a potent stimulus of tubulointerstitial inflammation and fibrosis (Sanchez-Lozada *et al*, 2005).

An international TLS consensus expert panel developed a classification model of oncological diseases with low, intermediate and high risk for TLS defined by the <1%, 1-5% or >5% risk of TLS development, respectively, and the associated prophylaxis recommendations (Cairo *et al*, 2010). ALL with WBC <100 x 10^9/L and lactate dehydrogenase (LDH) less than two times upper limit of normal (ULN) was assessed to the intermediate risk, and ALL with WBC ≥100 x 10^9/L and/or LDH more than two times ULN was assessed to the high risk of TLS development group (Cairo *et al*, 2010).

Traditionally, hyperhydration, urine alkalinization, allopurinol and delay in antileukemic therapy or small initial corticosteroid (CS) doses had been a generally accepted strategy applied to prevent and manage TLS (Jones *et al*, 1995; Ten Harkel *et al*, 1998). Since the introduction of urate oxidase, the risk of TLS has been markedly reduced, and the strategy for prevention and managing of TLS has changed (Goldman *et al*, 2001; Pui *et al*, 2001; Renyi *et al*, 2007; Wossmann *et al*, 2003). However, clinicians may still choose initial dose reductions of anticancer agents or delays in antileukemic therapy to avoid TLS, and the impact hereof on the risk of early complications related to hyperleukocytosis or of later relapse is uncertain, since there is a lack of large studies. Furthermore, most published reports are single centers analysis (Lowe

et al, 2005a; Bunin & Pui, 1985; Eguiguren *et al*, 1992a; Harousseau *et al*, 1980a; Maurer *et al*, 1988b; Wald *et al*, 1982).

A large international Nordic/Baltic population-based multicenter study of 221 children with ALL and WBC ≥200 x 10^9/L at diagnosis was constructed to explore in depth clinical presentation, complications and risk of early morbidity and mortality upon application of different initial treatment strategies. The study is presented in this dissertation.

2.9 Antileukemic treatment

ALL was one of the first cancers to respond to chemotherapy and the first disseminated cancer that became curable (FARBER & DIAMOND, 1948; Masera *et al*, 2013). Contemporary therapy for childhood ALL is risk-adapted, based on the clinical, biological, and genetic factors, and on early treatment response. More aggressive and therefore more toxic treatment may be required for patients who have a lower probability of long-term survival. In contrast, patients who are projected to have a good outcome should receive a less intensive and toxic treatment to protect them from early and late-term toxicities.

Up to twelve different agents are used in the first line therapy given for two to three years. Interestingly, no new agents have been added to initial therapeutic modalities during the last 30 years (Hudson *et al*, 2012; Kersey, 1997). Furthermore, intracranial radiation therapy was reduced or completely omitted by most of the contemporary treatment protocols, and some attempts were even made to reduce intensity of chemotherapy regimens for the lower risk group patients without demonstrating an increase in CNS involving relapses or decrease in survival rates (Sirvent *et al*, 2011; Marwaha *et al*, 2010b; Pui & Thiel, 2009; Sallan *et al*, 2009; Vora *et al*, 2013).

ALL treatment consists of remission induction, consolidation, reinduction and maintenance phases (Figure 5).

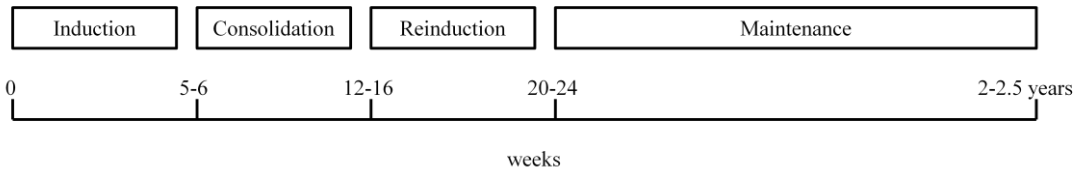

Figure 5. Schematic picture of ALL treatment protocol

Note. Time indicated in the figure is approximate. It depends on ALL risk group and the treatment protocol used.

Remission induction chemotherapy consists of three or four drugs: corticosteroid (prednisolone of dexamethasone), vincristine (VCR), L-asparaginase (delayed until consolidation phase in NOPHO protocols) and anthracycline. *Consolidation* phase is based on antimetabolites - high dose methotrexate (HD MTX) and oral 6-mercaptopurine (6-MP). *Reinduction* therapy resembles induction and is designed to eradicate the already present resistant leukemic sub-clones adding non-cross resistant drugs (cyclophosphamide, cytosine arabinoside and 6-thioguanine (6-TG)). *Maintenance* phase is designed to eradicate the residual dormant leukemic cells and consists of small doses of methotrexate (MTX) and 6-MP depending on the protocol up to two to three years from diagnosis (Tucci & Arico, 2008). Such maintenance therapy is considered to be superior to the other maintenance therapy regimens (Schmiegelow *et al*, 2009b). Preventive therapy for overt CNS leukemia consists of craniospinal irradiation or intensive intrathecal therapy together with HD MTX.

3. Outline of this dissertation

As no long-term population-based studies on childhood ALL in Lithuania were available, collection of such data and exploration of risk factors for survival was necessary. The population-based long-term treatment results of childhood ALL in Lithuania in 1992-2012 were analyzed and the benefits of joining a prospective clinical trial were evaluated in Study I and described in **chapter 6.1.** The role of WBC as a surrogate marker of both the leukemic clone and host characteristics was explored in Study II in the retrospective population-based study of 2666 Nordic patients as an independent predictor of risk of relapse in contemporary ALL treatment protocols when adjusting for other prognostic features and described in **chapter 6.2.** A population-based multicenter study of 221 Nordic/Lithuanian children with ALL and WBC ≥200 x 10^9/L at diagnosis with in-depth analysis of clinical presentation and early complications as well as the potential impact of antileukemic therapy and supportive care on early morbidity and mortality is described as Study III in **chapter 6.3.**

4. Patients and methods

4.1 Patients

Children treated for ALL in 1992–2012 in Lithuania and five Nordic countries (Denmark, Finland, Iceland, Norway or Sweden) were included into the studies presented in this doctoral dissertation. In total, data on 3159 patients were analyzed. Some overlap between the studies exists, i.e. patients >1.0 year old, who presented with WBC ≥200 x 10^9/L at diagnosis (34 Lithuanian and 125 Nordic patients) were included respectively into Study I on childhood ALL in Lithuania or Study II on the prognostic impact of WBC among the Nordic patients, and also into Study III on hyperleukocytosis in children with ALL (Figure 6).

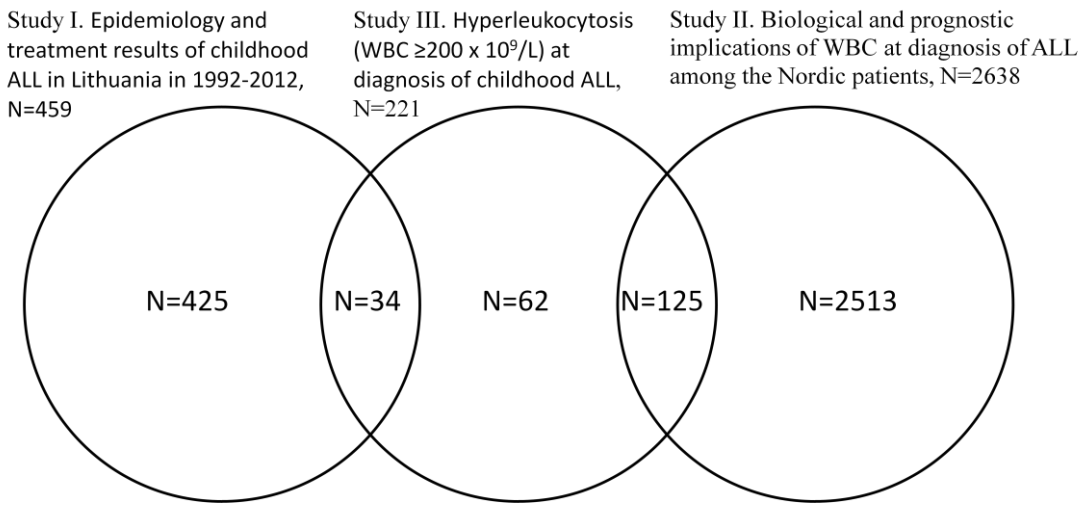

Figure 6. Distribution of patients among the studies

Inclusion criteria for specific studies

Study I. Epidemiology and treatment results of childhood ALL in Lithuania in 1992-2012

All 459 BCP or T-ALL patients treated during the period from January 1992 to December 2012 at the Center for Oncology and Hematology, Children's

Hospital, Affiliate of Vilnius University Hospital Santariskiu Klinikos (COH CH) were included. COH CH was the only center in Lithuania for treatment of children with ALL. The study cohort comprised of patients aged 0-15.9 years until January 2003, and aged 0-17.9 years in the subsequent period reflecting the age limit for patients in pediatric departments in Lithuania. Patients aged 0-15.9 years (N=446) were included into calculations of the incidence of childhood ALL in Lithuania, and all 459 patients including six Down syndrome patients were included into the rest of analyses.

Follow-up monitoring of children with cancer in COH CH was started five years ago, and current status for each of the patient was checked before including them in the current study.

Study II. Biological and prognostic implications of high WBC at diagnosis of ALL among the Nordic patients

BCP or T-ALL patients aged 1.0–14.9 years who were diagnosed and treated in the Nordic countries in 1992-2008 and enrolled in the NOPHO ALL-92 or -2000 protocols were included. Out of the 2666 newly diagnosed ALL patients twenty-eight were excluded from the study because of missing (n=9) or ambiguous (n=19) data on immunophenotype leaving 2638 patients for analysis.

Status of each patient in the NOPHO leukemia register is updated at least annually. Within 10 years from diagnosis of ALL (median: 6.7 years), 18 patients from ALL-92 cohort were lost to follow up in the NOPHO ALL register because of emigration outside the Nordic countries (N=3, median 2.8 years), change of Nordic address (N=4, median 5.2 years), transfer to an adult department (N=5, median 6.8 years), or cessation of clinical control or not-otherwise-specified (N=6, median 7.9 years). Similarly, a total of eight patients from ALL-2000 cohort were lost to follow-up in the NOPHO ALL register at a median of 2.5 years from diagnosis.

Study III. Hyperleukocytosis (WBC $\geq$200 x 10^9/L) at diagnosis of childhood ALL

Children with BCP or T-ALL aged 0-14.9 years and WBC ≥200 x 10^9/L at diagnosis, who were diagnosed and treated in Denmark, Finland, Iceland, Lithuania, Norway or Sweden from January 1992 to October 2011 were included. Out of 3985 newly diagnosed ALL patients, 241 patients (6%) had an initial WBC ≥200 x 10^9/L. Patient files from 224 patients (93%) could be retrieved for review. Two patients started ALL treatment outside the Nordic/Baltic countries, and information on initial treatment was lacking for one additional patient. The remaining 221 patient was included in the analysis.

4.2 Methods

Clinical, laboratory, treatment and outcome data were collected retrospectively or prospectively, and statistical analysis was performed.

4.2.1 Study-specific methods

Study I. Epidemiology and treatment results of childhood ALL in Lithuania in 1992-2012

Data on patient age, gender, WBC, CNS involvement, immunophenotype, karyotype, antileukemic therapy, response to treatment, the first event (induction failure, relapse, death in first remission, or second malignant disease, whichever occurred first) were collected.

Data were retrieved retrospectively from paper forms for patients diagnosed until April 2009, or were retrieved from the prospective NOPHO ALL-2008 Register for subsequently diagnosed patients. To evaluate trends in treatment results, the study period was divided into four time-periods: 1992-1996 (N=132), 1997-2002 (N=136), 2003-2008 (N=109) and 2009-2012 (N=82) based on available diagnostic and therapeutic possibilities. 1992-1996 period was excluded from survival analysis for patients with different immunophenotype, since few patients had their immunophenotype determined in 1992-1996 (BCP, N=1 or T-ALL, N=3).

Study II. Biological and prognostic implications of high WBC at diagnosis of ALL among the Nordic patients

For the Nordic patients, data on patient age, gender, WBC, CNS involvement, immunophenotype, karyotype, antileukemic therapy, response to treatment and the first event were retrieved from the NOPHO-92 and -2000 Leukemia Registries.

Study III. Hyperleukocytosis (WBC ≥200 x 10^9/L) at diagnosis of childhood ALL

To register the clinical symptoms or complications caused by hyperleukocytosis and TLS, and the effect of different initial management strategies on early- and long-term outcome, the Questionnaire was conducted (Appendix II). The structure of the Questionnaire was discussed at several NOPHO ALL-2008 protocol Events group meetings, and approved by the NOPHO Scientific Committee and the Board. Questionnaires were circulated among the 22 centres of pediatric oncology/hematology.

Data collected using the Questionnaire:

i. Initial peripheral blood counts and coagulation parameters, as well as initial and daily peak values of WBC and electrolytes by the introduction of full induction treatment with VCR and doxorubicin (Doxo).
ii. Hyperleukocytosis related clinical symptoms present at admission or developed within three days after admission to hospital, including neurological symptoms, respiratory distress or bleeding disorders. All complications were assessed as grade 2-4 of the NCI Common Terminology Criteria for Adverse Events (CTCAE) (2012). Early morbidity and mortality were defined as adverse events that occurred within the first month after admission.

iii. Initial hydration, urine alkalinisation, use of allopurinol and urate oxidase, packed red blood cell transfusions during the period when WBC was still ≥200 x 10^9/L, CS prephase treatment, timing of intrathecal methotrexate (i.th. MTX) and induction therapy with VCR and Doxo.

To avoid biases as much as possible in interpreting clinical symptoms or complications caused by hyperleukocytosis or TLS, Questionnaires were filled by myself in ten out of 22 centers. Data in the rest of the centers were collected or data collection was closely coordinated by the National Principal Investigators. Some information was missing or was difficult to interpret in this retrospective study, and this was thoroughly described in the results section in chapter 8.3.

4.2.2 Definition of tumor lysis syndrome

TLS was defined and categorized as laboratory TLS or clinical TLS according to the definitions proposed by M. S. Cairo and M. Bishop (Cairo & Bishop, 2004) (Table 1).

Table 1. Criteria used for diagnosis and classification of TLS.

Criteria for laboratory TLS	
Uric acid	≥476 µmol/L or 25% increase from baseline
Potassium	≥6.0 mmol/L or 25% increase from baseline
Phosphates	≥2.1 mmol/L or 25% increase from baseline
Calcium	≤1.75 mmol/L or 25% decrease from baseline
Criteria for clinical TLS	
Creatinine	≥1.5 upper limit of normal (ULN for <1 years 38.0; >1 <12 years 61.6 µmol/L; >12 years 88 µmol/L)
Cardiac arrhythmia/sudden death	
Seizure	

As defined by Cairo and Bishop (Cairo & Bishop, 2004)

Laboratory TLS was classified when two or more abnormal serum metabolite levels were detected simultaneously, and clinical TLS was classified if in addition one clinical criterion had developed.

All studies were approved by the Lithuanian Bioethics Committee, State Medicines Control Agency of Lithuania, Vilnius Regional Biomedical Research Ethics Committee or the National Ethics Committees of Nordic countries in concordance with national laws and regulations (Appendix I).

4.3 Diagnostic procedures used for study patients

Diagnosis of ALL was established if >25% lymphoblasts were present in diagnostic bone marrow based on morphological evaluation in combination with immunophenotyping with panels of monoclonal antibodies directed towards lineage-associated antigens according to well-established criteria (Bene *et al*, 1995). G-band karyotyping and molecular genetics techniques by fluorescence *in situ* hybridization (FISH) and/or reverse transcriptase polymerase chain reaction (PCR) were used to detect cytogenetic rearrangements for treatment stratification. Methods used differed among the countries and developed over time:

- In Lithuania diagnosis of ALL was established by cytomorphological evaluation of bone marrow smears stained by Romanowsky-Giemsa and peroxidase staining methods by the year 2001. Since 2001 routine immunophenotyping and since 2007 routine G-band karyotyping were introduced. Detection of translocations t(9;22)(q34;q11)*[BCR-ABL]* or t(1;19)(q23;p13)*[E2A-PBX1]*, and dic(9;20)(p13;q11), ic21amp or 11q23/*MLL* aberrations by PCR and/or FISH methods as well as establishment of DNA-index by flow cytometry became mandatory since 2009 as the risk stratifying factors in the NOPHO ALL-2008 protocol.

- In the Nordic countries only G-band karyotyping was mandatory in the ALL-92 protocol. The ALL-2000 required also directed analysis by FISH and/or PCR for translocations t(9;22)(q34;q11)[*BCR–ABL*] and t(1;19)(q23;p13)[*E2A–PBX1*], and for 11q23/*MLL* aberrations. The ALL-2008 protocol required in addition directed analysis for dic(9;20)(p13;q11) and ic21amp aberrations to exclude these patients from the standard risk group. Furthermore, many leukemic samples have been examined by comparative genomic hybridization, spectral karyotyping, and nearly all samples have been explored for translocation t(12;21)*[ETV6-RUNX1]*, although the presence of this translocation did not influence treatment stratification.

Extramedullary disease

CNS disease at diagnosis was defined as an increased number of mononuclear cells ($\geq 5 \times 10^6$/L) with leukemic blasts in the diagnostic spinal tap and/or peripheral nerve palsy or confirmation of CNS disease on imaging. In NOPHO ALL-2008 protocol the categories of CNS 1/2/3 defined as: i) no blasts on cytospin or ii) ≥1 and <5 cells/µl with blasts or iii) ≥5 cells/µl with blasts, respectively, and traumatic tap defined by ≥10 red blood cells/µl were introduced.

Testicular leukemia was defined as swelling of testicles with leukemic masses confirmed by ultrasonography and/or biopsy.

4.4 Antileukemic treatment used for study patients

Both in Lithuania and in the Nordic countries children were treated according to the protocols based on the strategy that was developed by BFM-group in 1960-1970's (Schrappe *et al*, 2010). Consequently, chemotherapeutic drugs and treatment phases were basically the same in all protocols with some differences in drugs combinations or time-schedules. The principles of such treatment were described in chapter 2.10.

An outline of chemotherapy used in Lithuania and the Nordic countries in different time-periods is depicted in Figure 7.

In Lithuania BFM-90, BFM-95 or BFM-2000 protocols were used depending on time-period by the year 2009. The protocols are described in detail by the BFM-group (Reiter *et al*, 1994; Moricke *et al*, 2008; Schrappe *et al*, 2000b; Moricke *et al*, 2010; Schrappe *et al*, 2013). In Lithuania the protocols were adapted according to diagnostic and treatment possibilities available in different time periods, and the details are described in the text below. In the Nordic countries children were treated according to NOPHO ALL-92 or -2000 protocols depending on time-period (Gustafsson *et al*, 2000a; Schmiegelow *et al*, 2010a; Toft *et al*, 2013; Vaitkeviciene *et al*, 2011) by the year 2008.

Since 2008 in the Nordic countries and since 2009 in Lithuania patients were treated according to the NOPHO ALL-2008 protocol (Schmiegelow *et al*, 2010a; Toft *et al*, 2013).

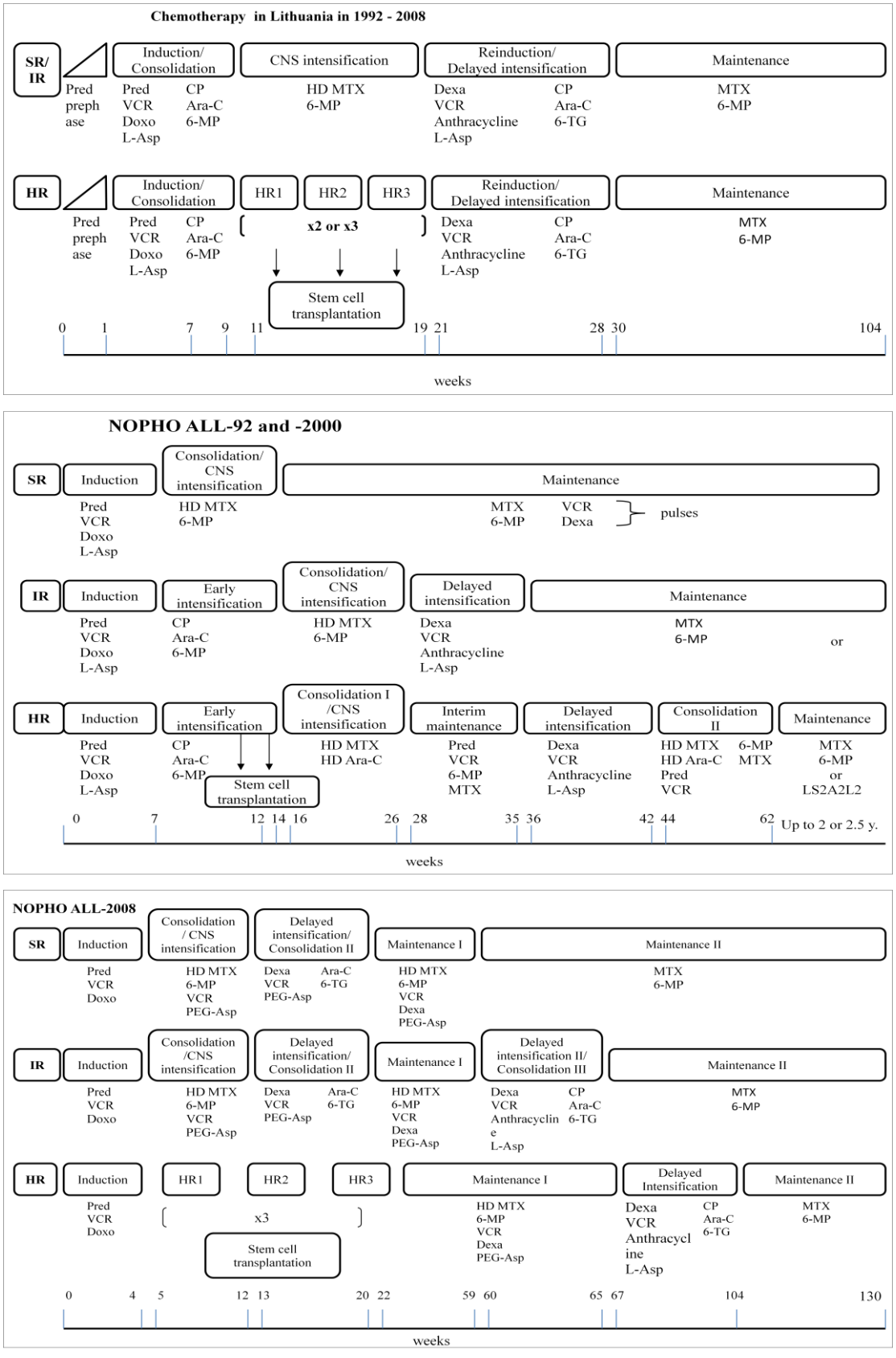

Figure 7. Outline of treatment used for children with ALL in Lithuania and the Nordic countries.

