Vascular density and bone marrow fibrosis in childhood acute lymphoblastic leukemia

Ulrika Norén Nyström
Cover pictures: Sections from bone marrow biopsies taken at diagnosis from children with acute lymphoblastic leukemia. Section to the left: vessels stained with von Willebrand-factor. Section to the right: silver impregnation staining of reticulin fibers (fibrosis).
“Life is too short to drink bad wine”
Anonymous

To Hanna, Viktor and Fredrik
ABSTRACT

Background: In childhood acute lymphoblastic leukemia (ALL), the cure rate has now reached 80% in the western world. Even so, 15–20% will die from the disease or treatment-related causes, among them children who did not present any known unfavorable features at diagnosis. Treatment of childhood ALL is risk-adapted, meaning that certain factors that are related to the child or the leukemic blasts stratifies to more or less intensive treatment. In this thesis, characteristics of the bone marrow (BM) stroma, reflecting the interaction between the leukemic cells and their microenvironment, were evaluated. The aims were to investigate these factors in relation to other known data in order to further understand the biology of leukemia, and to suggest additional risk factors that would further improve decision making for the treatment of individual children diagnosed with ALL.

Methods: We retrospectively investigated microvessel density (MVD), blast-congested vessel fraction (BCVF), and degree of fibrosis – reticulin fiber density (RFD) – in sections from diagnostic BM biopsies from children diagnosed in Umeå, Uppsala, and Stockholm. RFD was also studied in BM sections from treatment day 29.

Results: RFD had prognostic impact in patients with high-hyperdiploid (HeH) leukemia. Moreover, rapid reduction of RFD during induction treatment was associated with a favorable prognosis compared to slow reduction, in B-cell precursor (BCP) ALL patients. There was also a correlation between RFD at diagnosis and minimal residual disease (MRD) measured by flow cytometry on treatment day 29 in BCP patients. BCP patients with high RFD and high MVD had an unfavorable outcome compared to all other BCP patients. In addition, MVD and RFD were both associated with immunophenotype, and MVD with cytogenetic aberrations. There was a correlation between MVD and WBC count in BCP high-risk patients. There was also a strong correlation between BCVF and WBC count in all BCP patients, but not between BCVF and MVD or RFD.

There was a negative correlation between MVD and in vitro cellular resistance to several drugs in BCP patients. A drug-resistance score combining the drugs most strongly correlated to MVD – cytarabine, doxorubicin, and dexametasone (ADD score) – identified the prognostic potential of ADD score in HeH patients with no unfavorable features.

Conclusions: Taken together, these studies indicate that stroma factors in leukemia are related to both phenotypic and genotypic features of acute leukemia. Stroma factors also seem to influence the response to induction treatment, in vitro drug resistance, and outcome in certain subgroups of childhood ALL patients. The results emphasize the importance of BM stroma in leukemia and the need for greater use of BM biopsy at diagnosis.
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This thesis is based on the following papers, which are referred to in the text by their Roman numerals:


ABBREVIATIONS

ALL Acute lymphoblastic leukemia
AML Acute myeloid leukemia
Ara-C Cytarabine
BCVF Blast-congested vessel fraction
bFGF Basic fibroblast growth factor
BM Bone marrow
CCR Complete continuous remission
CML Chronic myeloid leukemia
CNS Central nervous system
ECM Extra cellular matrix
EFS Event free survival
EPC Endothelial progenitor cell
FMCA Fluorometric microculture cytotoxicity assay
HeH High-hyperdiploid (51 – 61 chromosomes)
HCL Hairy cell leukemia
HR High-risk
LR Low-risk
MDR Multi-drug resistance
MMP Matrix metalloproteinase
MRD Minimal residual disease
MVD Microvessel density
NCI National Cancer Institute
NOPHO The Nordic Society of Paediatric Haematology and Oncology
pDFS Probability of disease free survival
PDGF Platelet-derived growth factor
pRFS Probability of relapse free survival
RD Resistant disease
RFD Reticulin fiber density
SI Survival index
SJCRH St Jude Children’s Research Hospital
SMN Secondary malignancies after anti-cancer chemotherapy
TGF-β Transforming growth factor-β
WBC White blood cell
VEGF Vascular endothelial growth factor
INTRODUCTION

Childhood acute lymphoblastic leukemia (ALL)

General background and short history
ALL is the most common malignancy in childhood. The incidence in the Nordic countries – 3.9 per 100,000 children per year – has been stable over the last three decades (1). In Sweden, this means that roughly 75–85 children are diagnosed with ALL every year. This disease can occur at any age in childhood, with an incidence peak at two to seven years of age (1,2). Boys have a slightly higher incidence than girls (3). Internationally, a considerable degree of geographic variation is found, and from birth to 14 years of age the annual incidence rates range from 3-4 to 46 per million. The highest incidences reported have been in Costa Rica, Canada, Hong Kong, USA (among Caucasians) and the Nordic countries. The lowest incidence has been reported in India and in some African countries, but reporting from the developing countries, if it exists at all, is most likely uncertain (4). This geographic variation is also due to ethnic differences. The incidence is higher in white children than in black children, and appear to be highest in children of Hispanic origin (5).

Leukemia as a distinct disease, “white blood”, was described for the first time in 1845–1846 by Virchow (6), Bennett (7), and Craigie (8) in separate reports. Until the 1960s, childhood ALL was invariably fatal. Farber et al. reported temporary remissions with antimetabolite treatment for the first time in 1948 (9). During the next two decades, cortisone, 6-mercaptopurine, 6-thioguanine, cyclophosphamide, and vincristine were introduced – with remissions as a result but very disappointing cure rates. In the mid-1960s, low-dose cranio-spinal irradiation was introduced and some years later this treatment was combined with intrathecal methotrexate to prevent meningeal relapse. In 1971, a cure rate of 50% was documented for the first time using these combinations (10). Since then, the progress and outcome of leukemia treatment have been described as a success story. Several new chemotherapeutic drugs have been introduced, or already existing drugs have been refined (for example, cytarabine, anthracyclins, asparaginase). Improvements in supportive care and risk-stratified treatment regimes developed through consecutive controlled trials have also contributed to the positive development. At the beginning of the 21st century, the cure rate had reached 80% in the western world (3,11-22). The diagnostic procedures and treatment regimes are, however, too complex and expensive for many undeveloped countries, leaving the majority of the world’s children with ALL without any chance of cure.

Leukemia – definition and clinical symptoms
Leukemia is a clonal disease originating from lymphoid precursor cells in the BM. Maturation and differentiation is blocked, and the malignant clone proliferates and expands. Blasts can also invade extramedullary sites such as the lymph nodes, the spleen, the liver, the kidneys, the central nervous system (CNS), and the testes, and are frequently found in peripheral blood. Normal hematopoiesis is eventually suppressed and the clinical picture reflects the degree of BM suppression and extramedullary involvement. Patients often present with fever, fatigue, bone pain,
and other symptoms of a non-functioning BM such as paleness, bruises, and infections. Most cases have an acute, clear-cut onset, while in others indistinct signs and symptoms can prevail for months before the definitive diagnosis.

**Diagnosis – immunophenotype**

When leukemia is suspected due to some or all of the symptoms described above, the diagnosis is based on a BM sample. The diagnostic course first involves classification of the leukemic blasts according to lineage and degree of differentiation. BM smears are always examined morphologically, and can sometimes be used to distinguish lymphoid from myeloid leukemia. To establish the exact lineage and degree of differentiation of the leukemic blast, immunophenotyping by flow cytometry is essential. By defining the expression of a range of surface markers on the lymphoblasts, it is possible to determine the origin of the leukemia by identifying the relevant differentiation stage in B cell development (23). For the clinical management of patients, however, it is only necessary to distinguish between T-cell cases and mature B-cell cases from all other B-cell lineage (B-cell precursor, BCP).

**Diagnosis - cytogenetic aberrations**

The second part in the diagnostic process of characterizing the leukemic blast is detection of genetic aberrations. The malignant transformation of the lymphoid precursor cell arises due to specific genetic damage. Thus, ALL can be classified according to genetic changes – numerical changes (gain or loss of a chromosome) and structural changes such as, for example, translocations, deletions, insertions, etc. In some cases, these cytogenetic abnormalities are of prognostic importance. The most common cytogenetic changes in childhood BCP-ALL are illustrated in Figure 1. In a study of all patients diagnosed in the five Nordic countries between 1993 and 2003, 78.5% of patients with childhood ALL had detectable genetic aberrations; the remaining 21.5% had unsuccessful cytogenetic investigations (8%) or were cases with a normal (diploid) result (13.5%) (2). Cytogenetic abnormalities probably give rise to all known forms of leukemia. In many cases, a normal (diploid) result from the karyotyping results from growth of normal cells and is not derived from the leukemia clone, and it should thus be considered unsuccessful. Cryptic aberrations, so that molecular biological techniques are required for detection since conventional G-banding is not sufficient, are another explanation for “normal” karyotypes. High-hyperdiploidy (HeH, 51–61 chromosomes) and t(12;21)(p13;q22) are the two most common cytogenetic subgroups. When no unfavorable feature is present, both are generally associated with good prognosis when low-intensity treatment is used (24-26). t(12;21) is a cryptic aberration that was first recognized in 1994 (27). Routine diagnostic methods for detection of t(12;21) were introduced in Sweden in 1998.