Note. Treatment in more detail is described in the text below.

4.4.1 Antileukemic treatment used in Lithuania

Risk grouping strategy in Lithuania in 1992-2008

Prednisolone good response or prednisolone poor response was documented and established if <1000/μL or ≥1000/μL blasts, respectively, were found in peripheral blood after seven days of a prephase with prednisolone and i.th. MTX, and bone marrow response was evaluated at the end of induction therapy on day 33.

In 1992-1996, risk group assignment was based on presence of hepatosplenomegaly, CNS involvement, response to prednisolone prephase and response to induction therapy (Standard risk, SR: no hepatosplenomegaly, no CNS involvement, and no high risk criteria; Intermediate risk, IR: hepatosplenomegaly and/or CNS involvement, and no high risk criteria; High risk, HR: prednisolone poor respnse and/or >25% blasts in bone marrow at the end of induction on day 33).

In 1997-2002, the same criteria were used, except in addition: i) SR patients had to be 2.0 to 11.9 years of age and have initial WBC <20 x 10^9/L; ii) patients aged <2.0 or ≥12.0 years and/or initial WBC >20 x 10^9/L were treated as IR. T-ALL were excluded from SR group.

In 2003-2008, MRD measured by flow cytometry was implemented for risk stratification. Patients defined as SR if MRD_{d33} negative; IR if MRD_{d33} positive, but MRD_{d79} <10^{-3}; and HR if MRD_{d79} ≥10^{-3}. Other risk criteria remained the same as in previous periods.

Chemotherapy in Lithuania in 1992-1996

SR and IR groups. Induction/Consolidation: seven days of prephase with increasing dose of prednisolone and intrathecal (i.th.) MTX was mandatory, followed by oral prednisolone 60/mg/m^2/d, for 21 days, then tapered; weekly

VCR 1.5 mg/m^2 (max. 2.0 mg) concomitantly with daunorubicine 30 mg/m^2/d i/v infusion, four doses, (8-29 d.); L-asparaginase (Medac) 10 000 UI/m^2 i/v infusion, 8 doses, every 2-3 days (12 – 33 d). Cyclophosphamide 1000 mg/m^2 i/v infusion on d. 36 and 64; four blocks of four days of cytarabine 75 mg/m^2/d, subcutaneously (d. 38-62), and oral 6-MP 60 mg/m^2/d, given in the evening (d. 36-63); i.th. MTX on d. 1, 15, 29, 45, 59. Extra i.th. MTX on d. 8 and 22 for patients with CNS involvement. **Extra-compartment therapy (M protocol)** for SR and IR: MTX 1.0 g/m^2/24 h infusion with concomitant i.th. MTX on d. 8, 22, 36, 50; oral 6-MP 25 mg/m^2/d. (d. 1-57). **Reintensification (Protocol II):** the same as Indtuction/Consolidation except that: (i) adriamicin was given instead of daunorubicine; (ii) L-asparaginase only 3 doses given; (iii) cyclophosphamide was given once, on d. 36; (iv) cytarabine two blocks instead of four; (v) oral 6-thioguanine (6-TG) 60 mg/m^2/d instead of 6-MP on d. 36-49 and (vi) i.th. MTX on d. 38 and 45 only. **Maintenance** with oral 6-MP 50 mg/m^2/d and oral MTX 20 mg/m^2/dose, once per week with doses adjusted according to peripheral blood counts up to two years after diagnosis. IR patients older than 1.0 year additionally received **cranial irradiation** 12 Gy before maintenance.

HR patients started block therapy after the 33 d. of Induction. **Block HR-1:** oral dexamethasone 20 mg/m^2/d (d. 1-5); oral 6-MP 100 mg/m^2/d (d. 1-5); VCR 1.5 mg/m^2/d (max 2.0 mg) (d. 1, 6); MTX 1.0 g/m^2/24 h infusion (d. 1); cytarabine 2.0 g/m^2/dose, x 2 (d. 5); L-asparaginase 25 000 IU/m^2 (d. 6); i.th. TIT (d. 1). **Block HR-2:** oral dexamethasone 20 mg/m^2/d (d. 1-5); oral 6-TG 100 mg/m^2/d (d. 1-5); vindesine 3.0 mg/m^2/d (max 5.0 mg) (d. 1); MTX 1.0 g/m^2/24 h infusion (d. 1); daunorubicine 50 mg/m^2/d (d. 5); ifosfamide 400 mg/m^2/d i/v infusion (d. 1-5); L-asparaginase 25 000 IU/m^2 (d. 6); i.th. TIT (d. 1). **Block HR-3:** oral dexamethasone 20 mg/m^2/d (d. 1-5); cytarabine 2.0 g/m^2/dose, x 2 (d. 1, 2); VP-16 150 mg/m^2/dose (d. 1, 3, 5); L-asparaginase 25 000 IU/m^2 (d. 6); TIT (d. 1). Blocks were consequently repeated three times making nine HR blocks altogether. **Maintenance** with oral 6-MP 50 mg/m^2/d and oral MTX 20 mg/m^2/dose, once per week with doses adjusted according to peripheral blood

counts up to two years after diagnosis. **Cranial irradiation**: for ≥1.0 year patients 12 Gy after the 3rd HR-3 block.

For all risk groups patients with initial CNS involvement cranial irradiation was given dependent on age: <1.0 y. 0 Gy; 1-<2.0 y. 18 Gy, and ≥2.0 y. 24 Gy.

Chemotherapy in Lithuania in 1997-2002

Treatment was the same as in previous protocol except that: (i) L-asparaginase dose was redused to 5 000 UI/m^2; (ii) HR blocks were reduced from nine to six blocks; (iii) cranial irradiation for patients with initial CNS involvement was reduced to: <2.0 y. 12 Gy, and ≥2.0 y. 18 Gy.

Chemotherapy in Lithuania in 2003-2008

The treatment was the same as in 1997-2002, except that: (i) the dose of high dose MTX was increased from 1.0 g/m^2/24 h to 5.0 g/m^2/24 h; (ii) adriamycine was replaced by daunorubicine in Reintensification (Protocol II); (iii) prophylactic cranial irradiation was restricted only for T-ALL.

NOPHO ALL-2008 protocol was used in Lithuania since April 2009 and is described in chapter 4.3.2.

Hematopoietic stem cell transplantation in Lithuania

In Lithuania allogeneic stem cell transplantation for children became available in 2002. No strict criteria for hematopoietic stem cell transplantation (HSCT) existed before the NOPHO-era. In the NOPHO ALL-2008 protocol HSCT is indicated based on treatment response criteria only (described in chapter 4.3.2.). Overall, twenty patients received allogeneic HSCT during the study period in Lithuania in CR1 (N=8) or ≥CR2 (N=12). Only one patient is

alive from the latter group while out of those eight patients who were transplanted in CR1, six patients are alive without the disease with a median (range) follow-up of 2.0 (0.2-9.0) years.

4.4.2 Antileukemic treatment used in the Nordic countries

In the Nordic countries patients were treated according to the NOPHO ALL-92, -2000 or -2008 protocols depending on the time-period.

Risk grouping in the NOPHO ALL-92 and -2000 protocols

In ALL-92, the risk grouping was based on age and WBC at diagnosis (SR: age 2.0-9.9 y. and WBC <10 x 10^9/L; IR: age 1.0-1.9 or ≥10 y. and/or WBC 10.0-49.9 x 10^9/L; higher risk: WBC ≥50.0 x 10^9/L and the presence of higher risk features such as T-ALL, the presence of CNS or testicular involvement, translocations t(9;22)(q34;q11) or t(4;11)(q21;q23), lymphomatous leukemia or mediastinal lymphoma and/or a poor treatment response (≥25% blasts in bone marrow at day 15 or >5% blasts at day 29). The risk grouping was unchanged in ALL-2000 except that (i) all children aged 1.0-9.9 were eligible to the SR-arm, if their WBC was <10.0 x 10^9/L, and they had no HR group features, (ii) t(1;19)(q23;p13), hypodiploidy (<45 chromosomes), and all 11q23/*MLL* rearrangements were allocated to higher-risk groups; and (iii) it was optional to offer HSCT in first remission to patients with MRD levels ≥10^{-3} after 3 months of antileukemic therapy. Cranial radiotherapy was restricted to the VHR patients <5 years age.

Risk grouping in the NOPHO ALL-2008 protocol

Therapeutic risk group assignment was based on WBC < or ≥100 x 10^9/L, immunophenotype BCP vs T-ALL, cytogenetics and MRD at days 15, 29 and

79 (SR: WBC <100 x 10^9/L, BCP phenotype, day 29 MRD <10^{-3}, and no IR or HR cytogenetics; IR: i) BCP with WBC <100 x 10^9/L and MRD_{d29} ≥10^{-3}, but <5% or ii) BCP with WBC ≥100 x 10^9/L or T-cell ALL and <25% blasts in bone marrow at day 15 and MRD_{d29} <10^{-3}, and no HR cytogenetics; HR: i) any patient with day 29 ≥5% blasts in bone marrow or ii) BCP with WBC ≥100 x 10^9/L or T-ALL and day 15 ≥25% blasts in bone marrow and/or MRD_{d29} ≥10^{-3} or iii) presence of 11q23/*MLL* rearrangement or hypodiploidy (≤44 chromosomes or DNA index <0.85), irrespectively of other factors. Patients having t(1;19), dic (9;20) or ic21amp aberrations were assigned to IR treatment unless HR features are present.

NOPHO ALL-92 and ALL-2000 treatment strategy (Vaitkeviciene *et al*, 2011;Schmiegelow *et al*, 2010a;Gustafsson *et al*, 2000a)

Induction therapy*:* In ALL-92 all patients received prednisolone (60 mg/m^2/day on days 1-36, then tapered), weekly VCR (2.0 mg/m^2 six times, maximum 2.0 mg), doxorubicin (40 mg/m^2 three times (SR and IR) or 4 times (HR)), Erwinia asparaginase (30.000 IU/m^2 daily on days 37-46), and i.t. MTX on four occasions. ALL-2000 induction therapy was identical to that of the ALL-92 protocol except that: i) one dose less of doxorubicin was given, ii) the maximum dose of VCR was set to 2.5 mg, and iii) Erwinase was substituted with E-coli asparaginase (6.500 IU/m^2 at three days intervals, times 4).

Early intensification consisting of cyclophosphamide (1000 mg/m^2 times 2, four weeks apart), i.t. MTX, oral 6-MP and low-dose cytarabine (75 mg/m^2/day for four days, times 4), was given to IR and HR patients immediately after the induction phase.

Consolidation therapy in ALL-92 included HD-MTX at 5 g/m^2/24 hours for SR and IR with i.t. MTX and leucovorin rescue, whereas patients with higher risk-ALL received HD-MTX 8 g/m^2/24 hours alternating with high-dose cytarabine (12 g/m^2) with 2-month intervening periods of oral MTX and 6-MP

with two VCR/prednisolone reinductions per period (Skarby *et al*, 2006). In ALL-2000, SR and IR patients received three HD-MTX courses alternating with low-dose cytarabine blocks (75 mg/m^2/day for four days, times 2) with concomitant 6-MP, whereas HD-MTX consolidation therapy for higher risk patients was identical to that of the ALL-92 protocol. ***Delayed intensification*** in both ALL-92 and ALL-2000 was given to IR and higher risk patients and consisted of oral dexamethasone, weekly VCR four times, weekly anthracycline 3 or 4 times and 4 doses of asparaginase given twice weekly (Erwinia asparaginase in ALL-92, E-coli asparaginase in ALL-2000), followed by cyclophosphamide at 1000 mg/m^2, low-dose cytarabine and 6-thioguanine (Gustafsson *et al*, 2000b;Schmiegelow *et al*, 2010b).

Classical oral 6-MP/MTX ***maintenance therapy*** continued until 2 years (for IR and HR in ALL-92 and for HR and very HR (VHR) in ALL-2000) or 2.5 years (for SR in ALL-92 and for SR and IR in ALL-2000) after diagnosis. During the first year of maintenance therapy SR or IR-ALL received in addition alternate pulses at four weeks intervals of VCR and corticosteroids and HD-MTX at 5 g/m^2/24 hours until five courses of HD-MTX had been given. HR and VHR received reinductions of VCR and corticosteroids. In ALL-92 patients with VHR ALL (and all Finish patients with HR ALL) had oral 6-MP/MTX maintenance, substituted with cyclic LSA_2L_2 maintenance therapy, while in ALL-2000 LSA_2L_2 was given two (HR) or three times (VHR) prior to the start of oral MTX/6MP maintenance therapy, or until SCT could be performed (VHR-ALL) (Gustafsson *et al*, 2000b;Schmiegelow *et al*, 2010b)

A subset of patients with higher risk ALL was offered ***cranial irradiation*** in the ALL-92 (N=158) and ALL-2000 (N=128) protocols. These included VHR ALL patients, who were 5 years of age and older.

Hematopoietic stem cell transplantation in the Nordic countries

There were no uniform Nordic criteria for HSCT in ALL-92. In ALL-2000, HSCT was indicated in case of WBC ≥200 x 10^9/L at diagnosis, hypodiploidy (<34 chromosomes) and 11q23/*MLL* rearrangement and optional if MRD ≥10^{-3} after 3 months of therapy. In ALL-2008 HSCT is indicated if: (i) BCP patients with WBC <100 x 10^9/L no remission detected on day 29 (≥5% blasts in BM) or MRD_{d79} ≥10^{-3}; (ii) T-ALL and BCP patients with WBC ≥100 have ≥25% blasts on day 15 and ≥5% after block A1, or ≥5% on day 29, or after block B1 MRD ≥10^{-3}.

Out of 2638 Nordic patients included into Study II on Biological and prognostic implications of high WBC at diagnosis of ALL, 119 patients received HSCT in first remission in ALL-92 protocol (N=57) or ALL-2000 protocol (N=62).

5. Statistical analysis

Since most variables were not normally distributed non-parametric methods were applied to compare the distribution of parameters between subgroups, and the median and 75% ranges were presented. Chi-square test was used for comparison of categorized variables, and Mann-Whitney *U*-test or Kruskal-Wallis test was used for continuous variables. To describe correlations between parameters, Spearman's rank correlation coefficient was calculated. Kaplan-Meyer method was applied for the estimation of remission duration and for generation of survival curves. The projected duration of event-free survival (pEFS) was calculated from diagnosis to the date of assessment of induction failure (IF), resistant disease, death in first complete remission (DCR1), relapse or development of a second malignancy (whichever occurred first) or the last known follow-up date for event free survivors. Projected overall survival (pOS) was calculated from diagnosis to death from any cause. To estimate the cumulative incidences of relapse, IF or DCR1, all these events were considered as competing events. Backward stepwise Cox proportional hazards regression analysis was performed to identify independent prognostic factors for differences in outcome. Two-sided p-values <0.05 were regarded as significant. In case of multiple comparisons Bonferroni correction was applied to adjust for random effect. Data were analyzed using the statistical package for the social sciences (SPSS) software for Windows, version 15.0 or 18.0.

Statistical analysis was performed by myself, and was revised by senior statisticians in Vilnius or Copenhagen.

6. Results

6.1 Study I. Childhood ALL in Lithuania in 1992-2012

6.1.1 Epidemiology

During the last two decades, annual figures of newly diagnosed ALL among children aged 0-15.9 years varied from 10 to 31 cases per year with a dominance of boys (55.6%) vs girls (44.4%) in Lithuania. It made the incidence of 3.2–3.6 new cases of ALL per 100 000 children per year, and the male : female ratio of 1.3:1.0 (Figure 8).

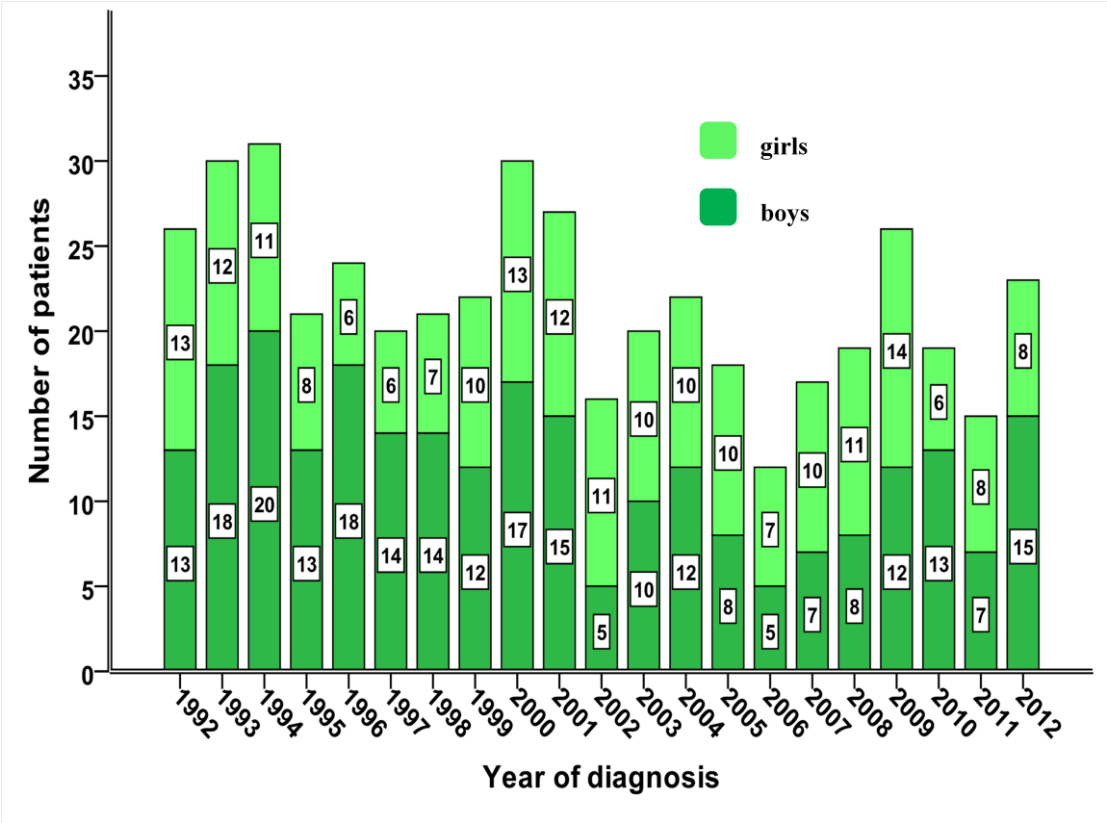

Figure 8. Distribution of annual cases of ALL for boys and girls in Lihuania during the study period.

Comparison of two decades (1992-2001 vs 2002-2011) shows a decline in incidence of childhood ALL with the median (75% range) incidence being 24 (18–31) and 18 (12–25) cases per year, respectively (p=0.005). However, there was a decrease in the population of Lithuania during the study period from 3.7

million in 1992 down to 3.0 million in 2012 (Statistics Lithuania, 2013) (Figure 9).

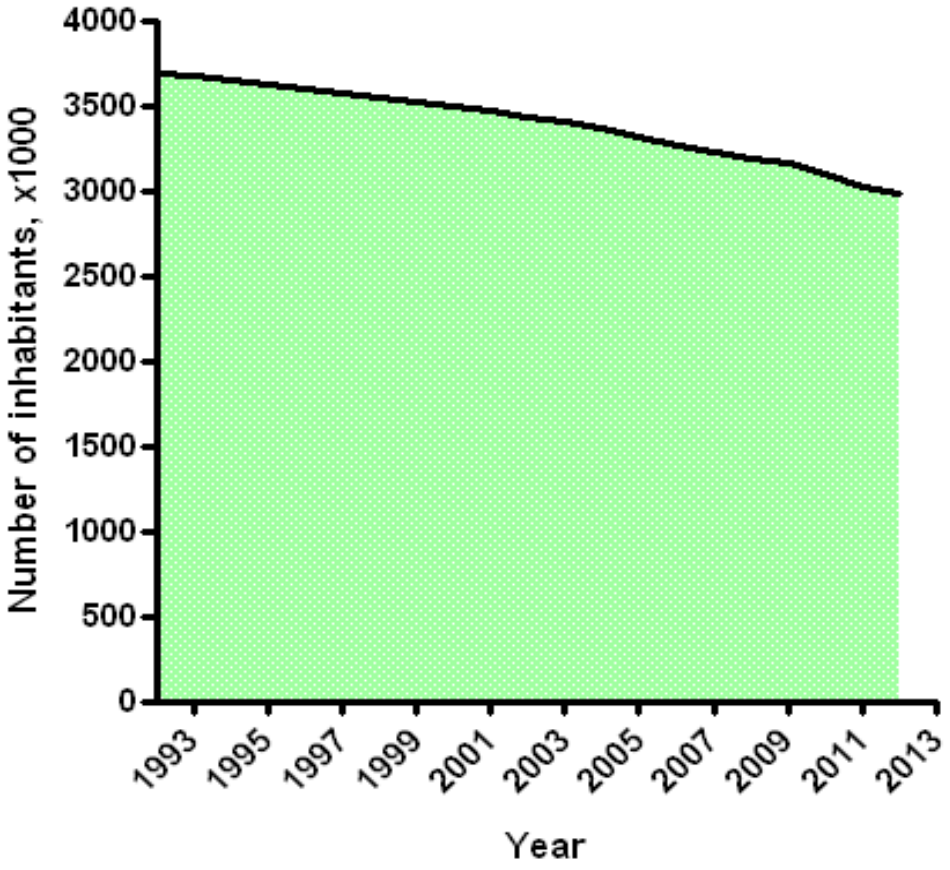

Figure 9. Lithuanian population during the 1990-2012 period. Source: (Statistics Lithuania, 2013).