Patients with HeH leukemia are a heterogeneous group with various trisomies (trisomy: one extra copy of a chromosome). Some trisomies are more frequent than others. The modal chromosome number peak is 55 in this group (25,28); thus nine extra chromosomes is most common. Gains of chromosomes 4, 6, 10, 14, 17, 18, 21 and X are the most frequent in general (28), although specific chromosome gains have been seen in a modal number dependent pattern (29). Outcome for HeH patients is generally good for children < 10 years of age, but slightly worse for older
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Figure 1. Cytogenetic sub-groups in 963 BCP-ALL patients, 0–14.9 years of age diagnosed in the five Nordic countries 1993–2003 (Patients with Downs syndrome excluded). "Other" represents all other colonal abnormalities including hypodiploid, pseudohypodiploid, near triploid and near tetraploid cases. Modified from Forestier and Schmiegelow, J Pediatr Hematol Oncol 2006.

children (26,30). Studies exploring the prognostic importance of certain trisomies have given different results. Harris et al. reported that combined trisomy of chromosomes 4 and 10 independently predicted a very favorable event-free survival (EFS) (31). Another American report stated that trisomy of chromosome 10 was the most significant prognostic factor for HeH patients, yet trisomy of chromosome 17 and a modal chromosome number of 54–58 were also independent factors (32). In the UK, girls aged 1–9 with trisomy of chromosome 18 had the best EFS (28), and in a third study from the US, NCI standard risk patients (see below) whose leukemic cells had simultaneous trisomies for 4, 10 and 17 had the best EFS in two independent study groups (33).

ALL patients positive for t(1;19)(q23;p13) previously had a poor prognosis, but since this was recognized, intensified treatment has been given and the prognosis is now very good (34,35).

MLL rearrangements/der(11q23) and t(9;22)(q34;q11) are associated with a very poor outcome despite intensive treatment (36,37). Most MLL rearrangements occur in infants, and these patients (infants with different 11q23-translocations) are treated using a separate international protocol. For several years, t(9;22) leukemia is treated according to a separate European treatment protocol – including treatment with Imatinib (Gleevec®). Hypodiploidy, defined as a modal chromosome number < 45, is associated with a poor outcome and is considered to be an unfavorable risk factor (25,38) (see below).

At diagnosis, a diagnostic BM sample is sent to the genetics laboratory for banded metaphase karyotyping (G-banding), fluorescence in-situ hybridization (FISH), and also for DNA and RNA preparation for molecular genetic analysis of defined
cytogenetic aberrations and to define the clonal immunoglobulin gene or T-cell receptor gene rearrangements (the latter analyses for minimal residual disease (MRD) measurements - see below). In the NOPHO ALL 2000 protocol, it is compulsory to take a BM sample for G-banding to obtain the modal chromosome number and for specific analysis (FISH or RT-PCR) for detection of t(9;22), t(1;19), and MLL rearrangements. Detection of t(12;21) is optional but recommended.

**BM samples**

Traditionally, in most centers the BM sample at diagnosis is obtained by an aspiration from the posterior iliac crest. In general, BM biopsies are taken only when there is difficulty in obtaining an aspirate. However, the advantage of taking also a biopsy is the opportunity to evaluate the BM stroma as well. In an aspirate sample, all stroma morphology is disrupted. In Umeå, both biopsy and aspirate have been routine samples at diagnosis and follow-up of childhood ALL since the late 1980s. In Uppsala this routine started in 1992, and it started in Stockholm in 2001. To our knowledge, this is not very common internationally.

**Etiology**

The etiology of ALL is unknown, but a prenatal origin of many cases has been established by the demonstration of t(4;11) and t(12;21) in Guthrie cards at birth from children who are diagnosed as having ALL later on (39,40). The occurrence of these aberrations at birth, however, is just a predisposing factor for ALL since the frequency of t(12;21) in cord blood is 100-fold greater than the risk of developing the corresponding leukemia (41). The general interpretation is that complementary and secondary genetic events that occur postnatally are required for development of the corresponding leukemia. How and why these secondary events occur is not understood at present, but a dysregulated immune response to infections in infancy is one suggested cause (42,43).

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**Figure 2.** Unfavorable features stratifying for treatment of childhood ALL. Modified from the NOPHO ALL 2000 protocol.
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Risk-stratification of treatment

Treatment is the most important prognostic factor in ALL. As treatment has improved, many factors previously useful as prognostic indicators have now disappeared. In Figure 2, the unfavorable features in the current NOPHO ALL 2000 protocol are illustrated. In Table 1, the stratifying features for the different treatment groups in the current NOPHO ALL 2000 protocol are compared to those in the previous NOPHO ALL 1992 protocol. The terms low-risk (LR) and high-risk (HR), used in the papers presented in this thesis, are also illustrated in Table 1. The National Cancer Institute (NCI)/Rome criteria stratify patients into subsets depending on a) age between 1 and 9.99 years and WBC < 50×10^9/L (standard risk), or b) age ≥ 10 years and/or WBC ≥ 50×10^9/L (higher risk) (44). The stratification in the NOPHO protocols is based on the NCI criteria and experiences from international and NOPHO clinical trials. Unfavorable features detected at diagnosis stratify the patients to treatment schedules with more aggressive treatment (Intensive, Very Intensive, and Extra Intensive). Cases with no such features comprise approximately 2/3 of the patients and – depending on age and WBC count – these are stratified to Standard or Intermediate Intensity schedules. Response to therapy is one of the most powerful prognostic indicators (45-47). Treatment response has so far been evaluated solely by morphological evaluation within the NOPHO ALL protocols (for definitions, see Table 1). Techniques are now available

Table 1. Stratifying features in NOPHO ALL protocols 1992 and 2000 and the corresponding risk groups in the studies.

<table>
<thead>
<tr>
<th>Risk groups in the thesis studies</th>
<th>NOPHO ALL 1992 – risk groups</th>
<th>NOPHO ALL 2000 treatment groups</th>
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<tr>
<td>Low-risk Group (LR)</td>
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<tr>
<td>Standard risk: age 2 - &lt; 10 and WBC &lt; 10×10^9/L, no high-risk criteria</td>
<td>Standard Intensity: age 1- &lt; 10, WBC &lt; 10×10^9/L, no high-risk criteria. Intermediate intensity: age ≥ 10 or WBC 10 - &lt; 50×10^9/L, no high-risk criteria.</td>
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<tr>
<td>Intermediate risk: age 2 - &lt; 10 and WBC 10 - &lt; 50×10^9/L, or age 1- &lt;2 or ≥ 10 and WBC &lt; 50×10^9/L. No high-risk criteria.</td>
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<tr>
<td>High-risk group (HR)</td>
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<tr>
<td>High: age ≥ 1 and at least one of the following criteria: WBC ≥ 50×10^9/L, T-cell, mediastinal mass, CNS or testis-ALL, t(9;22), 22q−, t(4;11), slow response (day 15 M3 or dag 29 M2/M3 marrow)</td>
<td>Intensive: WBC: 50–100×10^9/L, age &lt; 5 and WBC 50–200×10^9/L. Age &lt; 5 and CNS-ALL. Testis-ALL. t(1;19). Hypodiploidy (&lt; 45 chromosomes). T-cell. Poor treatment response (BM day 15: M3 and/or BM day 29: M2 or M3). Very intensive: Age &gt; 5 and WBC 100–200×10^9/L. Age &gt; 5 and CNS-ALL. Age &gt; 5 years and T-cell with mediastinal mass. Extra intensive: WBC ≥ 200×10^9/L. Very slow response (BM day 29:M3). 11q23, t(9;22). Hyperhaploidy (&lt; 34 chromosomes).</td>
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<tr>
<td>Very high: age ≥ 5 and at least one of the following criteria: lymphomatous features, T-cell leukemia with other high-risk criteria, CNS-ALL, slow response (day 15 M3 or dag 29 M2/M3 marrow)</td>
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that can detect the submicroscopic level of residual leukemia – minimal residual disease (MRD) – down to the level of one leukemia cell in a background of 1,000 to more than 1 million normal cells. MRD assessed by two different methods, detection of clone-specific genetic changes by PCR amplification and flow-cytometric immunophenotyping, has been shown to be very powerful in predicting relapse (48-52). Many current protocols already use MRD to select further therapy; these include AIEOP/BFM ALL-2000, the DCOG-ALL 10 protocols, and the current ALL protocol at St. Jude Children’s Research Hospital (SJCRH, Total Therapy Study XV). Within the NOPHO, MRD will be implemented in the next protocol.

The treatment-stratifying features used today are either patient-specific (age and gender), leukemia cell-specific (immunophenotype and cytogenetics), or related to tumor burden (WBC count and extramedullary leukemia) or treatment response (MRD and morphological evaluation during early treatment). Features representing the leukemia cell microenvironment or interactions between leukemic and surrounding cells have generally not been used or proposed as potential stratifying factors.

Characteristics proposed but not yet used in treatment stratification
Research covering many aspects of childhood ALL has resulted in new approaches to risk classification. One emerging topic is pharmacogenetics, the study of how inter-individual genetic variability affects differences in drug response. One example that is already known is the activity of thiopurine methyltransferase (TPMT), a key enzyme in the metabolism of 6-mercaptopurine. Determination of TPMT genotype is currently implemented in a few childhood ALL protocols (NOPHO, SJCRH, and the United Kingdom ALL (UKALL)) for stratification of mercaptopurine and thioguanin dosage. Additional genetic variations in the germ line that have been proposed to influence outcome after treatment of childhood ALL include polymorphisms affecting the expression of thymidylate synthetase (TS) (53), an essential enzyme in proliferating cells and the target of several anticancer drugs (e.g. methotrexate), and polymorphisms in the 5,10-methylene tetrahydrofolate reductase gene, the product of which is involved in the metabolism of methotrexate. Other examples have been reviewed by Ansari et al. (54).

Studies using gene expression profiling have found that the gene expression profiles of pediatric ALL cases cluster according to the recurrent cytogenetic aberrations associated with this disease (55-58). However, so far gene-expression profiles associated with treatment outcome have led to promising (59,60) but also conflicting results, largely because of differences in patient populations, in the analysis platforms used, and – most importantly – in the treatment given (61).

Cellular in vitro drug resistance
The level of cellular drug resistance at diagnosis is most likely one of the important determinants of the outcome of treatment. Several methods for testing cellular drug resistance in vitro have been described. In childhood leukemia, two methods are used: the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and the flurometric microculture cytotoxicity assay (FMCA), both of which are total cell-kill assays. The pattern of in vitro cellular resistance at diagnosis in some of the biological subtypes of childhood acute lymphoblastic leukemia (ALL)
has previously been characterized by these two methods. T-cell immunophenotype is associated with a generally higher in vitro resistance compared to B-cell precursor immunophenotype (62). t(12;21) is related to low in vitro cellular drug resistance to L-asparaginase (63), doxorubicin, and etoposide, and high in vitro resistance to cytarabine (64). Patients with hyperdiploid ALL are more sensitive to mercaptopurine, thioguanine, cytarabine, and asparaginase than non-hyperdiploid patients (65), and t(1;19)(q23;p13) is generally associated with a low in vitro drug resistance (66).