6.1.2 Biological characteristics

Baseline characteristics of all 459 study patients are presented in Table 2. Age of the patients (median (75% range): 5.3 (2.3-12.7) years) or initial WBC (median (75% range): 9.2 (2.6-46.4) x 10^9/L for the 211 BCP patients and 90.7 (12.5-382.5) x 10^9/L for the 55 T-ALL patients) were in consistence with the findings of other childhood ALL study groups (Vaitkeviciene *et al*, 2011; Moricke *et al*, 2008) (Table 2).

Table 2. Baseline characteristics of patients treated in Lithuania at different time periods, N=459

	1992-1996, N=132	**1997-2002, N=136**	**2003-2008, N=109**	**2009-2012, N=82**	**p**
Median (75% range) FU, years for alive patients	19.0 (16.6-20.4), N=71	12.7 (11.3-15.2), N=89	7.5 (4.7-9.5), N=84	2.2 (0.5-3.6), N=70	
Boys, N (%)	82 (62%)	77 (57%)	50 (46%)	47 (57%)	0.09
Girls, N (%)	5% (38%)	59 (43%)	59 (54%)	35 (43%)	
WBC, $x10^9$/L, median (75% range)	9.9 (2.3-56.0)	9.0 (2.8-43.8)	9.2 (2.3-58.4)	18.2 (2.9-101.3)	0.025
Age, median (75% range)	4.9 (2.1-10.3)	5.3 (2.2-10.9)	6.3 (2.5-13.1)	5.2 (2.3-11.5)	0.14
CNS, N (%)					
CNS1	95 (72)	125 (92)	100 (92)	78 (95)	
CNS 2/3	7 (5)	8 (6)	8 (7)	4 (5)	0.90
NA	30 (23)	3 (2)	2 (2)	0	
Immunophenotype, N(%)					
BCP	1 (1)	54 (40)	89 (82)	67 (82)	
T-ALL	4 (3)	17 (13)	20 (18)	15 (18)	
NA	127 (96)	65 (48)	0	0	
Cytogenetics, N (%)					
Normal karyotype	-	2 (1.5)	11 (10.1)	20 (2.4)	
HeH	-	-	19 (17.4)	11 (13.4)	
t(12;21)	-	-	2 (1.8)	19 (23.2)	
t(1;19)	-	-	-	6 (7.3)	
iamp(21)	-	-	-	3 (7.3)	
11q23/*MLL*$^-$	-	-	-	1 (1.2)	
t(9;22)	-	-	3 (2.8)	2 (2.4)	
Hypodiploid	-	-	-	1 (1.2)	
Other	-	-	11 (10.1)	20 (24.4)	
NA	132 (100)	134 (98.5)	63 (57.8)	0	

Note. p value = determined after pooled comparison of the values among different time-periods; FU = follow-up; NA = not available; HeH = high hyperdiploid karyotype (modal chromosome number >50); hypodiploid karyotype = modal chromosome number <45; 11q23/*MLL* = 11q23/*MLL* rearrangement; iamp21 = intrachromosomal *AML1* gene amplification; other = non-stratifying cytogenetic aberrations.

Higher proportion of T-ALL patients (18%) in the recent two periods may reflect patients' age up to 18 years. Incidence of T-ALL increase with age was also shown in the large Nordic/Baltic study which included ALL patients aged 1-45 years. It revealed frequency of T-ALL being 11%, 27% and 34% among patients aged 1.0-9.9, 10.0-17.9 and 18.0-45 years respectively (Toft *et al*, 2013).

Routine cytogenetic analyses for all ALL patients were available in the recent period only. Pattern of cytogenetic aberrations showed almost forty percent of patients to have high hyperdiploid karyotype or translocation t(12;21), and about five percent of patients to have unfavorable aberrations such as translocation t(9;22)*[BCR-ABL]*, 11q23/*MLL* gene aberration or hypodiploid karyotype (Table 2). Such distribution is similar to the findings published by other groups (Sutcliffe *et al*, 2005; Arico *et al*, 2000; Heerema *et al*, 2000; Harrison *et al*, 2004b).

6.1.3 Survival rates and risk factors

Progressive improvement in pEFS and pOS was observed over time (Table 3, Figures 10 and 11).

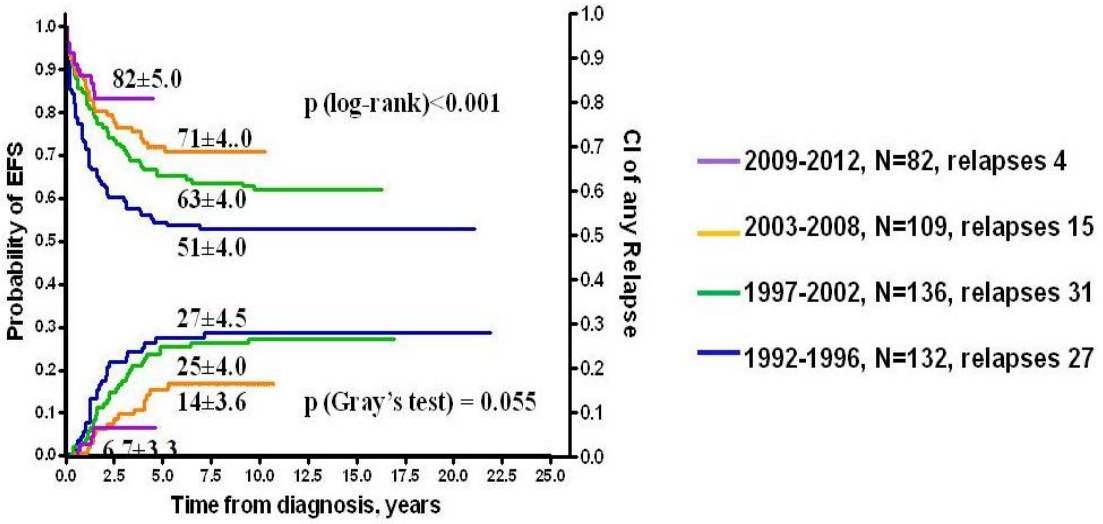

Figure 10. Probability of event-free survival and cumulative incidence of any relapse in four time-periods, N=459.

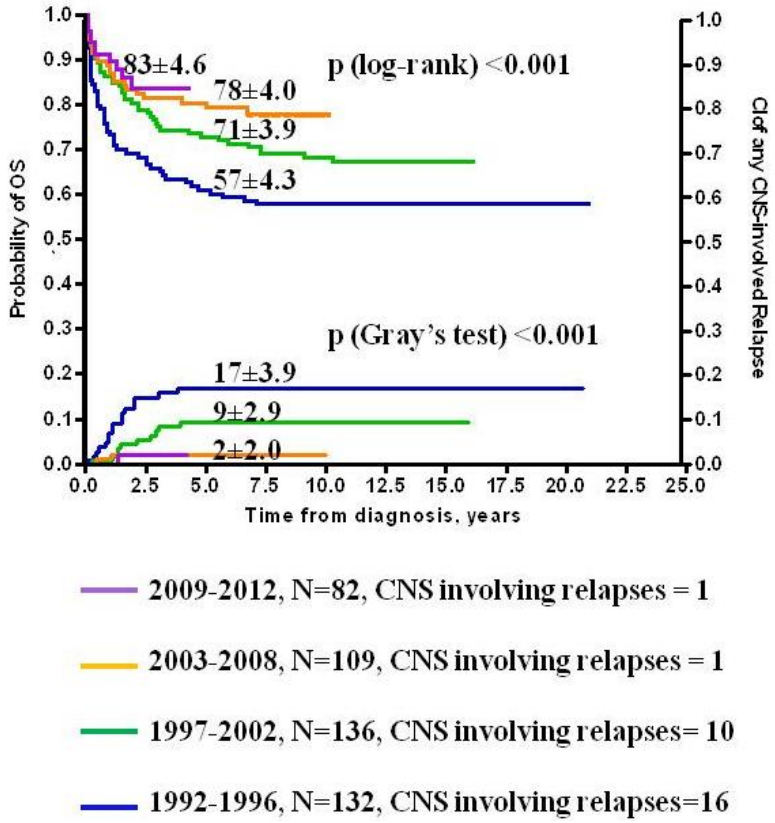

Figure 11. Probability of overall survival and cumulative incidence of isolated or any CNS-involving relapse in four time-periods, N=459.

The 5-year pEFS improved from 50±4% in 1992-1996 to 71±4% in 2003-2008 (pooled p <0.001), and the 5-year pOS improved from 57±4% to 78±4% for these periods, respectively (pooled p<0.001). There was a trend for further improvement in survival in 2009-2012, however it did not reach significant difference, and follow-up time was short (Table 3, Figures 10 and 11).

Table 3. Treatment results of patients treated at different time periods, N=459

	1992-1996, N=132	1997-2002, N=136	2003-2008, N=109	2009-2012, N=82	p
Primary events, N (%)					
IF	18 (13)	8 (6)	6 (6)	2 (2)	0.01
DCR1	20 (15)	14 (10)	11 (10)	7 (9)	0.41
Rel	28 (21)	30 (22)	15 (14)	4 (5)	0.003
CR1	65 (49)	82 (60)	76 (70)	69 (84)	<0.001
SMN	1 (0.8)	2 (1.5)	1 (0.9)	0	
Relapses, N (%)					
BM	9 (6.8)	18 (13.2)	13 (11.9)	3 (3.7)	
CNS	13 (9.8)	7 (5.1)	0	1 (1.2)	
BM+CNS	3 (2.3)	3 (2.2)	1 (0.9)	0	
Testis	1 (0.8)	2 (1.5)	1 (0.9)	0	
Other	0	1 (0.7)	0	0	
Event-free survival ± s.e.					
3-year	0.55 ± 0.04	0.70 ± 0.04	0.76 ± 0.04	0.82 ± 0.05	
5-year	0.50 ± 0.04	0.63 ± 0.04	0.71 ± 0.04	-	<0.001
10-year	0.50 ± 0.04	0.61 ± 0.04	0.70 ± 0.04	-	
Overall survival ± s.e.					
3-year	0.61 ± 0.04	0.73 ± 0.04	0.80 ± 0.04	0.83 ± 0.05	
5-year	0.57 ± 0.04	0.71 ± 0.04	0.78 ± 0.04	-	<0.001
10-year	0.54 ± 0.04	0.66 ± 0.04	0.76 ± 0.04	-	
WBC, x 10^9/L					
<10, 3-year pEFS±s.e.	0.57 ± 0.07	0.79 ± 0.05	0.86 ± 0.05	0.89 ± 0.06	
<10, 5-year pEFS±s.e.	0.52 ± 0.07	0.70 ± 0.05	0.82 ± 0.05	-	0.002
<10, 10-year pEFS±s.e.	0.50 ± 0.07	0.68 ± 0.06	0.80 ± 0.05	-	
10-99.9, 3-year pEFS±s.e.	0.58 ± 0.08	0.67 ± 0.07	0.76 ± 0.07	0.84 ± 0.06	
10-99.9, 5-year pEFS±s.e.	0.56 ± 0.08	0.62 ± 0.07	0.70 ± 0.08	-	0.18
10-99.9, 10-year pEFS±s.e.	0.56 ± 0.08	0.60 ± 0.07	0.70 ± 0.08	-	
≥100, 3-year pEFS±s.e.	0.36 ± 0.13	0.27 ± 0.13	0.40 ± 0.13	0.67 ± 0.14	
≥100, 5-year pEFS±s.e.	0.36 ± 0.13	0.27 ± 0.13	0.33 ± 0.12	-	0.19
≥100, 10-year pEFS±s.e.	0.36 ± 0.13	0.27 ± 0.13	0.33 ± 0.12	-	

Note. p value = determined after pooled comparison of the values among different time-periods; NA = not available; IF = induction failure; DCR1 = death in first complete remission; Rel = relapse; SMN = second malignant disesase.

Advance was more prominent with a borderline significance for BCP patients with 5-year pEFS increasing from 69±6% in 1997-2002 to 77±4.5% in 2003-2008 (p = 0.065), with a trend for further improvement in 2009-2012 (pooled p = 0.043) (Figure 12).

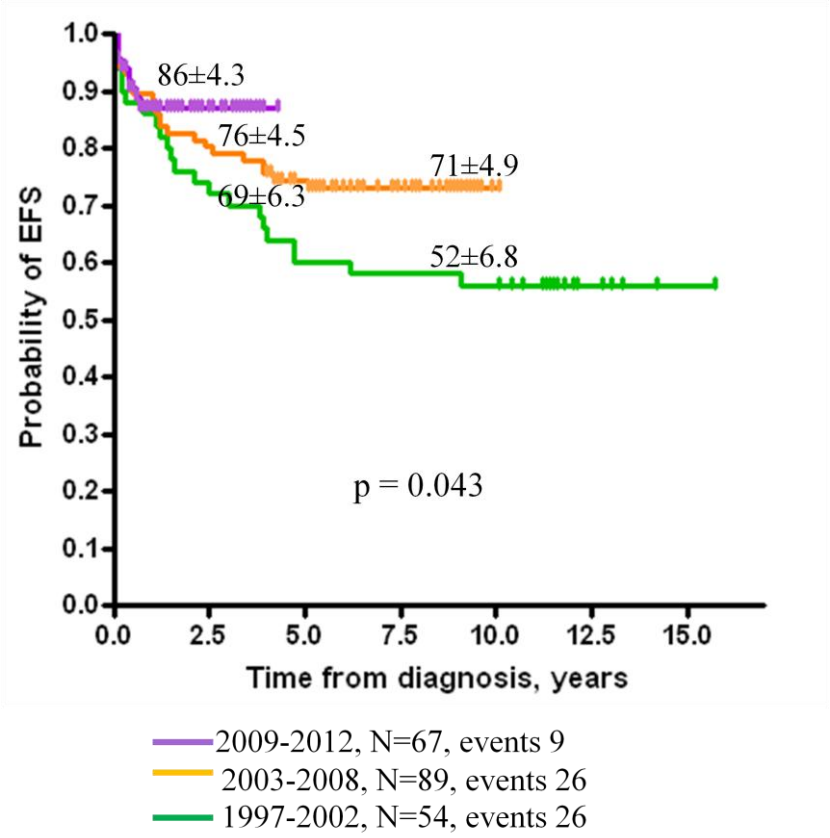

Figure 12. Probability of event-free survival for B-cell precursor ALL patients in different time-periods, N=210

In contrast, for T-ALL survival improvement rates were less prominent (Figure 13).

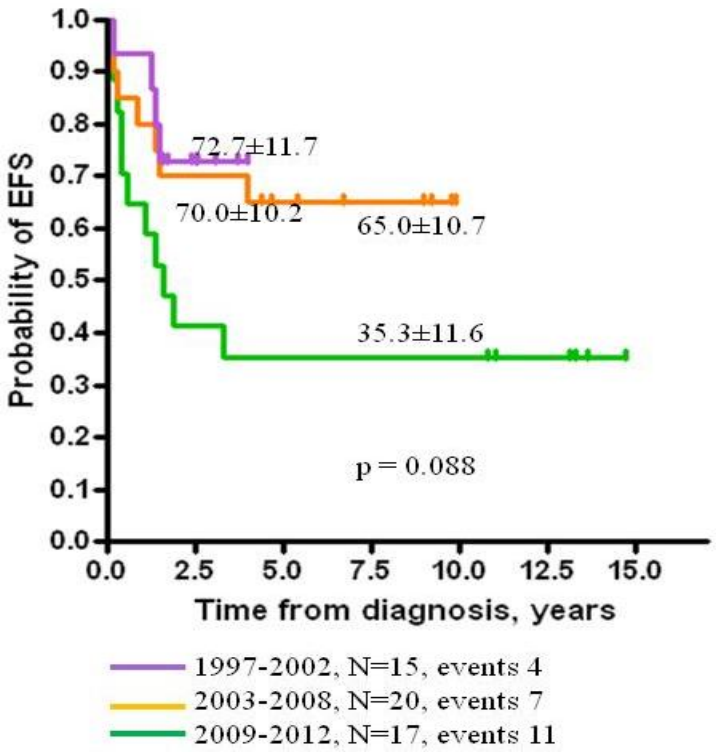

Figure 13. Probability of event free survival for T-lineage ALL patients during different time-periods, N=52

However, number of T-ALL patients was small. Out of four events for T-ALL patients in the recent period, two deaths appeared for HR patients due to septic complications during the block therapy induced myelosuppression (N=1) or cytomegalovirus pneumonia during maintenance phase (N=1). The latter patient could possibly be overtreated since MRD for risk-group assignment was measured by flow cytometry method instead of PCR which was recommended by the NOPHO protocol. The rest two events were relapses which are described below.

Five-year cumulative incidence of relapses reduced from 27 ± 4.5% in 1992-1996 to 14 ± 3.6% in 2003-2008 (p=0.042) (Figure 10). In the recent 2009-2012 period four patients out of 82 developed relapses so far (5%) after 0.6 – 1.4 years from diagnosis. Two out of these four patients developed a relapse due to possibly insufficient treatment. One of them (IR, BCP) developed an isolated bone marrow relapse after parents abandoned the treatment. Another (IR, T-ALL) developed an isolated CNS relapse, however,

he could be mistakenly treated according to IR instead of HR treatment arm (flow cytometry method was used for MRD measuring instead of PCR which was recommended by the NOPHO protocol). The remaining two isolated bone marrow relapses occurred for HR ALL patients (T-ALL with very high WBC and BCP with *MLL*$^{-}$ gene rearrangement).

Incidence of CNS disease at diagnosis remained stable (Table 2), while the 5-year cumulative incidence of CNS involving relapses decreased from 17±3.9% in 1992-1996 to 9±2.9% in 1997-2002 (p=0.077) and after HD MTX of 5 g/m^2 was introduced into practice in 2003-2008, it decreased further down to 1±1% (p <0.015) (Figure 11). Importantly, Cum Inc of CNS involving relapses did not increase in 2009-2012 period (3-year cumulative incidence 2%) after cranial irradiation was omitted for all patients (Table 2, Figure 11). However, follow-up time is short for the recent period.

In contrast, cumulative incidence of IF and DCR1 remained high, albeit decreasing, all over the study period (Table 3). IF was considered as death during induction phase (N=30) or later if remission was not achieved (N=1) or remission status in bone marrow was not evaluated (N=3). Death was induced by infectious complications (N=22), profuse bleeding (N=3), hyperleukocytosis caused complications (N=3), ALL progression (N=3), or the exact cause was difficult to establish in this retrospective study (N=3). Steady improvement in diagnostic work-up which in turn led to better risk classification, and intensification of initial treatment combined with improved supportive care contributed to reduction in five-year cumulative incidence of IF from 14 ± 3.0% in 1992-1996 to to 2.5 ± 1.7% in 2008-2012 (p=0.008).

Cumulative incidence of DCR1 has not changed significantly over time (pooled p = 0.237). Almost 80% of 38 patients who died in DCR1 and for whom information on death cause was available, died of infectious complications during chemotherapy induced myelosuppression (N=30). Other patients died because of bleeding (N=4), HD MTX induced gastroenteritis

(N=2), hemorrhagic pancreatitis (N=1), or cerebral venous sinus thrombosis (N=1). The exact cause of death for 11 patients was difficult to assess in this retrospective study.

Univariate Cox regression analysis revealed initial WBC, CNS involvement in presentation and two earliest time-periods to have a significant prognostic impact for event (Table 4).

Table 4. Results of univariate and multivariate logistic regression analyses to evaluate the risk of different factors for the development of any event

	HR (95% CI), *p* value			
Analysis	Univariate	*p*	Multivariate	*p*
WBC	1.003 (1.002-1.004)	**<0.001**	1.003 (1.002-1.004)	**<0.001**
Age	1.010 (0.97-1.04)	0.71		
Gender	0.88 (0.65-1.20)	0.41		
CNS involvement	2.4 (1.4-4.0)	**0.001**	1.68 (0.96-2.95)	0.07
Time-period				
1992-1996	2.80 (1.54-5.08)	**0.001**	2.76 (1.46-5.19)	**0.002**
1997-2002	1.89 (1.03-3.47)	**0.041**	2.22 (1.19-4.11)	**0.012**
2003-2008	1.42 (0.74-2.70)	0.29	1.53 (0.79-2.93)	0.21
2009-2012	1.00		1.00	

HR = hazard ratio; CI = confidence interval.

However, CNS involvement lost its significance in multivariate Cox regression analysis in which WBC was included as continuous variables, and CNS involvement and time-period as categorical variables, leaving WBC at diagnosis and the two earliest time-periods to be independent significant factors for an event (Table 4).

6.2 Study II. Biological and prognostic implications of high white blood cell count at diagnosis of ALL among the Nordic patients

In-depth retrospective study of 2371 BCP (89.9%) and 267 T-ALL patients (10.1%) treated in the Nordic countries in 1992-2008 and included into the NOPHO -92 or -2000 protocols revealed significant differences in WBC distribution both among different biological ALL subsets defined by immunophenotype or cytogenetical aberrations of leukemic blasts, and among patients of different age or gender.

6.2.1 Host factors

Median (75% range) WBC at diagnosis for BCP and T-ALL patients was 8.6 (2.3 - 56.5) x 10^9/L and 86.8 (8.8 – 394.5) x 10^9/L, respectively, with slightly higher WBC among boys than girls both within the BCP and T-ALL subsets (Table 5).

Except for the cases with 11q23/*MLL* rearrangement, an inverse correlation of WBC at diagnosis with age was detected for all cytogenetic subsets in BCP, both for boys and for girls. It was most significant for the patients with translocation t(12;21) and HeH karyotype (r_s = -0.27 (p = 0.01) and r_s = -0.28 (p = 0.01), respectively (p-values after Bonferroni correction) (Table 5). The latter two subsets are characteristic to childhood ALL with a prominent incidence peak between 2 and 7 years of age, and together they encompassed 78% of all BCP ALL cases with an aberrant karyotype in this age group.

Table 5. White blood cell count in peripheral blood at diagnosis in T-lineage ALL and cytogenetic subgroups of B-cell precursor ALL, N=2638

	No. of patients, (%)	Median WBC x 10^9/l (75% range)	*P* value vs. normal karyotype	Correlation between WBC and age, r_s
T- ALL	267	86.8 (8.8 – 394.5)		-0.06, p=0.33
boys	197 (73.8)	97.2 (9.3 - 388.8)		-0.09, p=0.23
girls	70 (26.2)	70.0 (4.5 – 435.6)		-0.006, p=0.96
BCP	2371	8.6 (2.3 – 56.5)		-0.17, p<0.001
boys	1246 (52.6)	9.0 (2.5 – 60.9)		-0.15, p<0.001
girls	1125 (47.4)	6.2 (2.2 – 87.5)		-0.20, p<0.001
Normal karyotype	445 (21.3)	7.6 (2.2 – 43.9)		-0.09, p=0.05
HeH	664 (31.8)	6.7 (2.2 – 32.1)	0.16	-0.28, p<0.001
t(12;21)	375 (18.0)	10.4 (2.9 – 60.7)	0.003	-0.27, p<0.001
t(1;19)	47 (2.2)	17.0 (6.2 – 72.0)	<0.001	-0.04, p=0.80
dic(9;20)	31 (1.5)	22.0 (5.0 – 93.7)	<0.001	-0.24, p=0.20
amp(21)	14 (0.7)	6.6 (1.7 – 24.0)	0.37	-0.30, p=0.30
11q23/*MLL*	29 (1.4)	52.5 (3.6 – 475.5)	<0.001	0.41, p=0.03
t(9;22)	41 (2.0)	29.6 (2.6 – 229.8)	<0.001	-0.11, p=0.48
Hypodiploid	19 (0.9)	12.3 (3.3 – 100.4)	0.17	-0.26, p=0.28
Other	345 (16.5)	11.4 (2.8 – 67.0)	<0.001	-0.13, p=0.02
Down syndrome	54 (2.1)	16.1 (2.2 – 66.6)	0.007	-0.04, p=0.77

Note. r_s = coefficient of Spearman's rank correlation; HeH = hyperdiploid karytotype (modal chromosome number >50); hypodiploid karyotype = modal chromosome number <45; other = all non-stratifying cytogenetic aberrations. p = values without Bonferroni correction.

6.2.2 Leukemic karyotype

An informative normal or aberrant karyotype was recorded in 2323 patients (88.1%). Different pattern of initial WBC distribution for ALL subsets with the well defined cytogenetic rearrangements was detected (Figure 14, Table 5).

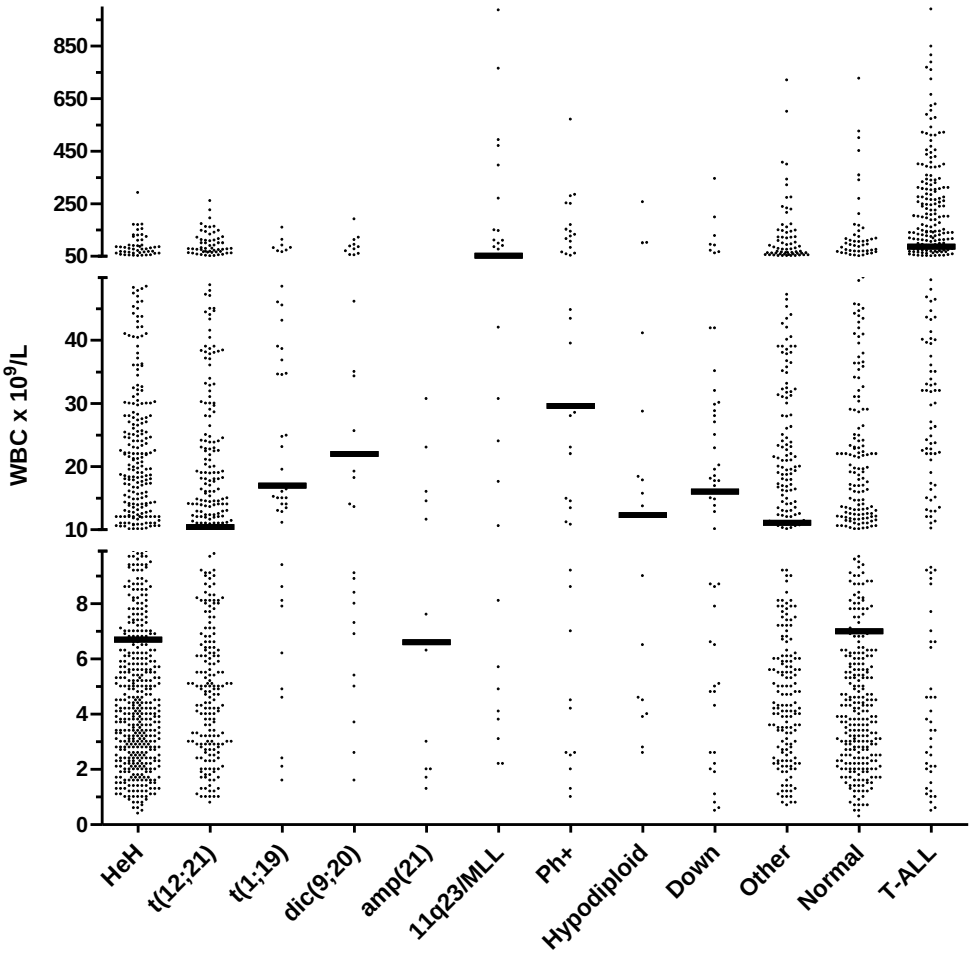

Figure 14. Distribution of initial white blood cell count within subsets of ALL with different cytogenetic aberrations.

Note. Each dot represents one patient. Horizontal bars represent median WBC values within the subgroups.

There was an increase in fractions of BCP subgroups with unfavourable cytogenetics [N=89; 11q23/MLL aberrations, translocation t(9;22) or hypodiploidy (modal chromosome number <45)] with increasing WBC. They comprised only 2.6% of BCP cases with WBC <50.0 x 10^9/L, and 11%, 18%

and 30% of BCP patients with WBC ≥50.0, ≥100.0 or ≥200.0 x 10^9/L, respectively. While the 1039 cases with translocation t(12;21) or HeH karyotype together comprised 46% of BCP cases with WBC <50.0, and 7.5% of those with WBC ≥200.0 x 10^9/L.

6.2.3 Extramedullary disease

The WBC for the 67 patients with CNS disease (2.6%) and for the 15 patients with overt testicular leukemia at diagnosis (1.1% of the 1425 evaluable male patients) was higher than for the patients without such extramedullary disease. The tendency was more pronounced in T-ALL than in BCP. Median WBC was 164.0 x 10^9/L for CNS positive T-ALL patients (N = 27) and 73.0 x 10^9/L for 240 CNS negative patients (p = 0.03). The frequency of CNS involvement for T-ALL patients with WBC ≥100 x 10^9/L was more than twice as high as for those with WBC <100 x 10^9/L (16.6% vs. 6.4%, p = 0.03). That was not the case in BCP ALL (3.5% vs 1.6%, p = 0.20). Median WBC was 25.1 vs. 11.0 x 10^9/L respectively for patients with and without overt testicular involvement (p = 0.03). Among the 127 T-ALL patients with a WBC ≥100 x 10^9/L at diagnosis, those 19 patients with extramedullary desease did significantly worse than the remaining 108 patients (the 10-year pEFS was 0.37 and 0.63, respectively, p = 0.004). Low patient number did not allow such comparison in BCP patients.

6.2.4 WBC impact on response to therapy and outcome

The 2048 patients who stayed in first remission had a median follow-up of 8.5 years (75% range 2.7 – 14.5 years). In total, 36 patients have died during induction therapy (1.4%), 22 had resistant disease (0.8%), 48 patients have died in CR1 (1.8%), 26 patients (1.0%) had developed a second malignant neoplasm, and 458 patients (17.4%) had experienced a leukemic relapse at a

median of 2.5 year from diagnosis (75% range: 9 – 53 months). The 10-year pEFS and 10-year pOS were 0.75 ± 0.01 and 0.85 ± 0.01, respectively. Remission rate was higher for BCP ALL (98.4%) than for T-ALL (92.5%) ($p < 0.001$). Patients who achieved remission had lower WBC at presentation than patients with resistant disease or induction failure for BCP ALL (median WBC: 8.3 vs 24.8 x 10^9/L, $p = 0.001$), but that was not the case for T-ALL (median WBC: 86.8 vs 127.8 x 10^9/L, $p = 0.5$).

Treatment for patients in the NOPHO ALL-92 and -2000 protocols was stratified according to initial WBC, meaning that patients with higher WBC received more intensive treatment. Even in such approach univariate survival function analysis revealed high WBC to have significant impact for events in BCP (Table 6).

Table 6. Log-rank test for the probability of 10-year event free survival for cytogenetic subgroups of BCP and T- ALL patients stratified by WBC at diagnosis

	$pEFS_{10y}$ (N)				
WBC, x10^9/L	<10.0	10-49.9	50-99.9	>100.0	*P* value
BCP	0.80 (1241)	0.80 (748)	0.70 (189)	0.49 (139)	<0.001
HeH	0.82 (410)	0.81 (205)	0.68 (35)	0.48 (13)	0.004
t(12;21)	0.77 (180)	0.82 (130)	0.83 (33)	0.70 (21)	0.41
t(1;19)	0.80 (10)	0.84 (28)	0.80 (5)	-	0.64
dic(9;20)	0.78 (9)	0.64 (9)	0.63 (8)	0.67 (3)	0.53
amp(21)	0.44 (9)	0.50 (4)	-	-	0.52
11q23/*MLL*	0.88 (8)	0.80 (5)	0.50 (6)	0.31 (10)	0.009
t(9;22)[*BCR/ABL*]	0.53 (11)	0.39 (13)	0.80 (5)	0.17 (12)	0.19
Hypodiploid	0.50 (8)	0.50 (8)	-	0.67 (3)	0.94
Normal karyotype	0.82 (248)	0.83 (132)	0.71 (25)	0.46 (22)	<0.001
T-ALL	0.58 (38)	0.70 (64)	0.65 (38)	0.58 (127)	0.33
Down syndrome patients	0.59 (20)	0.58 (26)	0.40 (5)	0.33 (3)	0.05

Note. HeH = high hyperdiploid karyotype (modal chromosome number >50); hypodiploid karyotype = modal chromosome number <45; 11q23/*MLL* = 11q23/*MLL* rearrangement. p-values without Bonferroni correction.

To eliminate the impact of the known risk factors for survival, we excluded from further analysis the patients who according to the NOPHO protocols were considered to have high risk factors: (i) unfavourable cytogenetic aberrations (11q23/MLL aberration, translocations t(9;22) or t(1;19) or hypodiploid karyotype (<45 chromosomes)), (ii) patients with extramedullary disease (CNS or testis involvement, mediastinal mass), and (iii) patients with Down syndrome. The risk for an event still correlated with higher WBC in BCP ALL (p <0.001) (Figure 15).

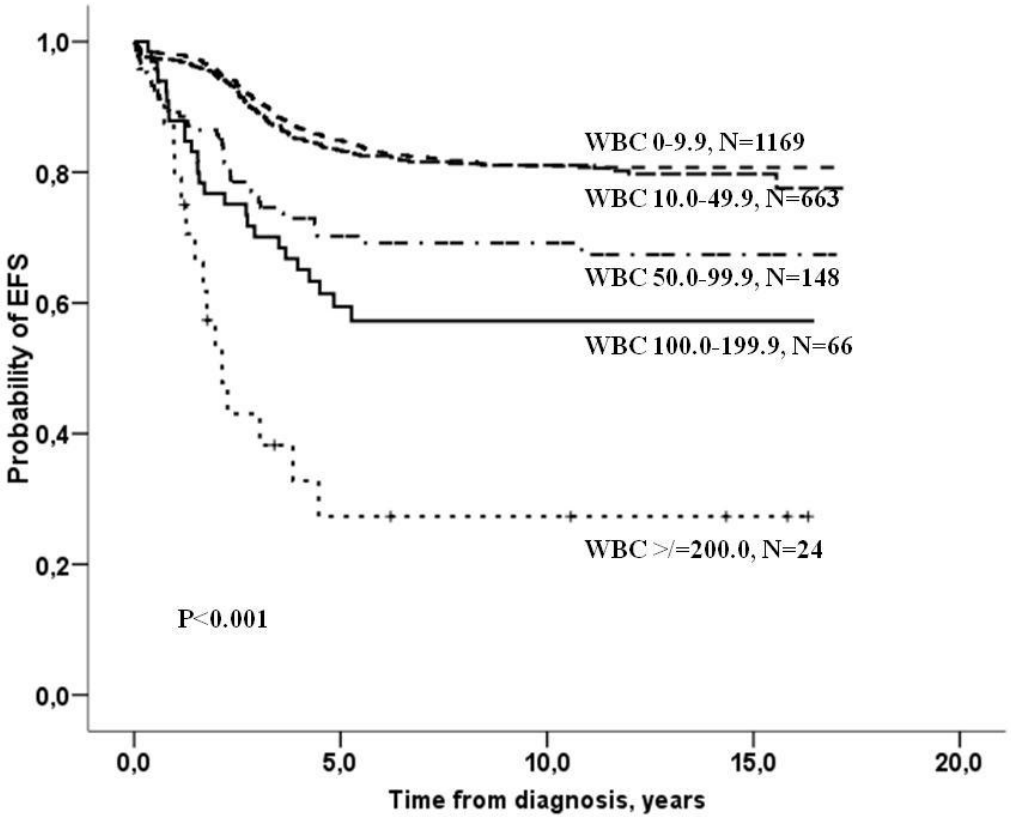

Figure 15. Probability of event-free survival for B-cell precursor ALL by white blood cell count in peripheral blood at diagnosis (excluding patients with CNS or testis involvement, mediastinal mass, lymphomatous disease, translocations t(9;22)[*BCR/ABL*] or t(1;19)(q23;p13), 11q23/*MLL* aberration or a hypodiploid karyotype (<45 chromosomes), and patients with Down syndrome).

Furthermore, there was a trend towards more events for the patients with higher WBC within some of the relatively homogeneous cytogenetic subgroups, but this reached significance only for the patients with HeH (p = 0.04) or a normal karyotype (p <0.001) (P-values after Bonferroni correction) (Table 6). In contrast, we could not identify such impact of initial WBC within the subset of T-ALL, but their overall pEFS was relatively low.

To explore the impact of initial WBC to the outcome when the other risk factors used in contemporary protocols were taken into account, we included early response to therapy defined by the minimal residual disease at day 29 (MRD_{d29}) into analysis. Patients were subdivided into groups of WBC ≥ or < 100.0 x 10^9/L as this was a stratifying factor in the NOPHO ALL-2008 protocol. For the good initial responders (MRD_{d29} ≤10^{-3}), pEFS rates were excellent for all patients irrespective of WBC both for BCP ($pEFS_{5y}$ 0.86 and 0.85) and for T-ALL ($pEFS_{5y}$ 0.86 and 0.85) (Figure 16).

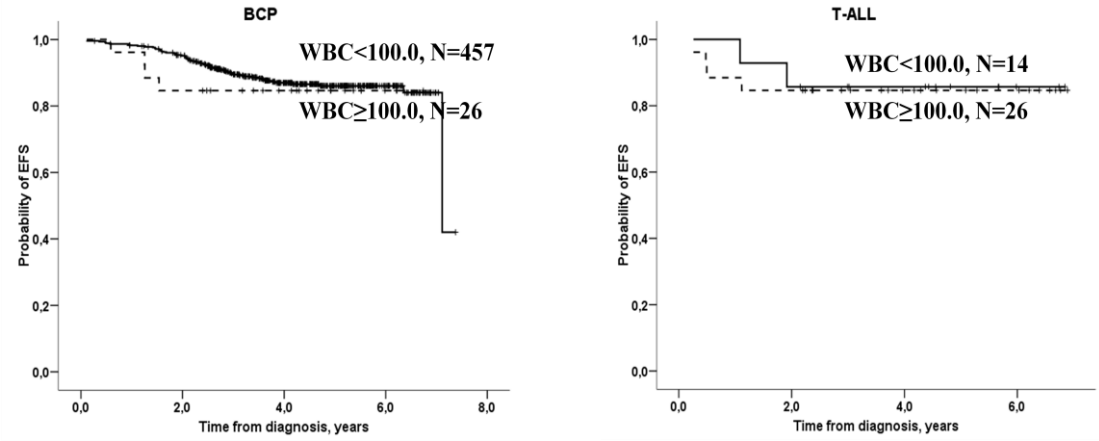

Figure 16. Probability of event free survival for B-cell precursor and T-ALL with minimal residual disease at day 29 less than 10^{-3}, in respect to white blood cell count in peripheral blood at diagnosis, p = 0.61.

In contrast, for the slower responders (MRD_{d29} ≥10^{-3} but in morphological remission at day 29 (<5% blasts in bone marrow)) patients with high tumor burden (WBC ≥100 x 10^9/L) did worse compared to the patients with lower WBC ($pEFS_{5y}$ 0.50 vs 0.75, p = 0.001) and that was the case both

for BCP ($pEFS_{5y}$ 0.58 (N=11) and 0.76 (N=145)) and for T-ALL ($pEFS_{5y}$ 0.38 (N=8) and 0.71 (N=7)) (Figure 17).

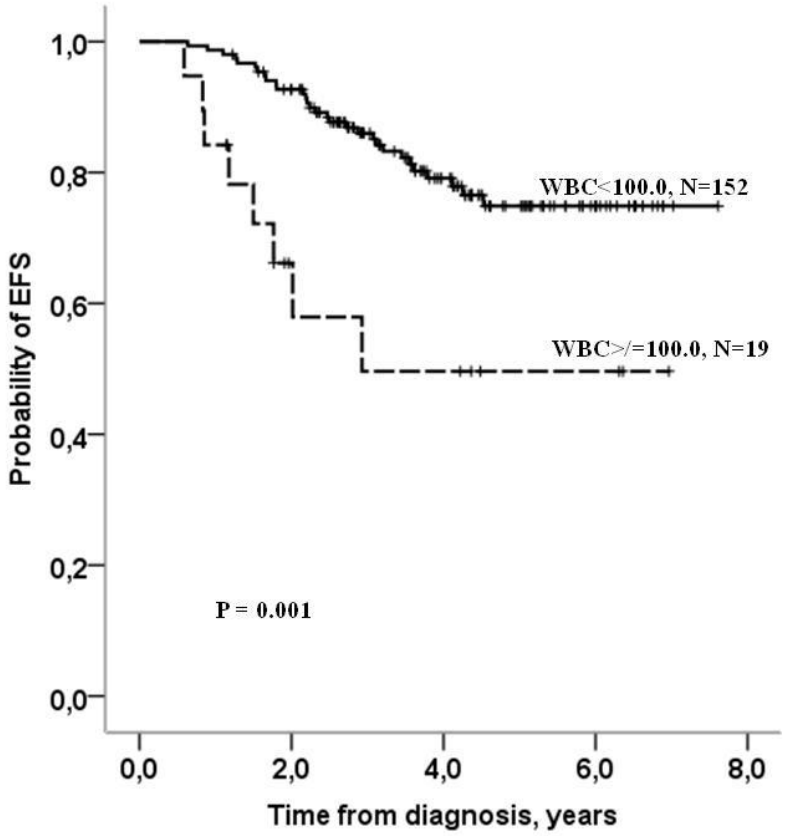

Figure 17. Probability of event free survival for B-cell precursor and T-ALL with minimal residual disease at day 29 more than 10^{-3}, and in remission (less than 5% leukaemic blasts in bone marrow), in respect to white blood cell count at diagnosis

To explore the overall prognostic impact of WBC to the outcome we created a model of the multivariate regression analysis in which we included gender, age, WBC, MRD_{d29} and cytogenetic aberrations for BCP. Cytogenetic aberrations were grouped to: (i) normal karyotype; (ii) favourable cytogenetics (included HeH and translocation t(12;21)); (iii) unfavourable cytogenetics (included hypodiploid karyotype (<45 chromosomes) or 11q23/*MLL* rearrangement or translocation t(9;22) or dic(9;20)) and (iv) other (included all patients with an aberrant, but uninformative karyotype). Multivariate logistic analysis stratified by immunophenotype was performed including age, WBC

and MRD_{d29} as categorical variables, and gender as well as groups of cytogenetic aberrations as categorical variables. MRD_{d29} data were available almost exclusively (except four cases) for the ALL-2000 protocol patients, and the protocols had several differences in the therapy approach therefore only ALL-2000 patients were included. We excluded also the patients for whom remission was not achieved (15 induction failures (1.5%) and 14 resistant disease (1.4%)) and 24 patients with Down syndrome (2.4%). Of the remaining 965 cases, MRD_{d29} data were unavailable for 246 patients leaving 719 patients (74.5%) available for the analysis. The stratified backward stepwise Cox multivariate regression analysis showed WBC to be a significant independent risk factor (hazard ratio being 1.002, p=0.006). Other significant independent risk factors were MRD_{d29} (hazard ratio 1.055, $p<0.001$) and for the BCP patients there was an increased risk for those with poor cytogenetics (hazard ratio 3.5, $p<0.001$).