The mechanisms by which ALL cells develop drug resistance are not understood, but several have been suggested (67). The expression of multidrug resistance-related efflux pump P-glycoprotein (P-gp) at diagnosis has been associated with unfavorable outcome (68). There are also accumulating evidence that the BM microenvironment mediate de novo drug resistance (tolerance to chemotherapy during initial drug exposure) in hematological malignancies through soluble factors or cell adhesion (reviewed by Li et al.) (69). However, to our knowledge, morphological characteristics of the BM stroma in childhood ALL have not been investigated in relation to in vitro drug resistance previously.

The bone marrow stroma in leukemia

In solid tumors, the importance of the tumor stroma is well established (70). Leukemias have previously been considered to be “liquid” tumors, i.e. leukemic cells were thought to live in the BM like a suspension culture in vitro, independently of stroma functions such as blood flow (71). This view began to change at the beginning of the 1990s. Besides the hematopoietic BM cells (pluripotent hematopoietic progenitor cells and all further stages in differentiation), the normal BM stroma consists of stromal cells (such as fibroblasts, mesenchymal stem cells, osteoblasts, macrophages, and adipocytes), blood vessel cells, and the extracellular matrix (ECM) (72). In hematological diseases, such as leukemias, there are increasing evidence to suggest that the microenvironment in the BM stroma takes an active part in the pathogenesis (73). Prolonged survival of B-lineage leukemic cells has been shown when cultured in direct contact with bone marrow-derived stromal cells in vitro (74). Others have found that genetic alterations, such as p53 mutations, in stromal cells can increase stroma-derived support of leukemia growth (75). It is also known that an abnormal BM stroma can predispose an individual to development of leukemia, such as in cases of Shwachman-Diamond syndrome (76). Mudry et al. showed that stromal co-culture sustained the proliferation of B-lineage leukemic cells and reduced apoptosis when they were exposed to cytarabine or etoposide (77). In addition, the survival ability of B-lineage ALL cells co-cultured with BM-derived stromal cells has been found to be an independent predictor of outcome (78).

The ECM is a network of molecules supporting tissues; it is also able to influence and interact with cells in its environment (79). Matrix metalloproteinases (MMPs) are a family of endopeptidases capable of cleaving several macromolecules in the ECM. MMPs have roles in angiogenesis, tumor growth, and metastasis of solid tumors (80). A basal secretion of MMP-9 and induced secretion of MMP-2 after
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stimulation with vascular endothelial growth factor (VEGF) or basal fibroblast growth factor (bFGF) has been found in childhood ALL cells (81). Moreover, the balance between MMP and the "tissue inhibitor of MMP" has been shown to be closely related to extramedullary involvement in infant ALL (82).

Angiogenesis

Blood vessels are critical for maintaining the homeostasis in nearly all cells of the human body and all cells must be located within 100–200 µm of a capillary – the diffusion limit for oxygen (83). Basically, vessels can form in two different ways: vasculogenesis and angiogenesis. Vasculogenesis refers to the formation of blood vessels from endothelial progenitor cells (EPCs). For many years, it was thought that vasculogenesis occurred only during embryonal life. There is, however, accumulating evidence that EPCs take part in both physiological and pathological vessel formation and maintenance in adult life as well. Physiological maintenance of mature vessels (normal replacement of endothelial cells undergoing apoptosis) has been shown to be executed by BM-derived EPCs (84). Moreover, EPCs have been shown to contribute to vessel growth under pathological conditions such as in ischemic, malignant, or wounded tissues to a varying level (85-88). This process is termed postnatal vasculogenesis. Angiogenesis is defined as the development of new capillaries from mature endothelial cells in existing blood vessels, and it occurs in both embryonic development and postnatal life (89). Angiogenesis is tightly regulated in adult life, and is induced during the female reproductive cycle, wound healing, and tissue repair. In addition, angiogenesis plays an essential part in a variety of pathological conditions such as rheumatoid arthritis, psoriasis, diabetic retinopathy, and cancer (90).

Table 2. Selected endogenous angiogenic and anti-angiogenic proteins.