6.3 Study III. Hyperleukocytosis (WBC ≥200 x 10^9/L) of childhood ALL

We performed a population-based multicenter study of 221 children aged 0-14.9 years with ALL and WBC ≥200x10^9/L at diagnosis treated in Denmark, Finland, Iceland, Lithuania, Norway or Sweden from January 1992 to October 2011. This constituted 92% of all 241 ALL patients with WBC ≥200x10^9/L during that period, and 6% of all 3985 newly diagnosed ALL patients.

6.3.1 Biological features

Median WBC (75% range) for the 221 study patients was 344 (226-695) x10^9/L (Table 7).

Table 7. White blood cell count and age distribution for the subsets of study patients.

	No. patients (%)	WBC $x10^9$/L Median (75% range)*	*p*	Age, years Median (75%)*	*p*
Infants**	48 (22)	454 (258-1058)		0.5 (0.2-0.8)	
boys	20 (39)	476 (223-990)		0.5 (0.2-0.7)	0.34
girls	28 (61)	439 (258-1127)	0.73	0.4 (0.1-0.8)	
BCP, ≥1.0 y.	49 (22)	306 (222-564)		5.7 (1.7-12.9)	
boys	26 (51)	394 (221-602)		4.2 (1.8-14.0)	0.78
girls	23 (49)	284 (222-525)	0.12	7.1 (1.2-10.8)	
T-ALL, ≥1.0 y.	120 (54)	340 (227-614)		7.9 (2.8-13.3)	
boys	87 (72)	325 (230-577)		7.8 (2.7-13.4)	0.77
girls	33 (23)	424 (210-678)	0.12	7.9 (2.7-12.2)	

Note. *median, minimal and maximal values are provided when number of cases is less than eight. **for two infants and four non-infant ALL patients immunophenotype was unavailable or ambiguous.

The incidence of BCP and T-ALL was almost equal (95 and 120 patients, respectively), however half of the BCP patients (N=46) were infants. Among non-infants, the 120 T-ALL patients were slightly older than the 49 BCP patients, median age (75% range): 7.9 (2.8-13.3) vs. 5.7 (1.7-12.9) years, respectively (p=0.02) (Table 6). Hyperleukocytosis was more pronounced in the 48 infants compared to the 173 older patients (median (75% range): 454 (258-1058) $x10^9$/L vs. 328 (225-604) $x10^9$/L, respectively, ($p<0.001$)) (Table 7). However, among patients without 11q23/*MLL* aberrations, the 14 infants did not differ from the 137 older patients (WBC median: 325 vs. 330; p=0.71).

11q23/*MLL* rearrangement was detected in 44 out of 195 analysed cases (23%) (29 infants and 15 older children), whereas other high risk cytogenetic aberrations: translocation t(9;22)(q34;q11) or hypodiploid karyotype were identified only in 1/48 infant (2%), 6/49 non-infant BCP (8%) and 1/120 T-ALL patients (1%) (Table 8).

Table 8. White blood cell count and age distribution for the subgroups of different cytogenetic aberrations

Cytogenetic aberrations	No. patients (%)	WBC $x10^9$/L, median* (75% range)	Age, median* (75% range)
Normal karyotype	64 (29)	328 (230-677)	8.1 (2.0-13.4)
11q23/*MLL*	44 (20)	490 (242-1022)	0.6 (0.3-9.1)
t(9;22)*[BCR/ABL]*	5 (2)	284 (213-570)	6.4 (1.9-7.2)
Hypodiploid	3 (1)	546 (256-556)	8.6 (0.2-8.8)
t(12;21)	4 (2)	283 (225-514)	3.5 (1.9-12.1)
t(1;19)	1 (0.5)	297	0.8
Other	67 (30)	338 (225-621)	6.0 (1.2-13.5)
Not available	34 (15)	321 (203-567)	6.5 (0.6-10.9)

Note. *median, minimal and maximal values are provided when number of cases is less than eight. Hypodiploid karyotype = modal chromosome number <45; other = non-stratifying cytogenetic aberrations.

Strong inverse correlation between WBC and age was found for the 14 infants without 11q23/*MLL* rearrangement (r_s= -0.71, p=0.004), but not for the infants with 11q23/*MLL* rearrangement or for older patients.

6.3.2 Hyperleukocytosis related complications

Forty percent of patients (N=92) experienced one or more complications associated with hyperleukocytosis (Figure 18).

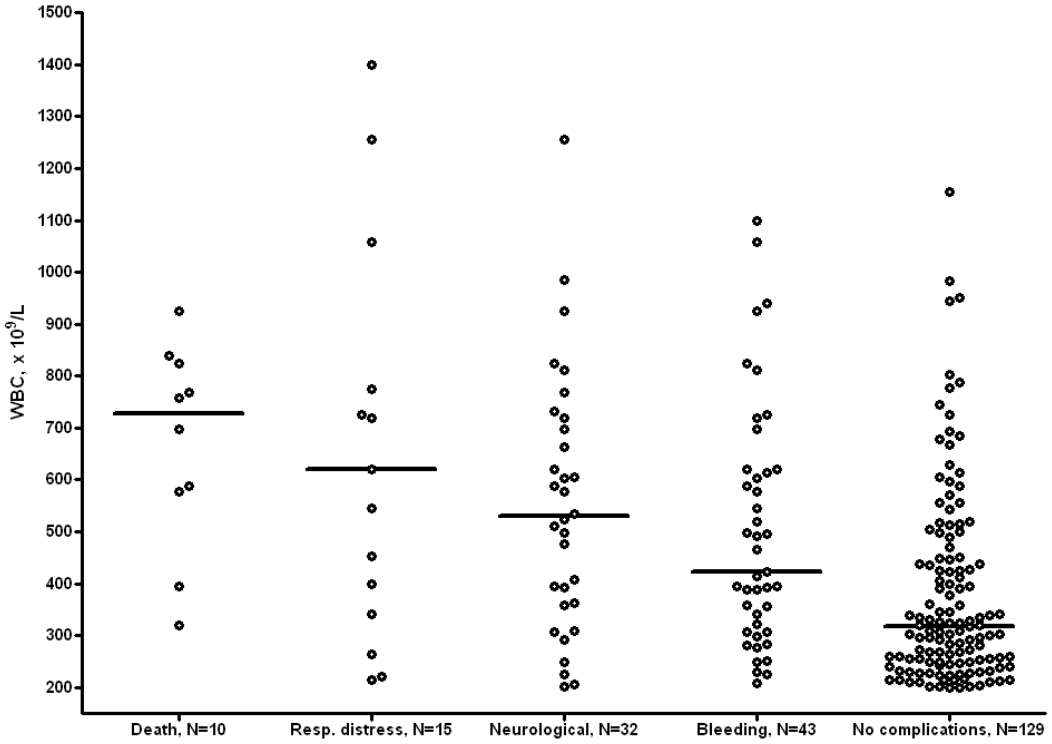

Figure 18. Distribution of white blood cell count at admission within subsets defined by hyperleukocytosis related complications.

Note. Each dot represents one patient; 40 patients had two or more complications and are included in all corresponding subsets. Horizontal bars represent median values.

Initial WBC for the patients for whom complications developed was moderately although statistically significantly higher than for patients without such complications (median (75% range): 396 (245-794) $x10^9$/L vs. 317 (219-603) $x10^9$/L, (p=0.001)) (Table 9).

Table 9. Hyperleukocytosis related clinical symptoms and complications

Complications	No. patients (%)	WBC x 10^9/L Median (75% range)	p	Age, median (75% range)	p
Neurological complications[a)]					
Yes	33 (15)	530 (256-823)		8.7 (1.8-14.3)	
No	188 (85)	327 (225-624)	<0.001	5.3 (0.6-11.3)	0.007
Respiratory distress[b)]					
Yes	15 (7)	620 (222-1400)		0.6 (0.1-8.6)	
No	206 (93)	336 (225-637)	0.006	6.1 (0.6-12.6)	0.001
Bleeding complications[c)]					
Yes	43 (19)	420 (261-821)		9.3 (1.1-14.5)	
No	179 (81)	327 (223-666)	0.005	5.2 (0.5-11.2)	0.001
Severe complications requiring treatment at ICU					
Yes	24 (11)	522 (248-914)		4.1 (0.2-13.1)	
No	197 (89)	334 (225-625)	0.002	6.0 (0.6-12.3)	0.62
Dialysis					
Yes	11 (5)	310 (202-528)		6.5 (3.1- 12.6)	
No	211 (95)	357 (227-714)	0.28	5.8 (0.6-12.3)	0.34
Any complication					
Yes	92 (42)	396 (245-794)		6.1 (0.7-13.3)	
No	130 (58)	317 (219-603)	0.001	5.3 (0.5-10.9)	0.03
Renal infiltrations					
	10 (5)	398 (254-690)		3.0 (0.3-11.6)	
Skin infiltrations					
	7 (3)	446 (230-720)		0.6 (0.0-12.7)	
Priapism					
	2 (0.9)	621; 394		3.9; 6.4	

Note. a) severe headache, dizziness, irritability, consciousness disturbances, peripheral nerve palsies, seizures; b) dyspnea or adults respiratory distress syndrome; c) epistaxis, metrorrhagia, gastrointestinal or intracranial bleeding; ICU = intensive care unit. *P* value = determined after comparison by Mann-Whitney *U*-test.

Hyperleukocytosis related neurological symptoms, respiratory distress or bleeding complications assessed as grade 2-4 of the NCI Common Terminology Criteria for Adverse Events (CTCAE) (2013b) were registered. Complications were present at or developed within three days from admission.

Ten patients (5%) died within the first month of treatment. Eight patients with early deaths were older than one year at diagnosis and seven of these had T-ALL (Table 10). Six of ten patients died because of intracranial hemorrhage (N=5) or massive intracranial infiltrates with secondary brain edema and herniation (N=1). Four of these six (patients no. 1, 2, 3 and 5 (Table 10)) presented with severe CNS symptoms at admission. Two patients developed such symptoms on the third day. In one patient the WBC increased from 305 to $625x10^9$/L because of no effect of a low initial prednisolone dose of 11.7 mg/m^2/24 h (patient no. 7) and in one patient there was a limited reduction in WBC from 395 to $291x10^9$/L (patient no. 4). Efforts to carry out leukapheresis were the reason for not starting or delaying the start of corticosteroids in two patients (no. 3 and 2) (Table 10). The remaining four patients died because of septic complications during neutropenia and both patients for whom day 15 bone marrow was available had >25% leukemic blasts in an otherwise aplastic bone marrow.

Twenty four patients (11%) (5/48 infants, 2/49 non-infant BCP, and 17/120 T-ALL patients) were transfered to intensive care units (ICU) within the first six days because of complications related to hyperleukocytosis, including respiratory insufficiency or neurological injuries leading to the need for mechanical ventilation (N=12), renal impairment (N=7), extracranial grade 4 bleeding (according to the NCI CTCAE grading score (2013b)) (N=2), convulsions (N=2), cardial arrhythmia (N=1), or complications after pericardiocentesis (N=1) (Table 9).

Table 10. Main characteristics of patients who died within one month from diagnosis.

Patient no.	Age, years	Phenotype	WBC, $x10^9$/L (centile)	PLT, $x10^9$/L	Hb, g/L	PRBC transf.	Coagulation	Therapeutic measures	Time to start of antileukemic therapy	CS initial dose* (mg/m^2/24h)	Time to death	Cause of death
1.	7.8	T-ALL	577 (80-90%)	135	82.2	no	FII/FVII/FX decreased	CS	within 24 h	9.8	24 h	IC hemorrhage
2.	1.7	T-ALL	925 (90-100%)	104	86.0	no	DIK	i.th. MTX and CS; LPh planned. Not done due to venous access	24-48 h	1.7	3 days	IC hemorrhage
3.	2.5	T-ALL	768 (90-100%)	47	53.2	no	Not checked	LPh performed, w/o WBC reduction	-	-	3 days	IC infiltrations, brain edema and herniation
4.	14.7	BCP	395 (50-60%)	77	120.0	no	D-dimers increased	i.th. MTX and CS	24-48 h	60.8	6 days	IC hemorrhage, brain edema and herniation
5.	0.3	BCP	825 (90-100%)	9	32.2	yes	Normal	CS	24-48 h	40.0	10 days	IC hemorrhage
6.	10.8	T-ALL	757 (90-100%)	57	54.8	no	Normal	Induction with CS+VCR+Doxo w/o prephase	24-48 h	60.0	13 days	Septic shock chemotherapy indused neutropenia
7.	10.6	T-ALL	305→625 (80-90%)	108	135.0	yes	Not checked	CS prephase and Induction with CS+VCR+Doxo	>48 h	11.7	14 days	IC hemorrhages
8.	14.3	T-ALL	588 (80-90%)	40	56.4	yes	D-dimers increased	CS prephase and Induction with CS+VCR+Doxo	NA	NA	25 days	Sepsis during chemotherapy indused neutropenia
9.	0.2	BCP	840 (90-100%)	93	119.0	yes	Not checked	CS prephase and Induction with CS+VCR+Doxo	Within 24 h	14.3	25 days	Sepsis during chemotherapy indused neutropenia
10.	6.5	T-ALL	320 (40-50%)	108	86.0	yes	Not checked	CS prephase and Induction with CS+VCR+Doxo	24-48 h	20.8	27 days	Sepsis during chemotherapy indused neutropenia

Note. *CS initial dose was calculated as equivalent prednisolone dose. PLT = platelet count in peripheral blood at admission; Hb = hemoglobin; PRBC transf. = packed red blood cell transfusion within a period when WBC was ≥200.0; CS = corticosteroids; IC = intracranial; LPh = leukapheresis; VCR = vincristine; Doxo = doxorubicin; NA = not available.

The 61 patients with neurological complications and/or a serious bleeding episode present at admission or developed within three days had moderately higher WBC and older age compared to the remaining 160 patients, whereas the 15 patients with respiratory distress were younger, but also tended to have higher WBC at diagnosis (Table 9 and Figure 18). In a multivariate logistic regression analysis the risk of neurological complications was significantly associated with WBC (OR (95% CI): 1.004 (1.002-1.005), (p<0.001)) and age (OR (95% CI): 1.2 (1.1-1.3), (p<0.001)), but not with gender, immunophenotype or the leukemic karyotype. Similarly, the risk of respiratory distress or severe bleeding was significantly associated with WBC (OR (95% CI): 1.003 (1.001-1.006), (p=0.005), and borderline with age (OR (95% CI): 0.8 (0.7-1.0), (p=0.062) and 1.2 (0.9-1.4), (p=0.047), respectively), but not with gender, immunophenotype or karyotype.

Prognostic impact of potential risk factors for early death was explored by multivariate logistic regression analysis including as continuous variables: i) age, ii) WBC and iii) hemoglobin at admission, and as categorical variables: i) presence of neurological symptoms at admission, ii) gender, iii) immunophenotype, iv) leukemic karyotype, v) administration of antileukemic therapy within 24 hours after admission or later, and vi) administration of packed red blood cell transfusion when WBC was still ≥200x10^9/L. Only WBC (OR (95% CI): 1.004 (1.001-1.006), (p=0.007)) and presence of neurological symptoms at admission (OR (95% CI): 5.8 (1.3-25.2), (p=0.018)) were independently and significantly associated with risk of early death.

6.3.3 Initial treatment and tumor lysis syndrome

Initial therapy was heterogeneous and center-dependent since there was no common Nordic/Baltic tumor burden reducing strategy for patients with hyperleukocytosis. The majority of the patients (N=178, (85%)) were initially hydrated with ≥3000 ml/m^2/24 h. The remaining 26 patients for whom

information was available were hydrated with a median of 2183 ml/m^2/24 h (75% range: 1408-2975 ml/m^2/24 h). Out of 141 patients who did not receive urate oxidase, the patients urine was alkalinised and allopurinol given to 125 and 136 patients (89% and 97%), respectively.

Figure 19 depicts the different treatment modalities given before administration of full induction with VCR and Doxo (N=219).

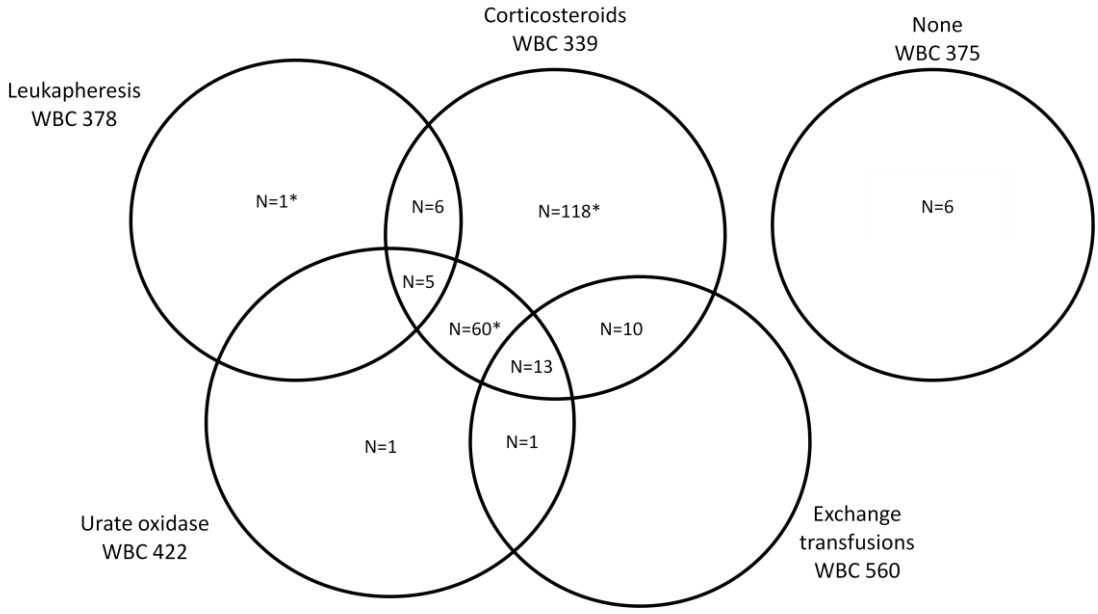

Figure 19. Treatment modalities before administration of induction with vincristine and doxorubicin and median white blood cell count within subsets.

Note. *1 leukapheresis patient, 3 patients in corticosteroids group and 1 patient in corticosteroids with concomitant urate oxidase group died before administration of induction with vincristine and doxorubicin.

Urate oxidase was given upfront or on the same day as the administration of any antileukemic treatment for 96% of the 71 patients who received this treatment and had information on timing of the first dose available.

A total of 36 patients (16%) received either exchange transfusion (N=24) or leukapheresis (N=12). Fifteen of these patients started such mechanical cytoreduction as their first treatment modality, whereas the remaining 21 patients had mechanical cytoreduction after or concomitantly with

administration of CS and/or i.th. MTX. Median (75% range) absolute and relative reduction in WBC per procedure for exchange transfusions was 298 (81-674) $x10^9$/L and 57% (26-82%), and for leukapheresis it was 165 (67-337) $x10^9$/L and 48% (26-68%), respectively (p=0.11). Antileukemic therapy was delayed more often for the patients with mechanical cytoreduction as first treatment modality (N=15) compared to all the remaining patients (N=191); median (75% range) time to administration was: 1.5 (1.0-2.4) vs. 1.0 (0-2.0) days, respectively (p=0.009).

TLS developed in 27 patients (12%): 5 infants, 1 non-infant BCP and 21 T-ALL patients. Four patients developed laboratory TLS, and the remaining 23 patients had clinical TLS. TLS was present at admission (N=8) or developed within three days after initiation of any antileukemic therapy (N=19). Four out of the latter 19 patients developed TLS after only i.th. MTX had been administered. Patients who developed TLS had significantly higher initial uric acid level than those who did not, both within the cohort of all patients (median (75% range): 652 (519-1243) vs. 460 (270-676) µmol/L; p<0.001) and within the T-ALL group (661 (533-1486) vs. 490 (279-666) µmol/L (p<0.001)). In contrast, initial uric acid level did not differ for the patients who received and did not receive urate oxidase (median: 484 vs. 474 µmol/L; p=0.99). After initiation of antileukemic therapy, TLS developed in 16 of 118 patients (14%) who received only CS compared to 2 of 60 patients (3%) who recieved both CS and upfront urate oxidase (p=0.03). Eleven patients (5%) were dialysed (ten patients with T-ALL and one non-infant BCP patient) because of metabolic derangement (hyperkalemia or hyperphosphatemia), increase in serum creatinine or marked reduction in urine output. However, the exact reasons for dialysis were in some of the cases difficult to identify with certainty in this retrospective analysis. Only one of the dialysed patients had received urate oxidase. No patients died due to TLS or its treatment.

Initial CS dose (calculated as a prednisolone equivalent dose) did not differ significantly when patients who developed TLS were compared to those

who did not, with medians of (75% range) 9.2 (1.7-43.5) vs. 12.3 (2.7-52.3) mg/m^2/24 hours, respectively (p=0.19). Furthermore, none of the patients who started antileukemic therapy with 60 mg/m^2/24 h of prednisolone as prephase (N=11, median WBC 303x10^9/L) or with full induction therapy including prednisolone, VCR and Doxo (N=6, median WBC 375x10^9/L) developed TLS. Urate oxidase had been given to four out of these seventeen patients.

Multivariate logistic regression analysis revealed uric acid level at admission to be the only significant risk factor for TLS (OR (95% CI): 1.005 (1.001-1.008), p=0.009). In contrast, age, gender, initial WBC or LDH, time to start of antileukemic therapy, initial CS dose or mechanical cytoreduction procedures were not significantly associated with the risk of TLS.

6.3.4 Impact of initial treatment strategy to long-term survival

After a median follow-up of 5.1 years for patients who stayed in remission, twelve patients had died during induction therapy, ten had been registered with resistant disease, 23 had died during first complete remission, 71 had developed a relapse (32% involving the CNS) at a median of 13.5 months from diagnosis, and two had developed a second malignant neoplasm. Five and ten-year pEFS were 0.43+/-0.04. The 5-year pEFS did not seem to improve significantly over time and was 0.35+/-0.05 for the 84 patients diagnosed before 2002 and 0.51+/-0.05 for the 137 patients diagnosed in the latter period (p=0.07).

Median (75% range) time from initiation of CS therapy to administration of VCR and Doxo was 6 (2-7) days, and the median WBC at the time of administration of VCR and Doxo was 10.0 x10^9/L (75% range: 2.2-210). Neither the interval of CS-prephase, nor the WBC at the time point of administration of VCR and Doxo had any impact on the long-term results: when the patients were divided in four subsets defined by the median duration of CS-prephase, and median WBC at the time of administration of VCR and

Doxo, the 5-year pEFS did not differ significantly between the groups with superimposable survival curves (p=0.63) (Figure 20).

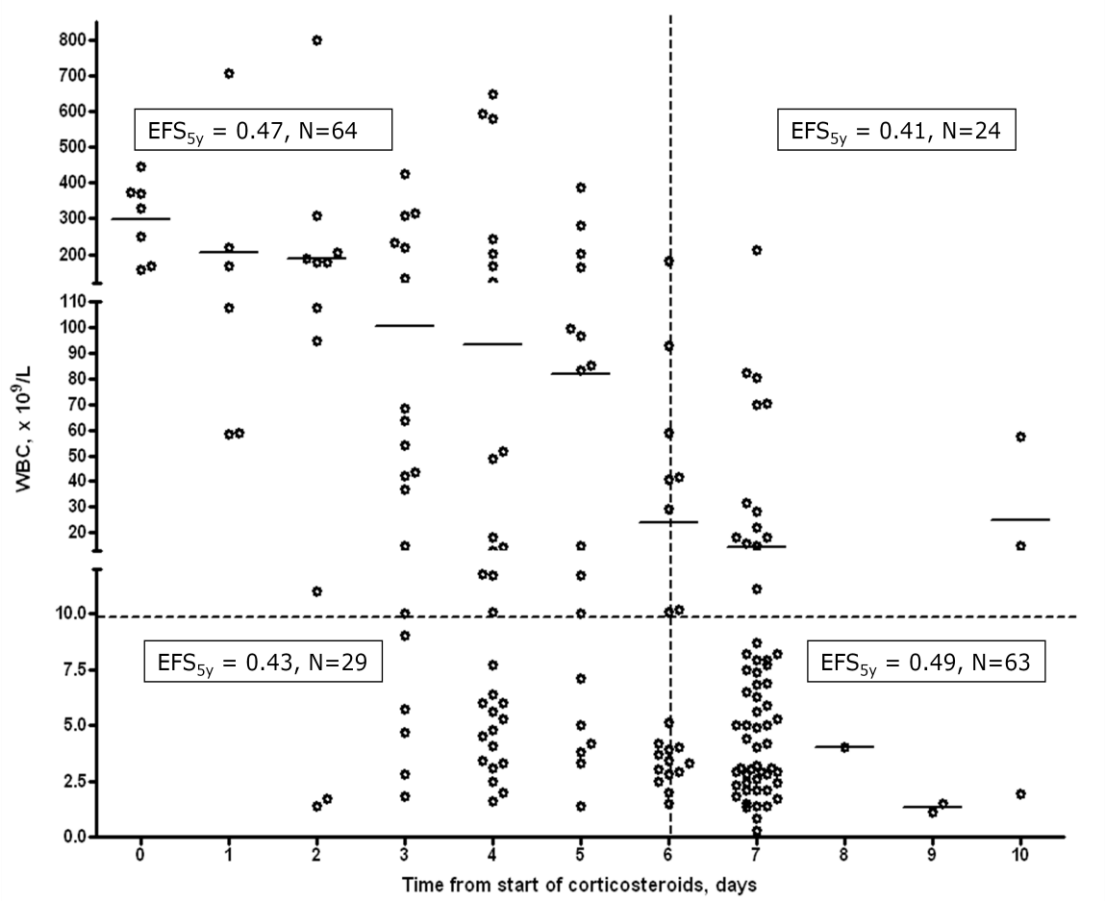

Figure 20. Distribution of white blood cell count in peripheral blood on the day of introduction of induction with vincristine and doxorubicin.

Note. Each dot represents one patient. Horizontal bars represent median WBC values. Dashed lines mark median WBC at the day of introduction of induction and median duration of corticosteroids prephase.

In a backward stepwise Cox multivariate regression analysis that explored age and WBC as continuous variables; and gender, immunophenotype, genotype, administration of mechanical cytoreduction procedures, administration of CS prephase, and the development of TLS as categorical variables, the best-fit model to predict disease-free survival, revealed BCP-phenotype as the only prognostic factor (hazard ratio (95% CI) 1.9 (1.2-3.1); p=0.009).

In conclusion, study showed that tumor burden as such poses the highest risk for children with ALL and very high WBC at diagnosis, while tumor lysis syndrome is managable when applying contemporary supportive care methods. Based on the results of the study Nordic/Baltic guidelines for the initial management of ALL patients with high risk for the development of TLS (WBC >100 x 10^9/L) were developed (Appendix III). The guidelines were approved by the NOPHO ALL-2008 protocol Events group and NOPHO Leukemia and Lymphoma committee, and will be implemented as Nordic/Baltic guidelines.

7. General discussion and future perspectives

In this dissertation, results of the first in-depth study on epidemiology, cure rates and risk factors for an event of childhood ALL in Lithuania from the two recent decades (1992–2012) were presented. Study showed EFS rates in Lithuania in the beginning of the study period to be about 20% inferior to those reported by large international study groups. Steady improvement in survival has been observed over time approaching the rates reported by large international study groups in the recent period (2009-2012).

The prognostic impact of WBC at the time of diagnosis of ALL as a surrogate marker of both leukemic cells and the host in a large cohort of 2638 Nordic patients was further explored. Multivariate analysis revealed WBC to be a significant independent risk factor, however, only among slow-responding patients (MRD $\geq 10^{-3}$ at the end of induction).

The study on patients with very high WBC ($\geq$200 x 10^9/L) at diagnosis showed tumor lysis syndrome to be manageable by the use of contemporary supportive care measures. In contrast, delay in initial antileukemic therapy or dose reduction of antileukemic agents could contribute to the development of early deaths.

Not surprisingly, survival rates of childhood ALL in Lithuania during the study period were inferior to those reported by the groups from Western countries. Five-year pEFS in Lithuania in 1992-1998 and 1999-2002 was 50±4% and 63±4%, respectively, while five-year pEFS for the similar period reported by BFM, NOPHO or St. Jude Children's Research Hospital groups were 78±1%, 77±2%, and 80.1±2.6%, respectively (Pui *et al*, 2000a; Schrappe *et al*, 2000b; Gustafsson *et al*, 2000a; Moricke *et al*, 2010; Schmiegelow *et al*, 2010a; Pui *et al*, 2010). In 2009-2012, three-year pEFS in Lithuania reached 82±5% for all patients and for lower risk patients (WBC <100 x 10^9/L), it reached 84-89%. This was approaching the results reported by the best contemporary study groups, such as Children Oncology Group which has

recently reported its results for patients diagnosed in 2006-2009 period and showed five-year EFS for NCI SR patients to be 88-95% (Hunger *et al*, 2013). Such achievements have several implications.

First, during Soviet times, the Lithuania's health care system used to be characterized by inefficiency, poor health care and lack of universal access (Bankauskaite & O'Connor, 2008). The restricted access to internationally available research information and international collaboration led to the lack of professional information on the rapidly developing pediatric oncology among Lithuanian physicians. Nursing practice lagged also behind with nurses' duties limited to technical procedures such as delivering the prescribed drugs or procedures to the patients. However, with enormous progress in nursing since 1990, the curriculum is now close to the Western European standards (Toliusiene & Peicius, 2007). Similarly, quality of treatment improved by physicians' training at the pediatric oncology/hematology centers in Western countries, and also setting up of the unit for stem cell transplantation for children in 2000 at COH CH.

Second, in 1990 the total health care expenditure per capita in US dollars in Lithuania was only 10% of that in the European Union countries (Bankauskaite & O'Connor, 2008). During the earliest two study periods (1992-2002) there was a lack of both the supportive care measures such as broad spectrum antibacterial or antifungal drugs, and of antileukemic therapy. Due to health care reforms, health care expenditure per capita increased in 2011 to 1 292 $US, i.e. 35% of that in Western European countries (2013a). Furthermore, since the year 2000 approximately Litas 2 million (Euro 580 thousand) was additionally assigned on annual basis by the State for the treatment of children with cancer in Lithuania. This allowed the necessary antileukemic and supportive care drugs to be available for all childhood ALL patients and to perform all required diagnostic procedures in spite of increasing costs (van Litsenburg *et al*, 2011).

Third, the study indicated a further trend towards survival improval in the 2009-2012 period compared with the 2003-2008 period despite the fact that neither financial nor human resorces had improved significantly. Close collaboration with the NOPHO organization and finally joining the prospective international NOPHO ALL protocol could have played a significant role in several ways: (i) new laboratory methods for contemporary diagnostic work-up and monitoring of MRD in consistence with the NOPHO requirements were implemented. The Lithuanian flow cytometry lab had to standardize their diagnostic procedures and participate in the NOPHO validation program. Improved diagnostics and monitoring of response to therapy at the molecular level of MRD led to the better stratification of patients into the risk groups thus avoiding under- or over-treatment of patients. (ii) prospective registration of patients into the NOPHO ALL-2008 protocol Register enabled not only to easily retrieve our own data, but also to compare directly the results with other centers results. (iii) With the assistance of on-line NOPHO ALL-2008 protocol Helpdesk service, we have gained a quick access to the comments of the Nordic experts on questions regarding complicated cases. The cases are usually commented by Helpdesk experts within 24 hours including weekends or public holiday.

All the features listed above led to treatment standardization and a steadily increasing understanding of the biology of leukemic cells, mechanism of developing of adverse events, pharmacokinetics and pharmacodynamics of antiluekemic drugs which in turn may have improved clinical decisions.

In addition, joining the NOPHO protocol provided researchers in Lithuania with an opportunity to run international research by getting access to a large cohort of Baltic/Nordic data.

Furthermore, after noticing the benefits of such retrospective collection and prospective registration and thorough analysis of ALL data, the same process for the other groups of childhood cancers is planned. Preparatory work

and first registration steps have already started in Lithuania for acute myeloblastic leukemia, bone tumors and neuroblastoma patients.

The main challenge in Lithuania remains high rate of toxic deaths. Most of the patients who were reported both as IF or as DCR1 died of infectious complications which was also the main cause of therapy related deaths reported by other groups (Lund *et al*, 2011; Prucker *et al*, 2009; Rubnitz *et al*, 2004; Christensen *et al*, 2005). However, in most contemporary clinical trials, <5% of patients die during induction therapy or first complete remission (Prucker *et al*, 2009; Rubnitz *et al*, 2004; Hargrave *et al*, 2001; Slats *et al*, 2005; Moricke *et al*, 2008). S. P. Hunger *et al*. from SJCRH had proposed ALL treatment regimens for low-income countries and suggested that 10-15% non-relapse mortality rate would be unacceptable (Hunger *et al*, 2009). In Lithuania, we managed to achieve significant reduction in the rate of IF however rate of DCR1 still remains high. Several reasons to explain these processes may be named.

First, in the beginning of the study period (1992-2002), a high proportion of patients who died of infection during induction phase still had a high tumor burden at the time of death, and leukemia itself may be considered as immunosuppressive factor by suppressing normal hematopoiesis. Thus, intensification of antileukemic treatment in the following periods may have contributed to reduction in IF rate.

Second, economical development and changes in health care system that were described above may have considerably contributed to improvement in supportive care and to reduction of IF. However, it did not make an influence on DCR1 rate.

Third, delayed hospitalization in case of neutropenic fever may have had a role in several cases of death for patients in remission. Long traveling distances (the distance from COH CH to some regions in Lithuania is about 300 km), low some families' incomes to cover travelling expenses, or

insufficient education of the families could be the reason. Importance of social factors for survival had been also reported in the studies for patients both in medium-income and also in high-income countries (Lightfoot *et al*, 2012; Magrath *et al*, 2013).

Fourth, lack of analysis of our own data may have been one of the reasons for inappropriate quality of supportive care leading to high rate of treatment related deaths. Data collection during preparation of this dissertation as well as analysis of NOPHO ALL-2008 protocol interim toxic events at the NOPHO ALL-2008 protocol Events group may have already made a positive effect however follow-up period has been too short to show a difference.

Apart from the effect of early time-periods (1992-2002), the multivariate analysis of Lithuanian data revealed the WBC at diagnosis to be an independent significant risk factor for an event. However, we were unable to adjust these findings to other known risk factors such as immunophenotype, genetic rearrangements or the molecular level of response to therapy. This was impossible due to the short time-period when these tests were performed in Lithuania, and also because of a small number of patients.

Such analysis became possible in a large data cohort of 2638 Nordic patients. The study revealed heterogeneous distribution of WBC among biological subsets of patients defined by characteristics both of the host (age, gender) and of leukemic cells (immunophenotype, cytogenetic rearrangements).

An impact of age to WBC at diagnosis was determined. The highest WBC was observed among infants with all eight cases with initial WBC $\geq$1 000 x 10^9/L in the study on hyperleukocytosis (WBC $\geq$200 x 10^9/L) to be among the patients <1.0 year of age. Such finding may reflect biological differences of infant ALL which are characterized by the presence of *MLL*$^-$ gene rearrangements and very immature B-cell phenotype (CD10$^-$). These cell characteristics are associated with aggressive course of the disease presenting

with high WBC at diagnosis, spreading of leukemic cells to extramedullary compartment and poor outcome (van der Linden *et al*, 2009; Kosaka *et al*, 2004; Biondi *et al*, 2000). Study on Interfant-99 protocol data showed congenital ALL (of first month of life) to have even more aggressive course of the disease with significantly higher WBC and significantly poorer outcome compared with older infants (van der Linden *et al*, 2009).

However, our findings of a strong inverse correlation (r_s = -0.71, p=0.004) between WBC and age for infants without 11q23/*MLL* rearrangement as well as less strong but of a same pattern correlation for older patients particularly in those having high hyperdiploid karyotype or translocation t(12;21) which are characteristic of childhood ALL, indicate a possible impact of normal cell growth and proliferation factors to WBC at diagnosis of ALL. Same type of association is seen in normal B-cell precursor and B-lymphocyte compartment of healthy children. Percentage of B-cell precursors in bone marrow increases during the first months of life reaching the peak at six months, and from then on, rapidly declines. In parallel, the absolute number of circulating peripheral B cells reach plateau between two and 24 months of age, and then declines gradually (Jensen *et al*, 2010;Comans-Bitter *et al*, 1997). The extent to which hyperleukocytosis in children with ALL is determined by host factors or by leukemic karyotype remains to be established.

Study of the NOPHO ALL-92 and -2000 data, supported previous findings that patients with higher WBC show higher propensity for extramedullary and extralymphatic presentation, which may demonstrate their different abilities to evade apoptosis, survive outside the bone marrow microenvironment and infiltrate non-lymphatic organs. These clinical observations are in line with the laboratory findings of Hansson et al. (Hansson *et al*, 2008) who compared ALL cell differentiation level and expression of cell markers in peripheral blood and bone marrow. They found no difference in marker expression between the subset of leukemic cells in bone marrow and

peripheral blood suggesting that transition of ALL cells from bone marrow into circulation is not a result of progressed differentiation, but rather an inherent ability of a subset of leukemic blasts to exist in a microenvironment not supportive for normal cells at a corresponding maturational stage. The study also indicated that ALL cells may retain some ability to progress beyond the pre-B stage into more mature stages of development (Hansson *et al*, 2008). This questions, therefore, whether the presence of extramedullary disease carries an independent prognostic significance.

The study showed an increase in proportion of HR BCP cytogenetic subsets with increasing WBC. Accordingly, poor prognosis of the cases with high WBC is strongly associated with poor-risk karyotypes (Pui *et al*, 1990b; Vrooman & Silverman, 2009; Pui *et al*, 2011). However, our findings of inverse relationship between WBC and event-free survival among the patients without known unfavorable cytogenetics as well as an identified subset of patients among the slow responders ($MRD_{d29} \geq 10^{-3}$) with WBC $\geq 100 \times 10^9$/L that still had a significantly poorer prognosis even though they had received more intensive therapy may have several implications.

First, this could indicate the presence of rare cytogenetic aberrations which cause both poor prognosis and high WBC. Such as a subtype of aggressive so-called *BCR-ABL1*-like ALL which have been recently identified in genome-wide studies by den Boer *et al*. Most of the cases had deletions in genes involved in B-cell development, including *IKZF1*, *TCF3*, *EBF1*, *PAX5*, and *VPREB1* and were associated with both higher WBC and with an unfavorable outcome (Den Boer *et al*, 2009).

Second, prognosis in ALL is closely related to cellular resistance to chemotherapeutic agents. Several genes regulating DNA-repair, cell-cycle or cell apoptosis have been identified by several studies as being associated with response to antileukemic drugs and with ALL survival (Flotho *et al*, 2007; Silveira *et al*, 2013; Holleman *et al*, 2004; Kaaijk *et al*, 2003).

Third, inherited genetic polymorphisms of genes regulating drug distribution, metabolism, cellular transport or cell targets may influence antileukemic treatment intensity and have been shown to have an impact on outcome in childhood ALL (Borst *et al*, 2011; Borst *et al*, 2012; Schmiegelow *et al*, 2009a).

Fourth, inherited genetic polymorphisms of genes involved in function of immune or coagulation systems may have an impact on adverse events thus making a difference in outcome among the patients with similar WBC.

Survival improvement of childhood ALL in contemporary protocols had been achieved mainly through intensification of chemotherapy regimens. Despite improvement, many children are being overtreated. Understanding the underlying biological mechanisms associated with high WBC in complex with interindividual variability in response to therapy and in propensity to side effects would add to more precise risk stratification and implementation of more individualized treatment regimens.

In the so far the largest and the first international population-based study on ALL and hyperleukocytosis (WBC $\geq$200 x 10^9/L), we have analyzed the clinical presentation of childhood ALL with very high WBC for patient subsets defined by age, immunophenotype, or cytogenetics, and also compared the impact of different initial treatment strategies on early morbidity and mortality as well as event free and disease free survival.

Although the clinical presentation and the pattern of leukostasis or hyperviscosity associated complications were very similar to those previously reported (Eguiguren *et al*, 1992b; Harousseau *et al*, 1980b; Lowe *et al*, 2005b; Maurer *et al*, 1988a), we demonstrated a 1.5-fold absolute increased risk of complications with every WBC increase of 100x10^9/L.

Leukostasis is rarely seen in ALL, however, all six early deaths in our study were induced by hyperleukocytosis and occurred due to intracranial hemorrhage or massive leukemic infiltrates. Histological findings of brain

damage in case of hyperleukocytosis were described previously in brain biopsy or postmortem brain autopsy material (Fritz *et al*, 1959a; Freireich *et al*, 1960; Koenig *et al*, 2008). The studies demonstrated hemorrhagic lesions to be most centered at intravascular microscopic leukemic nodules and limited to the white matter of the brain. A subset of patients with hyperleukocytosis but without intracranial hemorrhages showed areas of brain white matter infiltrated with intravascular microscopic leukemic blast nodules occupying or all of the vascular lumen, with or without the presence of fibrin (FRITZ *et al*, 1959a; McKee, Jr. & Collins, 1974).

However, pathophysiology of leukostasis still have to be elucidated. Aggregation of leukocytic thrombi is thought to be due to up- and down-regulation of a number of specific adhesion molecules (Stucki *et al*, 2001; Opdenakker *et al*, 1998). Several studies suggest that hemodynamic flow conditions and local hypoxemia may modulate the expression of adhesion molecules and chemokines (Reinhardt & Kubes, 1998; Karakurum *et al*, 1994). In addition, recent studies focused on multiple sclerosis and animal models had described the mechanisms of immune T-cell migration per diapedesis through endothelial blood brain barrier by the sequential interaction of different adhesion and signaling molecules (Engelhardt & Ransohoff, 2012; Engelhardt & Ransohoff, 2005; Akers *et al*, 2010).

Mechanical cytoreduction (leukapheresis or exchange transfusions) is still generally recommended for initial reduction of hyperleukocytosis and leukostasis. However, the lack of evidence of the benefit of mechanical cytoreduction procedures in reducing early mortality (Haase *et al*, 2009a; Porcu *et al*, 1997a), the need for immediate presence of experienced personnel, the risk of catheter-related complications especially for young children and the current knowledge of the involvement of adhesion molecules in the pathology of leukostasis, make us argue whether mechanical cytoreduction should be used as first treatment modality in case of hyperleukocytosis for children with ALL.

Our study have strongly indicated that delay in initial antileukemic therapy or dose reduction of antileukemic agents could contribute to the worsening of the symptoms and development of early deaths at least for some of the patients. Decision to perform mechanical cytoreduction as first treatment modality could add to the delay of antileukemic therapy.