<table>
<thead>
<tr>
<th>Proangiogenic factors</th>
<th>Antiangiogenic factors</th>
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<tbody>
<tr>
<td>Vascular endothelial growth factor (VEGF) – A, B, C, D</td>
<td>Endostatin* (Phase II)</td>
</tr>
<tr>
<td>Basic fibroblast growth factor (bFGF)</td>
<td>Angiostatin</td>
</tr>
<tr>
<td>Platelet-derived growth factor (PDGF)</td>
<td>Anti-thrombin III (truncated)</td>
</tr>
<tr>
<td>Angiogenin</td>
<td>Thrombospondin* (Phase II)</td>
</tr>
<tr>
<td>Angiopoietin-1</td>
<td>Interferon-γ, -α and -β* (Phase III)</td>
</tr>
<tr>
<td>Epidermal growth factor (EGF)</td>
<td>2-methoxyestradiol* (Phase II)</td>
</tr>
<tr>
<td>Transforming growth factor (TGF)-α and -β</td>
<td>Troponin 1</td>
</tr>
<tr>
<td>Tumor necrosis factor (TNF)-α</td>
<td>Angiostatin</td>
</tr>
<tr>
<td>Granulocyte-macrophage colony-stimulating factor (GM-CSF)</td>
<td>Vasostatin</td>
</tr>
<tr>
<td>Granulocyte colony-stimulating factor (G-CSF)</td>
<td>Pigment epithelial-derived factor (PEDF)</td>
</tr>
<tr>
<td>Hepatocyte growth factor (HGF)</td>
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</tbody>
</table>

* Anti-angiogenic drug in ongoing clinical trials.

Tumor angiogenesis

In 1971–72, Judah Folkman proposed that malignant tumors are angiogenesis-dependent (91,92). A vast number of publications have since proved him right, and this field of research has exploded. A search of PubMed for “angiogenesis and cancer” resulted in 18,251 hits on April 20, 2008. The normally quiescent
vasculature surrounding tumor cells is activated to sprout new capillaries early in tumorigenesis, when the microscopic tumor has reached a critical size (93). This “angiogenic switch” is characterized by hypoxia- or oncogene-driven tumor expression of pro-angiogenic proteins (94) such as VEGF, bFGF, and others (Table 2). Tumors also produce angiogenesis inhibitors (Table 2); thus, the angiogenic switch is on when the net balance is tipped in favor of angiogenesis (83,93). For examples of endogenous angiogenic and anti-angiogenic proteins, see Table 2.

Quantification of angiogenesis in a biopsy specimen at diagnosis of cancer may help predict the risk of metastasis or relapse. The use of vascularity as a prognostic marker was first reported in skin melanoma patients (95). Microvessel hot-spot density (MVD) was first proven to be of independent prognostic value in breast cancer by Weidner et al. (96) and since then in many other solid tumors (reviewed by Sharma et al.) (97).

Anti-angiogenic therapy has been very promising in animal studies (98-100). Avastin (bevacizumab, an anti-VEGF antibody) was the first angiogenesis inhibitor to be approved in the USA for the treatment of colorectal cancer. Other anti-angiogenic drugs have now been approved in several countries for the treatment of colorectal cancer, lung cancer, macular degeneration, and multiple myeloma, and further drugs are presently in clinical trials (reviewed by Folkman) (101).

Angiogenesis in hematological disease

It is now established that angiogenesis plays a role in hematological malignancies as well as in solid tumors (71,102,103). In 1994, it was observed that the level of bFGF, was higher in the urine of newly diagnosed leukemic patients than in the urine of normal control subjects (104). Increased microvessel density in BM disease was first demonstrated in childhood ALL in 1997 (105). Enhanced BM vascularity has since been reported in chronic and acute leukemia of myeloid and lymphoid lineages in adults (106-108). In chronic myeloid leukemia (CML), increased MVD was found to be a significant predictor of patient survival (109). Also, in chronic lymphocytic leukemia (CLL) enhanced angiogenesis has been suggested as a marker of the risk of progression (110). The prognostic importance of increased angiogenesis at diagnosis has been shown in several studies in adult acute myeloid leukemia (AML) (111-113). The role of angiogenesis in adult ALL is more uncertain, and there have been few published studies (114,115).

In 1997, Perez-Atayde et al. reported high MVD and high levels of bFGF in the urine of childhood ALL patients compared to controls (105). Since then, several groups have found altered levels of angiogenic factors or increased MVD in pediatric ALL cases compared to controls (116). For example, both Yetgin et al. and Lyu et al. found lower serum and plasma levels, respectively, of VEGF in cases at diagnosis than in controls (117,118). On the other hand, Schneider et al. reported higher protein levels of bFGF in the urine of cases at diagnosis than in controls (119,120), but also that the bFGF level was significantly lower in patients with poor outcome than in patients in continuous complete remission (CCR) (121). The plasma levels of the endogenous angiogenesis inhibitor endostatin, have recently been shown to be higher in ALL cases than in controls (122). Others have analyzed
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VEGF mRNA levels in BM plasma by RT-PCR, both at diagnosis and in relapsed patients (123-125). In all of these reports, comparisons with controls and associations with high-risk criteria and outcome were done, but with divergent results. The number of study patients was generally low (below 50), however, and the patients were heterogeneous regarding immunophenotype, cytogenetic characteristics, and risk classification. In the largest study to date, Avramis et al. measured S-VEGF-A protein levels in 117 standard-risk ALL patients and found that patients with a 6-year event-free survival generally had lower S-VEGF levels at the end of induction treatment than relapsed patients (126). The morphological net result of the angiogenic process in the BM – vascular density – has since the first report by Perez Atayde et al., been evaluated in two studies in childhood ALL, prior to this thesis. Pulé et al. found that the difference in total MVD between controls and cases was entirely due to differences in small microvessels. No difference in MVD between standard-risk and poor-risk patients was found in a total of 41 patients (11 of whom were poor-risk) (116). Kalmanti et al. compared two methods, MVD and volume-corrected MVD index, and suggested that the latter is more representative (127). In summary, there are several studies supporting that angiogenesis is upregulated in childhood ALL, but the clinical relevance of this phenomenon has been controversial.