Interestingly, although it is generally accepted that low starting doses of CS and slow dose increments may protect patients from the development of TLS (Ozdemir *et al*, 2009; Howard *et al*, 2011), our study did not find the relation between TLS and WBC at diagnosis or initial CS dose. The main risk factors for TLS in our study revealed to be T-cell immunophenotype and an increased level of uric acid at diagnosis.

Our findings suggest the existence of two subtypes of leukemic cells: (i) a subtype of aggressive leukemic cells prone to induce formation of intravascular aggregations and leukemic microthrombus through inducing multiple adhesion reactions, and subsequently causing leukostasis and intraparenchymal hemorrhages. In some cases such cells may probably be able to migrate through endothelial blood brain barrier; (ii) a subtype of leukemic cells with very high cellular turnover that may induce development of TLS even after small chemotherapy doses.

Based on the results of this study, a common Nordic/Baltic guideline for the standardized management of ALL with hyperleukocytosis and high risk for TLS (WBC $\geq$100 x 10^9/L) has been developed (Appendix II). In this guideline we recommend initiation of antileukemic therapy within 24 hours as soon as all necessary diagnostic samples have been obtained and urate oxidase has been given. Initial full induction with dexamethasone 10 mg/m^2/24 h, VCR and Doxo is recommended except for patients who have metabolic derangements or clinical symptoms compatible with TLS at admission. For these patients a prephase consisting of prednisolone at a dose of 6.6 mg/m^2 every eight hours is suggested with rapid dose increment up to full induction

within 48-72 hours. Uric acid level are suggested to be monitored at eight hours intervals and urate oxidase re-administered when the urate level exceeds 100 μmol/L within the first three days (Appendix III). The prospective study for monitoring of the safety and effectiveness of such approach has been developed, and has already been approved by the NOPHO Scientific Committee.

8. Conclusions

1. Epidemiological and clinical findings in Lithuania during the study period were in consistence with the findings by other childhood ALL study groups. In the earliest two study periods (1992 – 2002) cure rates of childhood ALL in Lithuania were inferior to those reported by large childhood ALL study groups. However, through participation in international protocol survival rates of childhood ALL in Lithuania reached those in Western European countries.
2. WBC remained as a risk factor to have a significantly poorer prognosis both for BCP and T-ALL patients with a WBC $\geq 100 \times 10^9$/L, but only among slow-responding patients (MRD $\geq 10^{-3}$ at the end of induction).
3. A 1.5-fold absolute increased risk of early complications with every WBC increase of 100×10^9/L was demonstrated for patients with hyperleukocytosis ($\geq 200 \times 10^9$/L).
4. Very high WBC itself, but not tumor lysis syndrome was the main threat for early mortality in patients with hyperleukocytosis ($\geq 200 \times 10^9$/L). Delay in initial antileukemic therapy or dose reduction of antileukemic agents could contribute to the development of early deaths.
5. Start of antileukemic treatment within the first 24 hours for patients with hyperleukocytosis is necessary to possibly reduce early mortality rate. Based on the results of Study III, the Nordic/Baltic guidelines for initial treatment of ALL patients with hyperleukocytosis and a high risk for the development of tumor lysis syndrome (WBC $\geq 100 \times 10^9$/L) were conducted.

9. Publications

List of publications included to dissertation

- **Vaitkevičienė G**, Heyman M, Jonsson OG, Lausen B, Harila-Saari A, Stenmarker M, Taskinen M, Žvirblis T, Åsberg A, Ragelienė L and Schmiegelow K. Early morbidity and mortality in childhood acute lymphoblastic leukemia with very high white blood cell count. *Leukemia*. 2013 (Epub ahead of print).

- **Vaitkevičienė G**, Forestier E, Hellebostad M, Heyman M, Jonsson OG, Lähteenmäki PM, Rosthoej S, Söderhäll S and Schmiegelow K. On behalf of the Nordic Society of Paediatric Haematology and Oncology (NOPHO). High white blood cell count at diagnosis of childhood acute lymphoblastic leukaemia: biological background and prognostic impact. Results from the NOPHO ALL-92 and ALL-2000 studies. *Eur J Haematol*. 2011; 86: 38-46. (Publication)
- **Vaitkevičienė G**, Ragelienė L. Naviko lizės sindromas ir inkstų funkcijos nepakankamumas vaikams, sergantiems ūmine leukemija ir solidiniais navikais [Tumor lysis syndrome and renal impairment in children with acute leukemia and solid tumors]. *Medicinos teorija ir praktika* 2011; 18(1): 112-117. Article in Lithuanian. (Publication)
- **Vaitkevičienė G**, Matuzevičienė R, Stoškus M, Žvirblis T, Ragelienė L, Schmiegelow K. Cure results of childhood acute lymphoblastic leukemia in Lithuania and the benefit of joining the international collaboration. (Manuscript ready for submission)

List of publications not included to dissertation

- Coenen EA, Zwaan CM, Reinhardt D, Harrison CJ, Haas OA, de Haas V, Mihál V, De Moerloose B, Jeison M, Rubnitz JE, Tomizawa D, Johnston D, Alonzo TA, Hasle H, Auvrignon A, Dworzak M, Pession A, van der Velden VHJ, Swansbury J, Wong K, Terui K, Savasan S, Winstanley M, **Vaitkeviciene G**, Zimmermann M, Pieters R and van den Heuvel-Eibrink MM. Pediatric acute myeloid leukemia with t(8;16)(p11;p13): a distinct clinical and biological entity, a collaborative study by the International-Berlin-Frankfurt-Munster AML-study group. *Blood* 2013 (Epub ahead of print)

- Toft N, Birgens H, Abrahamsson J, Bernell P, Griškevičius L, Hallböök H, Heyman M, Holm MS, Hulegårdh E, Klausen TW, Marquart HV, Jónsson OG, Nielsen OJ, Quist-Paulsen P, Taskinen M, **Vaitkeviciene G**, Vettenranta K, Åsberg A, Schmiegelow K. Risk group assignment differs for children and adults 1-45 yr with acute lymhpoblastic leukemia treated by the NOPHO ALL-2008 protocol. *Eur J Haematol* 2013 90(5):404-12. (Publication)
- Simanauskiene E, Daugelaviciene V, Laurinavicius A, Mickys U, Simonyte V, **Vaitkeviciene G**, Verkauskas G. Unilateral hydronephrosis and renal damage after acute leukemia. Case Rep Med. 2012; Epub 2012 Apr 3. (Publication)
- Ragelienė L, **Vaitkevičienė G,** Tamašauskaitė I. Vaikų ne Hodžkino limfoma. Ilgalaikė 1990-2008 metų laikotarpio Vilniaus universiteto vaikų ligoninės Onkohematologijos skyriaus patirtis. [Childhood non-Hodgkin lymphoma treated in 1990-2008 at Vilnius University Children‘s Hospital, Center for Oncology and Haematology. Long-term single-institution experience]. *Visuomenės sveikata*, 2011 Nr. 3(54):43-50. Article in Lithuanian (Publication)

10. Summary in Lithuanian

Leukemija yra sisteminė piktybinė kaulų čiulpų ir periferinio kraujo liga, išsivystanti dėl kloninės nesubrendusių limfoidinės arba mieloidinės eilės ląstelių proliferacijos sutrikus šių ląstelių diferenciacijai bet kurioje jų vystymosi stadijoje. Disertacijos objektu pasirinkta vaikų ūminė limfoblastinė leukemija (ŪLL).

Išsivysčiusiose šalyse ilgalaikis vaikų, sirgusių ŪLL, išgyvenamumas siekia beveik 90%. Lietuvoje vaikų ŪLL epidemiologija ir gydymo rezultatai detaliai nagrinėti nebuvo.

Leukocitų skaičius ilgą laiką buvo laikomas vienu svarbiausių nepriklausomų rizikos veiksnių. Pastaruoju metu nustatyti nauji rizikos veiksniai, pvz., ląstelių genetinės aberacijos bei ligos atsako į gydymą dinamika. Leukocitų skaičius (LS) pradėjo prarasti savo prognostinę reikšmę, tačiau bendrame kontekste su naujaisiais rizikos kriterijais jo reikšmė populiaciniuose tyrimuose tyrinėta nebuvo.

Labai didelis LS periferiniame kraujyje yra ūminė pavojinga gyvybei klinikinė situacija onkologijoje susijusi su dideliu komplikacijų skaičiumi ir didesniu mirtingumu, nes didėja kraujo klampumas, vystosi leukostazė ir sutrikdoma mikrocirkuliacija. Prasidėjus masyviam didelio kiekio leukocitų irimui iš ląstelių išsiskiria įvairūs metabolitai, sukeldami naviko lizės sindromą. Didelių tyrimų, analizuojančių tokių ligonių kliniką ir gydymą publikuota nedaug, trūksta pradinio gydymo rekomendacijų.

Pirminis disertacijos tikslas buvo įsitraukti į tarptautinio gydymo protokolo veiklą ir tokiu būdu pagerinti vaikų ŪLL gydymo rezultatus Lietuvoje. Antrinis disertacijos tikslas buvo ištirti biologinius veiksnius, lemiančius pradinį leukocitų skaičių (LS) diagnozuojant vaikų ŪLL ir LS prognostinę reikšmę ankstyviems neigiamiems įvykiams bei ilgalaikiam išgyvenamumui, kai į tyrimą įtraukiami kiti žinomi prognostiniai veiksniai.

Disertacijoje aprašyti trys vienas kitą papildantys tyrimai, į kuriuos įtraukti 3159 ŪLL sirgę vaikai, 1992–2012 m. gydyti Lietuvoje ir Šiaurės šalyse (Danijoje, Islandijoje, Norvegijoje, Švedijoje, Suomijoje).

I tyrimo metu buvo išnagrinėta vaikų ŪLL epidemiologija ir gydymo rezultatai Lietuvoje 1992–2012 m. (N=459). Tiriamojo laikotarpio pradžioje vaikų, sergančių ŪLL, išgyvenamumas Lietuvoje maždaug 20%, atsiliko nuo tarptautinių rezultatų. 5 metų išgyvenamumas be neigiamo įvykio pagerėjo nuo 50 ± 4% 1992-1996 metų laikotarpiu iki 71 ± 4% 2003-2008 metų laikotarpiu ($p < 0,001$). Įsitraukus į tarptautinį gydymo protokolą NOPHO ALL-2008, vaikų ŪLL gydymo rezultatai Lietuvoje priartėjo prie išsivysčiusių šalių vaikų ŪLL tyrimo grupių skelbiamų rezultatų.

II tyrime analizuota biologinė pradinio LS sergant ŪLL reikšmė tarp 2638 iki 15 m. amžiaus vaikų, 1992–2008 m. gydytų Šiaurės šalyse. Nustatyta, kad ŪLL su skirtingomis genetinėmis aberacijomis pasireiškia skirtingu pradiniu leukocitų skaičiumi. Rasta neigiama koreliacija tarp ligonio amžiaus ir pradinio leukocitų skaičiaus. Įtraukus ir kitus žinomus prognostinius veiksnius, nustatyta, kad esant lėtam atsakui į gydymą ŪLL sergančių vaikų išgyvenamumas be neigiamo įvykio buvo reikšmingai blogesnis vaikams, kurie turėjo LS $\geq 100 \times 10^9$/L.

III tyrime analizuota hiperleukocitozės (LS $\geq 200,0 \times 10^9$/L) klinikinė ir prognostinė reikšmė tarp <15 m. amžiaus Lietuvoje ir Šiaurės šalyse 1992–2011 m. gydytų vaikų (N=221). Nustatyta, kad didelis leukocitų skaičius, o ne naviko lizės sindromas buvo pagrindinis ankstyvo mirtingumo rizikos veiksnys. Vėliau pradėtas specifinis ŪLL gydymas ar sumažintos pradinės chemopreparatų dozės galėjo turėti neigiamos reikšmės ankstyvų mirčių išsivystymui. Sudarytos bendros Baltijos ir Šiaurės šalių centrams skirtos rekomendacijos dėl pradinės ligonių su ŪLL ir hiperleukocitoze bei didele naviko lizės išsivystymo rizika (LS $\geq 100 \times 10^9$/L), gydymo taktikos.

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12.Appendices

Appendix I. Permissions to conduct biomedical trials given by authorities in Lithuania

Copy of a favourable opinion to conduct a clinical trial on medicinal products by Lithuanian Bioethics Committee

PATVIRTINTA
Lietuvos bioetikos komiteto
pirmininko 2008 m. gruodžio 31 d.
įsakymu Nr. V-19

LIETUVOS BIOETIKOS KOMITETAS

Valstybės biudžetinė įstaiga, Didžioji g. 22, LT-01128 Vilnius, tel. (8 5) 212 4565,
faks. (8 5) 260 8640, el. p. lbek@sam.lt , http://bioetika.sam.lt
Duomenys kaupiami ir saugomi Juridinių asmenų registre, kodas 188710595

PRITARIMO ATLIKTI KLINIKINĮ VAISTINIO PREPARATO TYRIMĄ LIUDIJIMAS

2009-03-05 Nr. P-09-009
Vilnius

Tyrimo pavadinimas: **NOPHO-ALL 2008 gydymo protokolas vaikų (1-17,9 metų amžiaus) ūmiai limfoblastinei leukemijai gydyti**	
EudraCT Nr.:	**2008-003235-20**
Protokolo Nr.:	**NOPHO ALL-2008**
Versija:	**1c-2**
Data:	**2009 m. vasario 06 d.**
Asmens informavimo forma lietuvių kalba (tėvams/globėjams):	
Versija:	**2**
Data:	**2009 m. vasario 13 d.**
Asmens informavimo forma lietuvių kalba (15-18 metų ligoniams):	
Versija:	**2**
Data:	**2009 m. vasario 13 d.**
Informuoto asmens sutikimas dalyvauti tyrime lietuvių kalba:	
Versija:	**1**
Data:	**2008 m. lapkričio 30 d.**
Pagrindinis tyrėjas:	**gyd. Goda Vaitkevičienė**
Tyrimo vieta (įstaigos pavadinimas):	**Vilniaus Universiteto Vaikų Ligoninė**
Adresas:	**Santariškių 4, Vilnius, Lietuva**

Pritarimo liudijimas išduotas Lietuvos bioetikos komiteto Biomedicininių tyrimų ekspertų grupės posėdžio, įvykusio **2009 m. sausio 20 d.**, sprendimu.

Vyriausioji specialistė,
l. e. pirmininko pareigas — Ingrida Narušytė-Daugėlienė

Copy of permission to conduct clinical trial by State Medicine Control Agency at the Ministry of Health of the Republic of Lithuania

VALSTYBINĖ VAISTŲ KONTROLĖS TARNYBA
PRIE LIETUVOS RESPUBLIKOS
SVEIKATOS APSAUGOS MINISTERIJOS

LEIDIMAS ATLIKTI KLINIKINĮ VAISTINIO PREPARATO TYRIMĄ

2009 m. balandžio 9 d. Nr. 12KL-68

Vilnius

Klinikinio tyrimo pavadinimas: **NOPHO-ALL 2008 gydymo protokolas vaikų (1-17,9 metų amžiaus) ūmiai limfoblastinei leukemijai gydyti**	
Protokolo Nr.	**NOPHO ALL-2008**
Data:	**2009 m. vasario 6 d.**
Versija:	**1c-2**
EudraCT Nr.	**2008-003235-20**
Pagrindinis tyrėjas:	**Gyd. Goda Vaitkevičienė**
Klinikinio tyrimo vieta (įstaigos pavadinimas):	**Vilniaus universiteto vaikų ligoninė**
Adresas:	**Santariškių 4, Vilnius**

Leidimo išdavimo pagrindas: VVKT Farmakologinio budrumo, geros laboratorinės ir geros klinikinės praktikos priežiūros komisijos 2009 m. balandžio 6 d. posėdžio sprendimas.

Viršininkas Mindaugas Būta

Copy of permission to conduct biomedical trial by Vilnius Regional Biomedical Trials Ethics Committee

VILNIAUS UNIVERSITETO MEDICINOS FAKULTETAS

Kodas211950810, M.K.Čiurlionio 21/27, 03101,Vilnius Tel.(85)2398701, 2398700,faks.2398705,El.p. mf@mf.vu.lt

VILNIAUS REGIONININIS BIOMEDICININIŲ TYRIMŲ ETIKOS KOMITETAS

M.K.Čiurlionio 21/27, LT-03101,Vilnius Tel.(85) 2686998, el.p.: rbtek@mf.vu.lt

LEIDIMAS ATLIKTI BIOMEDICININĮ TYRIMĄ

2011-10-04 Nr.158200-10-407-111

Tyrimo pavadinimas:

Leukeminių blastų subpopuliacijų ir ligonių genetinio polimorfizmo įtaka leukeminės masės išsivystymui bei šių biologinių faktorių prognostinė reikšmė vaikams, sergantiems ūmine limfoblastine leukemija

Protokolo Nr.: 001
Versija: 1
Data: 2011-09-09
Asmens informavimo forma tėvams/globėjams (lietuvių kalba):
Versija: 1
Data: 2011-09-09
Asmens informavimo forma 10-18 m. paaugliams (lietuvių kalba):
Versija: 1
Data: 2011-09-09
Asmens informavimo forma vaikams iki 10 m. (lietuvių kalba):
Versija: 1
Data: 2011-09-09
Pagrindiniai tyrėjai: L.Ragelienė (G.Vaitkevičienė)
Biomedicininio tyrimo vieta:
Įstaigos pavadinimas: Vaikų ligoninė, VšĮ Vilniaus universiteto ligoninės „Santariškių klinikos“ filialas
Įstaigos adresas: Santariškių g.1, Vilnius LT-08660

Leidimas išduotas Vilniaus regioninio biomedicininių tyrimų etikos komiteto posėdžio (protokolas Nr. 158200-2011/10), vykusio 2011 m. spalio 04 d., sprendimu.

Vilniaus regioninio biomedicininių tyrimų etikos komiteto ekspertų grupės nariai			
Nr.	Vardas, pavardė	veiklos sritis	dalyvavo posėdyje
1	doc. Dr.Laimutė Jakavonytė	filosofija	taip
2	doc. Dr. Kęstutis Žagminas	epidemiologija	taip
3	dr. Indrė Isokaitė	teisė	ne
4	dr. Marija Veniūtė	visuomenės sveikata	ne
5	doc.dr. Jolanta Gulbinovič	medicina	ne
6	prof.dr. Vytautė Pečiulienė	medicina, odontologija	taip
7	Laura Malinauskienė	medicina	taip
8	dr. Gražina Pastavkaitė	klinikinė psichologija	ne
9	Ugnė Šakūnienė	pacientų teisės	taip

Pirmininkė Vytautė Pečiulienė

VILNIAUS UNIVERSITETAS * Vilniaus regioninis biomedicininių tyrimų etikos komitetas * MEDICINOS FAKULTETAS

Appendix II.

Questionnaire for Study III. Hyperleukocytosis (WBC ≥200 x 10^9/L) at diagnosis of childhood ALL

*Please fill in the underscored boxes that expand while writing. Date format is **dd/mm/yyyy**.*

Questionnaire completed by: _____ ***Completion date:*** _____
Contact person in the department _____, ***e-mail:*** _____

I. Personal information on the patient

NOPHO number: _____ Sex: ☐M ☐F

Initials: _____

Date of birth: _____ Date of diagnosis: _____

Height at Dx, cm ____ Weight at Dx, kg ____

II. ALL biology concerning data

Phenotype: ☐PreB ALL ☐T-ALL ☐Biphenotypic Other: _____

Normal karyotype:	☐Yes	☐No	☐Not available
t(12;21)	☐Yes	☐No	☐Not available
t(1;19)	☐Yes	☐No	☐Not available
dic(9;20)	☐Yes	☐No	☐Not available
ic21amp	☐Yes	☐No	☐Not available
11q23/*MLL* rearrangement	☐Yes	☐No	☐Not available
t(9;22)	☐Yes	☐No	☐Not available
Hypodiploid (<44 crom.)	☐Yes	☐No	☐Not available

Other: _____

Comments: _____

III. Clinical symptoms (at presentation and/or if developed during the period until WBC redused to <50.0 x 10^9/L)

1. Extramedullary disease

a. Mediastinal mass ☐Yes ☐No

b. Liver enlargement ☐Yes ☐No

If yes, cm below costal margin: _____

If yes, and not measured in cm below costal margin,

then the size measured by US: _____

c. Spleen enlargement ☐Yes ☐No

If yes, cm below costal margin: _____

If yes, the size measuered by US: _____

d. Lymphnodes >3.0 cm ☐Yes ☐No

e. Testis involvement (boys only): ☐Yes ☐No

If yes: ☐Unilateral ☐Bilateral

If yes, diagnosis confirmed: (you may mark several options)

☐Clinically ☐US ☐Biopsy

Other (e.g. not examined, not sure about testis involvement, etc.) _____

Comments: _____

2. CNS and/or peripheral nerve affection

a. Clinical signs, if regarded to be related to high WBC (from mild confusion and somnolence to stupor and coma):

☐Yes ☐No If yes, date of onset: _____

If yes, please specify: _____

b. Date of the first lumbar puncture: _____

b. Number of leukocytes in CSF (cells/mkl): _____

c. Number of blasts in CSF (cells/mkl): _____

d. Number of RBC in CSF (cells/mkl) _____

d. Brain MRI and/or CT performed: ☐Yes ☐No

If yes, summary of the results: _____

Comments: _____

3. Major bleedings and thrombosis (e.g. retinal hemorrhage, retinal vein thrombosis, acute renal vein ischemia, disseminated intravascular dissemination, other):

☐Yes ☐No If yes, date of onset: _____

If yes, please specify: _____

Comments: _____

4. Pulmonary symptoms, if regarded to be related to high WBC (e.g., tachypnea, hypoxia, severe respiratory distress)

☐Yes ☐No If yes, date of onset: _____

If yes, please specify: _____

Infiltration determined by chest X ray and/or CT and/or MRI, regarded to be related to high WBC:

☐Yes ☐No If yes, date of first findings: _____

Comments: _____

5. Priapism (boys only) ☐Yes ☐No If yes, date of onset: _____

Comments:_____

6. Other serious symptoms, likely to be caused by high WBC:

☐Yes ☐No If yes, please specify: _____

Comments: _____

7.Peripheral blood and metabolites tests starting with the day of admission until full induction with VCR and Doxo

Date:	_____	_____	_____	_____	_____	_____	_____
WBC, $x10^9$/L	_____	_____	_____	_____	_____	_____	_____
Ca^2+(mmol/L)	_____	_____	_____	_____	_____	_____	_____
K+ (mmol/L)	_____	_____	_____	_____	_____	_____	_____
PO4- (mmol/L)	_____	_____	_____	_____	_____	_____	_____
Creatinine (mkmol/L)	_____	_____	_____	_____	_____	_____	_____
Uric acid (mkmol/L)	_____	_____	_____	_____	_____	_____	_____
LDH (U/L)	_____	_____	_____	_____	_____	_____	_____
CRP (mg/L)	_____	_____	_____	_____	_____	_____	_____
*Fever, T>38.5°, mark if Yes	☐	☐	☐	☐	☐	☐	☐
*The name of antibacterial drug, if	_____	_____	_____	_____	_____	_____	_____

given

Coagulation test abnormal: ☐Yes

V. Treatment

NOPHO protocol: ☐-92 ☐-2000 ☐-2008 Other: _____

1. Transfusions:

Hgb value at admittance to the hospital (use the dimension used in your institution):

_____g/L _____mg/mL _____ mmol/L

Packed red blood cells (PRBC) transfused until WBC <200.0 x 10^9/L: ☐Yes ☐No

If yes, the highest Hb value before transfusion:

_____g/L _____mg/mL _____ mmol/L

Number of PRBC transfusions until WBC <200.0 x 10^9/L _____

Comments: _____

2. Leukapheresis performed: ☐Yes ☐No

If yes, put the data on each leukapheresis procedure into the table:

	Date:	**WBC x 10^9/L before procedure**	**WBCx10^9/L within 4 hours after procedure, if measured**
1.	_____	_____	_____
2.	_____	_____	_____
3.	_____	_____	_____
4.	_____	_____	_____

Comments:_____

3. Exchange transfusions performed: ☐Yes ☐No

If yes, please put the data on exchange transfusions in the table below:

	Date	**WBC x 10^9/L before procedure**	**WBC x 10^9/L within 4 hours after procedure, if measured**
1.	_____	_____	_____
2.	_____	_____	_____
3.	_____	_____	_____

Comments:_____

4. Dialysis performed: ☐Yes ☐No

If yes, date of the first procedure: _____

Comments: _____

5. Treatment to prevent tumor lysis syndrome

a. Urinary alkalinisation ☐Yes ☐No

If yes, date of start: _____

b. Allopurinol ☐Yes ☐No

If yes, date of start: _____

c. Rasburicase ☐Yes ☐No

If yes, dates of Rasburicase given: _____ _____ _____ _____ _____ _____ _____

d. Acetazolamine ☐Yes ☐No

If yes, dose/kg _____

e. Hyperhydration until WBC <50.0 x 10^9/L:

Starting hydration volume:

☐<2500 ml/m^2/24 h

☐2500 - <3000 ml/m^2/24 h

☐3000 ml/m^2/24 h

☐>3000 – <3500 ml/m^2/24 h

☐3500 - <4000 ml/m^2/24 h

☐4000 - <4500 ml/m^2/24 h

☐≥4500 ml/m^2/24 h

f. Treatment in ICU within 4 weeks from diagnosis: ☐Yes ☐No

If yes, date of start _____

If yes, the reason for treatment in ICU _____

Comments: _____

6. Specific treatment

a.Glucocorticosteroid (GC) prephase:

☐Yes ☐No If yes, name of drug: _____

If yes, please fill in the table below. Please put the total dose of GC the patient received during the entire day starting from 0:00 to 24:00

Prephase day (presuming day of 1st VCR is day 0):	-7	-6	-5	-4	-3	-2	-1
Dose of GC, mg/ 24 hours	____	____	____	____	____	____	____

b. Date of 1st VCR _____

c. Date of 1st i.th. MTX _____

d. Date of 1st Doxorubicine _____

e. Other chemotherapy given not according to the protocol: ☐Yes ☐No

If yes, please specify: _____

If yes, date of first administration: _____

Comments: _____

VI. Other questions of relevance

1. General anaesthesia delayed because of high tumor burden: ☐Yes ☐No

Date of 1st general anaesthesia: _____

Complications because of general anaesthesia: ☐Yes ☐No

If yes, please specify: _____

Comments: _____

2. Date of first BM examination: _____

3. D15 BM analysed: ☐Yes ☐No If yes, date of analysis: _____

If performed, BM: ☐Hypocellular ☐Normo/hypercellular

☐M1 ☐M2 ☐M3

4. d29 BM analysed: ☐Yes ☐No If yes, date ofanalysis: _____

If analysed, BM ☐Hypocellular ☐Normo/hypercellular

☐M1 ☐M2 ☐M3

Comments: _____

VII. Major events within 4 weeks from diagnosis

1. Death ☐Yes ☐No If yes, date of death: _____

If yes, cause of death: _____

Comments: _____

Thank you very much for sparing your time to fill in the questionnaire. You may return the form:

1. by e-mail to goda.vaitkeviciene@rh.regionh.dk
2. by Fax.
3. by ordinary mail:

Goda Vaitkeviciene
Dep. of Oncology and Hematology
Vilnius University Children Hospital
Santariskiu 4
08406, Vilnius
Lithuania

My best regards,
Goda

Appendix III.

Guideline for initial treatment of ALL patients with hyperleukocytosis and high risk for TLS (WBC ≥100 x 10^9/L)

The guideline is addressed for ALL patients with hyperleukocytosis (WBC ≥100 x109/L) only and should not be used without modifications in case of other diseases with high tumor burden such as AML or Burkitt lymphoma. The guideline has been prepared based on the results of retrospective Nordic/Baltic study.

Hyperleukocytosis

About ten percent of children with ALL will have a WBC at diagnosis above 100 x10^9/L. These patients are at risk of tumor lysis syndrome (TLS) before or after initiation of antileukaemic therapy. In addition, a few percent of such children will have a WBC >400 x10^9/L and will have a high risk of life-threatening CNS complications (leukostasis with thrombosis or bleeding) during the first days after admission .

Hyperleukocytosis causes mainly neurological, pulmonary, bleeding or metabolic complications. CNS leukostasis may cause headache, mental changes, seizures, and brain haemorrhage. Pulmonary leukostasis may manifest as respiratory symptoms, hypoxia, and pulmonary infiltrates on X-ray. Metabolic complications are caused by tumour lysis (see below).

Increased blood viscosity is one of the threats in case of hyperleukocytosis. Unless the patient's condition is critical due to anaemia, red blood cell transfusions should be postponed, because it may substantially increase viscosity. In contrast, platelets may be given liberally since they do not contribute significantly to blood viscosity. A spurious elevation of the automated platelet count can occur due to the presence of fragments of white and red blood cells therefore platelets may have to be counted manually. Hydration decreases viscosity (see instructions below). Cytoreduction through

exchange transfusions or leukapheresis have been used in cases with very high leukocyte counts, but the benefit of this procedure remains uncertain. In this guideline the decision on whether to perform the cytoreduction procedure is left to the discretion of the treating centre, however cytoreduction can be initiated only after antileukemic therapy has been already started.

Tumor lysis syndrome

Acute tumour lysis syndrome (TLS) usually develops over 12-72 hours from the start of antileukemic therapy or in some cases spontaneously before the start of any antileukemic therapy due to the rapid release of intracellular products originating from lysis of leukemic cells. According to the internationally accepted definition of Bishop and Cairo the TLS is categorised as laboratory TLS (simultaneuos presence of two or more abnormal serum metabolite concentrations: uric acid ≥476 μmol/L, potassium ≥6.0 mmol/L, phosphates ≥2.1 mmol/L, or calcium ≤1.75 mmol/L) or clinical TLS (when laboratory derangements progress to organ dysfunction: creatinine ≥1.5 x upper limit of normal with or without urine retention, generalised seizures, or cardial arrhythmia) (Table 1).

Table 1. The criteria for diagnosis and classification of TLS.

Criteria for laboratory TLS	
Uric acid	≥476 μmol/L or 25% increase from baseline
Potassium	≥6.0 mmol/L or 25% increase from baseline
Phosphates	≥2.1 mmol/L or 25% increase from baseline
Calcium	≤1.75 mmol/L or 25% decrease from baseline
Criteria for clinical TLS	
Creatinine	≥1.5 upper limit of normal (ULN for <1 years 38.0; >1 <12 years 61.6 μmol/L; >12 years 88 μmol/L)
Cardiac arrhythmia/sudden death	
Seizure	

As defined by Cairo and Bishop

TLS will be aggravated by renal failure, which may be caused by preexisting renal disease, dehydration, precipitation of calcium-phosphate salts or uric acid or xanthine crystals in the kidneys, renal leukemic infiltration, uretheral compression by enlarged lymph nodes or leukemic infiltrates, and nephrotoxic drugs. Hyperphosphatemia is a common precipitating factor behind acute renal failure. Life-threatening cardiac arrhythmias may be caused by both hyperkalemia and hypocalcemia, and the latter may in addition cause muscle cramps and tetany. In addition, many patients experience nausea, vomiting, lethargy, edema, fluid overload and congestive heart failure, seizures, and sudden death.

General guidelines for ALL patients with WBC ≥100 x 10^9/L (Figure 1)

• **On the day of admission** (or within the first hours after hyperleukocytosis is detected):

1. Start hydration (without alkalinisation) ≥3000 ml/m^2/24 h. Hydration increases urinary output and thus increases excretion of potassium and phosphorus and reduces the risk of uric acid, xanthine, and/or calcium-phosphate precipitation in the kidneys. A high urinary output can be obtained by use of furosemide, but use of diuretics is contraindicated in patients with hypovolemia or obstructive uropathy.

2. Diagnostic bone marrow samples for cytomorphology, cytogenetics, karyotyping, flow cytometry should be taken. In rare cases with excessive hyperleukocytosis a general anaesthesia may be life-threatening and systemic antileukemic therapy may be necessary before the patient's general condition allows a bone marrow sample to be taken in general anaesthesia. In such cases the morphology, immunophenotype, cytogenetics, and molecular genetics can be done on peripheral blood. The lab should be asked to differentiate leukemic blasts as of lymphoid or myeloid lineage as soon as possible in order to promptly start proper antileukemic therapy.

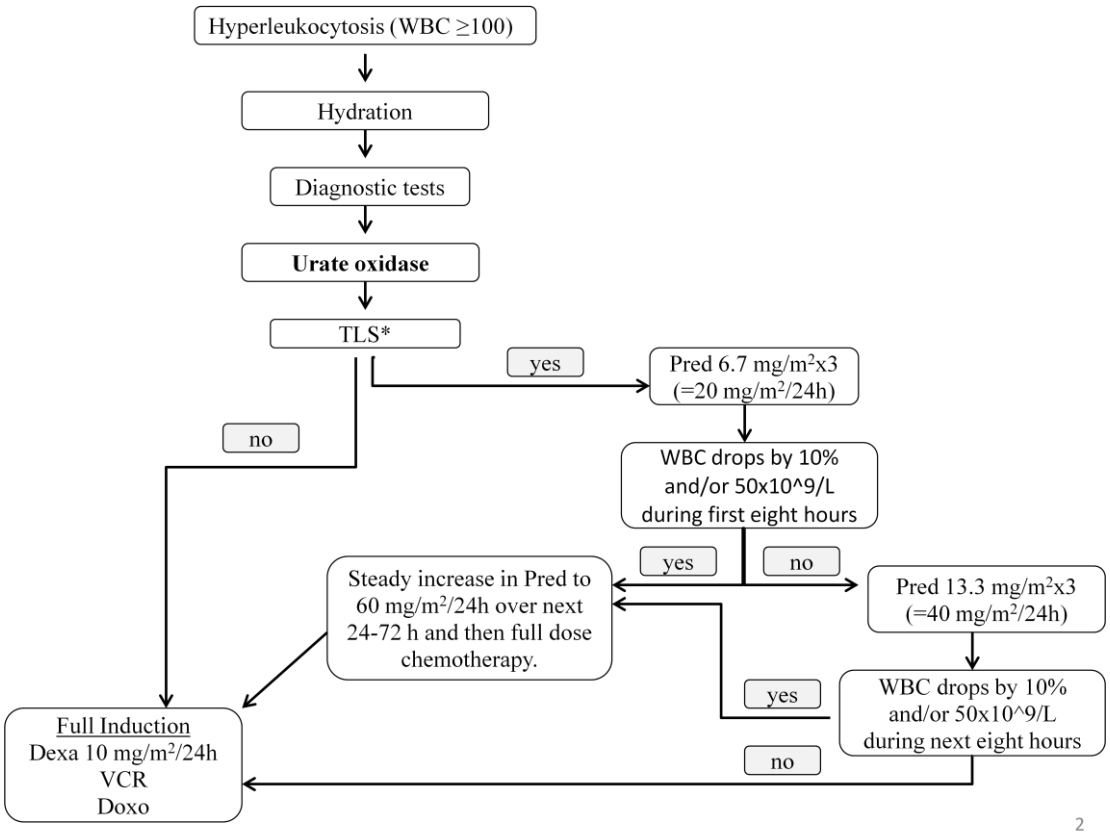
3. Biochemistry: uric acid, LDH, creatinine, electrolytes (K^+, PO_4^{2+}, Ca^{2+}, Na^+), coagulation tests; other - according to institutional guidelines.

4. Urate oxidase (Rasburicase) (0.1-0.2 mg/kg) should be given prophylactically, irrespective of uric acid concentration. One dose on the day of admission is given before the start of any antileukemic therapy. Rasburicase should be repeated if its level during the first three days rises above 100 µmol/L (see instructions below).

5. Corticosteroids should be started as soon as all above is fulfilled, i.e. diagnostic samples are taken, hydration is started and the first dose of urate oxidase (Rasburicase) is given.

6. Packed red blood cell transfusions should be avoided for the patients with WBC ≥200.0 unless the patient has critical anemia. Even in this situation it is often prudent to transfuse smaller volumes, e.g. 5 ml/kg.

Figure 1. Algorythm of initial treatment for ALL patients with WBC ≥100 x 10^9/L



*TLS = tumour lysis syndrome (see Table 1)

Start of antileukemic therapy

Systemic antileukemic therapy for patients with WBC $\geq$100 x 10^9/L should be initiated within the first 24 hours as soon as all necessary diagnostic samples have been obtained, hydration started and urate oxidase given.

Urate oxidase (Rasburicase)

Urate oxidase (*Rasburicase) in ALL-2008 is given prophylactically, irrespective of uric acid concentration for all ALL patients with WBC >100 x 10^9/L.* The recombinant urate oxidase catabolises uric acid to allantoin, which is 5-10 times more soluble in urine compared to uric acid. It should be given in a dose of 0.1-0.2 mg/kg as a 30-minute i.v. infusion once daily. After day 1, urate oxidase is administered if the plasma-urate concentration rises above 100 µmol/L during the first three days. Optimally, the first dose is administered about 4 hours before starting chemotherapy. Adverse reactions include allergy, fever, vomiting, nausea, headache, abdominal pain, diarrhea, mucositis and rash. Hemolysis and methemoglobinemia has been reported in patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency, and known G6PD deficiency is regarded as a contraindication for urate oxidase therapy.

Corticosteroids

Start with full induction with Dexamethasone 10 mg/m^2/24 h, VCR and Doxo is recommended within the first 24 hours for all ALL patients with >100 x10^9/L after hydration is started and urate oxidase given. However, for the patients who have metabolic derangements or clinical symptoms compatible with TLS at admission a prephase with Prednisolone 6.7 mg/m^2 x 3 (=20 mg/m^2/24 h) may be considered with rapid dose increase up to full induction within 48-72 hours. If the WBC does not drop by at least 10% or 50 x10^9/L within the next eight hours after the first Prednisolone dose, Prednisolone dose should be doubled to 13.3 mg/m^2 x 3 (=40 mg/m^2/24 h). If the WBC does not drop by at least 10% or 50 x10^9/L within the next eight hours after the second

Prednisolone dose, full induction with Dexametasone, VCR and Doxo should be started.

If oral administration is not possible, prednisolonesodiumsuccinate (at 1.33x the oral prednisolone dose) may be given i.v. If prednisolonesodiumsuccinate is not available for i.v. use, then give i.v. methylprednisolonesodiumsuccinate (the same dose as oral prednisolone) (see section 18).

Follow-up and monitoring guidelines

- WBC every 8 hours
- Uric acid*, creatinine, electrolytes (K^+, PO_4^{2+}, Ca^{2+}) every 6 - 8 hours
- **<u>*NB! Blood samples should be placed on ice for uric acid analysis after Rasburicase administration because of *ex-vivo* activity of enzyme</u>**
- Coagulation tests at least every 24 hours
- Careful monitoring of the urine output
- ICU and/or nephrologists should be notified in case of signs of severe metabolic disturbancies and/or renal impairment about possible necessity of dialysis

In case of WBC <100, and a large leukemic burden (e.g. extensive organomegaly) Allopurinol (10-15 mg/kg/day in 2-3 doses) should be given. The dose should be reduced down to 50% in case of renal insufficiency. Allopurinol will increase xanthine levels and the risk of xanthine-precipitation in the urine, since xanthine is less soluble than uric acid. Urate oxidase is optional, according to the center guidelines.

Management of TLS

Hyperkalemia should be verified with a second sample and ECG to rule out fictious hyperkalemia.

- For asymptomatic patients sodium polystyrene sulfonate 1 g/kg with 50% sorbitol (Kayexalate) orally or rectally
- For symptomatic patients:
  - Rapid-acting insulin (0.1 U/kg) and glucose (25% dextrose 2 ml/kg) infusion
  - Sodium bicarbonate (1 to 2 mEq/kg via IV push)
  - Calcium gluconate (100 – 200 mg/kg/dose via slow infusion with ECG monitoring)

Hyperphosphatemia if >2.1 mmol/L (1.5 mmol/L in adults).

- Avoid calcium infusions and increase hydration to 4500 or even 6000 ml/m^2/24h.
- Aluminium hydroxide 50 – 150 mg/kg/d orally every 6 hours (should be limited to 1 – 2 days)
- Other phosphate binders
  - Sevelamer hydroxide
  - Lanthanum carbonate
- In severe cases, continuous peritoneal dialysis or hemodialysis may be indicated. Hemodialysis is considered to be better as compared with continious veno-venous hemofiltration or peritoneal dialysis

Hypocalcemia. For asymptomatic patients no intervention is recommended.

- For symptomatic patients 10% calcium gluconate 50 – 100 mg/kg IV administration by slow intravenous infusion with ECG monitoring
- It usually resolves, when hyperphosphatemia is corrected.

Avoidance of nephrotoxic drugs, e.g. aminoglycosides.

Curriculum vitae

06-09-2013

Personal data. Goda Vaitkevičienė, born 1972-03-30. Married, three daughters

Education: *1996 graduated Vilnius University Faculty of Medicine*
1996 – 1999 residency of Pediatric diseases and Pediatric hematology

1998 passed exam for pediatric diseases at Vilnius University

1999 passed exam for pediatric hematology at Vilnius University

2009 October – 2013 October PhD student at Vilnius University.

2009 January – 2013 June research work as PhD student at Copenhagen University Hospital, Rigshospitalet at Bonkolab with Prof. Kjeld Schmiegelow as supervisor.

Main training programs and courses:

2002 May–July training at the Bone marrow Transplant Unit, at Wroclaw Medical University Children's Hospital, Wroclaw, Poland

2002 May Salzburg–Philadelphia seminar in pediatric hematology/oncology (organized by Children's Hospital of Philadelphia) in Salzburg, Austria

2004 April Advanced ICAS (International Center for Advanced Studies in Health Sciences and Services) training course 'Blood stem cell transplantation: State-of-the-arts, methods and perspectives' organized by Ulm University, Ulm, Germany

2003 – 2005 a cycle of NOPHO educational course on issues of pediatric oncology/hematology

2007 – 2008 special FECS (Federation of European Cancer Societies) project 'Nurses and doctors in pediatric oncology working together – special project'. SIOP – EONS project.

2005 – 2007 a cycle of course on pediatric oncology and hematology carried out by the Baltic Society for Pediatric Oncology and Hematology

2010 June 15-17 Educational course 'Regression and survival analysis using SPSS software'. IT-center for education and research of Denmark

2010 November 10-12 Research course in pediatric hematology. Århus University, Denmark

2011 October 13-15 NOPHO update course 2011, Copenhagen, Denmark

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Emploiment:

Since 2002 as pediatric oncologist and hematologist at Vilnius University Children Hospital, Department for Oncology and Hematology, Bone Marrow Transplantation Unit.

January 2009– June 2013 50% time as clinical assistant at JMC, Rigshospitalet, Copenhagen and 50% time as pediatric oncologist at Vilnius University Children Hospital

Field of research: Acute lymphoblastic leukemia in children.

Other activities:

1. Principle investigator in Lithuania for the NOPHO ALL 2008 protocol since 2009-
2. Member of the Events group of NOPHO ALL-2008 protocol since 2011-.
3. Member of NOPHO ALL-Relapse groups since 2013-
4. One of the founders of the Baltic Society for Pediatric Oncologists and Hematologists (BSPOH). General Secretary 2005-2008. Board member since 2008-
5. Board member of Lithuanian Society of Pediatric Oncologists and Hematologists since 2011-.
6. Member of European organization of bone marrow transplantation (EBMT) since 2002-.

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