Anti-angiogenic treatment in hematological malignancies

Anti-angiogenic treatment is currently being tested in clinical trials on pediatric patients with recurrent or refractory disease (http://www.cancer.gov/clinicaltrials/developments/anti-angio-table/). There are two classes of angiogenesis inhibitors that target blood vessels: a) direct (direct inhibition of endothelial cells) and b) indirect (prevention of the expression of, or blockade of, the activity of pro-angiogenic factors produced by tumor cells). Anti-angiogenic therapies in hematological malignancies hypothesized and tested so far have been reviewed by Ribatti et al. (128). Here are some examples:

- Thalidomide and thalidomide analogs have anti-tumor effects (129), but they also inhibit angiogenesis in several hematological malignancies such as multiple myeloma and AML (130-132). Thalidomide acts directly on endothelial cells, but the mechanism is unknown

- Conventional chemotherapeutics such as cyclophosphamide, have been used at low and non-cytotoxic concentrations as anti-angiogenic agents with very promising results in a murine leukemia model (133). Thus, the mechanisms of cytotoxic drugs already used in the treatment of childhood ALL may involve anti-angiogenic effects

- There are several therapies that block VEGF or its receptors. Inhibition of both paracrine and autocrine VEGF/VEGFR-2 signaling has been shown to induce long-term remissions in animal models with xeno-transplanted human leukemias (134). Clinical trials with inhibitors of VEGF receptor signaling have shown promising results in the treatment of AML (135,136)
INTRODUCTION

- TNP-470, a synthetic analog of the fungal antibiotic fumagillin (a potent inhibitor of endothelial cell proliferation), was found to have antitumor activity in a murine leukemia model (137). This drug has also been tested in a clinical trial involving treatment of refractory pediatric tumors and leukemias (http://www.cancer.gov/clinicaltrials/), but no results have been published.

- Other agents of recognized clinical importance in the treatment of leukemia patients, acting also via inhibition of angiogenesis, include arsenic (138,139) and all-\textit{trans} retinoic acid (ATRA) (140).

\textbf{Bone marrow fibrosis}

BM fibrosis is generally defined as increased deposition of reticulin and/or collagen fibers in the ECM (141). Reticulin is defined as “a scleroprotein present in the connective fibers of reticular tissue, closely related to collagen in composition” (142). In normal and fibrotic marrow, the distribution of reticulin fibers is identical to that of type III collagen and its precursor, type III procollagen (143,144). In addition to reticulin fibrosis, the glycoprotein fibronectin contributes to the fibrotic process in BM of hairy cell leukemia (HCL) patients (145). Growth factors and cytokines that have been shown or suggested to induce or stimulate fibrosis are transforming growth factor $\beta$ (TGF-$\beta$), bFGF, and platelet-derived growth factor (PDGF) (144,146,147). The exact components of reticulin fibrosis secondary to childhood ALL and the inducing factors/cytokines have not been identified.

Augmented BM reticulin is clinically used as an unspecific sign of abnormal marrow, and has been associated both with reactive conditions, such as autoimmune and granulomatous diseases (148), and a variety of neoplastic disorders including myelodysplastic syndromes (149), chronic myeloproliferative diseases (150), CML (151), and acute leukemias (152) – especially acute megacaryoblastic leukemia (153). The disease idiopathic myelofibrosis constitutes a response to a myeloid neoplastic clone, and is classified as a chronic myeloproliferative disease (154). In CML, recent studies have shown that increased BM fibrosis is predictive of disease progression and poor outcome (151,155,156). Reticulin fibrosis was already in 1964 seen associated with a high degree of dry taps (failed BM aspirations), and also with unresponsiveness to the therapy given at that time to adult and childhood ALL patients. Reduction of increased reticulin during remission was also observed (157). More recently, the degree of BM fibrosis has been found not to be a prognostic factor in adult ALL (158). In 1978, Hann \textit{et al.} demonstrated secondary myelofibrosis, defined as being moderately to considerably increased, in 21 out of 37 cases (57\%) of childhood ALL, but found no prognostic effect of this feature (159). In 1989, Wallis \textit{et al.} confirmed the frequency of BM fibrosis found by Hann \textit{et al.}, and demonstrated an association between B-lineage markers and presence of fibrosis – and also an association between absence of fibrosis and high WBC count – in a study of 63 childhood ALL patients (56 BCP and 7 T-cell) (160). Since then, and prior to the studies included in this thesis, the clinical importance of BM fibrosis in childhood ALL has not been studied.
**Methods for quantification of BM fibrosis**

In the clinical situation, scoring for grading of myelofibrosis is mainly based on subjective evaluations by individual pathologists using different scoring systems. The most frequently used scoring systems for assessment of BM fibrosis are based on the Bauermeister scale (161). A European consensus on the grading of BM fibrosis was reached in 2005, which involved four categories for assessment of fiber density (162). However, semiquantitative methods have several major limitations when used in research. As mentioned above, these methods are subjective and result in categories. The latter are a disadvantage in statistical evaluation of the result, and in research the importance of objective and reproducible methods is obvious. For research purposes, other methods have been used. In several studies, Kvasnicka *et al.* have used a method based on stereology – the point-intersection method (156,163). A method measuring the volume ratio of BM affected by fibrosis has been used by Buesche *et al.* (155,164).

**The NOPHO organization**

The Nordic Society of Pediatric Hematology and Oncology (NOPHO) was formally established in 1984, but the prospective registration of all children with ALL diagnosed in the five Nordic countries had already started in 1981. This was also when the first treatment protocol for children with standard-risk ALL was established within the NOPHO. The first uniform treatment protocol for all childhood ALL risk groups started in 1992 (NOPHO ALL 1992). The current protocol (NOPHO ALL 2000) started in 2002, and the new NOPHO ALL 2008 protocol will start in July, 2008.

The NOPHO registry is a database that covers all relevant diagnostic and follow-up data on all children diagnosed with ALL in the five Nordic countries. These data are summarized in the NOPHO report once a year.
AIMS and OBJECTIVES

General aims

Of the children diagnosed with ALL in the western world, as many as 15–20% will eventually die from the disease or from disease-related causes. Even though the treatment regimes are risk-adapted, some of the children without unfavorable features at diagnosis and who are therefore stratified to low-risk treatment will experience a relapse. Figure 3 illustrates the event-free survival, in January 2007, for children with ALL treated according to the NOPHO ALL 1992 protocol. This figure also demonstrates that the absolute number of relapses in low-risk patients is higher than the number of relapses in high-risk patients. Most features used for the treatment stratification are patient-specific or leukemia cell-specific. As mentioned previously, the goal of risk stratification is to individualize the intensity of treatment to suit each child in order to minimize both relapses and secondary malignancies (SMN) and other late effects. So, despite the promising prognosis, there is still a need for additional research – especially regarding low-risk patients – to be able to optimize the individualization (and thereby the outcome) for children with ALL. The general aims of these studies were to investigate BM stroma factors that mirror the interface between leukemia blasts and the patient. This was with a view to examining them in relation to other known data, in order to gain better understanding of leukemia biology and to be able to suggest additional risk factors to further improve the individualization of treatment in children diagnosed with ALL.

Figure 3. Probability of event-free survival in the NOPHO ALL-92 protocol. Graph from the NOPHO report, Reykjavik 2007. Events = resistant disease, induction failure, secondary malignancy, relapse or death in remission. The majority of events = relapses, 82% in low-risk and 76% in high-risk groups.
The specific aims of the studies in this thesis were as follows:

- to evaluate and describe microvessel density (MVD), the fraction of vessels filled with leukemia blasts: blast-congested vessel fraction (BCVF), and reticulin fiber density (RFD) in diagnostic bone marrow biopsies from children with ALL

- to find correlations between MVD, BCVF, and RFD, and also biological factors such as immunophenotype, WBC count, flow cytometric MRD measurements, and specific cytogenetic aberrations

- to investigate the prognostic importance of MVD, BCVF, and RFD in defined clinical, immunophenotypic, and cytogenetic subgroups of childhood ALL

- to investigate the possibility that correlations exist between MVD and RFD on the one hand and cellular in vitro drug resistance at diagnosis on the other.
PATIENTS AND METHODS

**Patients**

All patients were diagnosed with ALL at the oncology departments in Umeå, Uppsala, and Stockholm and their diagnostic and follow-up data were entered in the NOPHO registry. Paper I describes a local study in patients diagnosed in Umeå; 81 patients out of 119 who were eligible were studied. In papers II–IV, patients diagnosed in Uppsala and Stockholm were also included. In paper II, patients were aged from 1 to 14 years; 433 patients were eligible and 166 were studied. In paper III, 475 patients, 1–17 years of age, were eligible for the study and 185 were studied. Paper IV included 95 patients 1–17 years of age for whom *in vitro* resistance testing was successful. The number of patients tested for each drug varied from 51 to 77. For further details of patients, the reader should refer to the relevant paper. The numbers of patients who participated in one or several of the studies included in this thesis are given in Figure 4.

Infants (age < 1 year) and patients with Down’s syndrome were excluded in all papers. In paper II, patients positive for t(9;22)(q34;q11) were also excluded.

**Figure 4.** The number of patients participating in paper I–IV.

Diagnosis was established by morphology, immunophenotyping, flow cytometric DNA-index, and cytogenetic analysis (G-banding and FISH). Flow cytometric MRD (MRD-flow) measurements in paper II were part of the ongoing evaluation of MRD in Sweden. Evaluation of cellular *in vitro* drug resistance was done in Uppsala and is part of an ongoing NOPHO study.

Patients in papers II–IV were treated according to NOPHO ALL protocols 1992 and 2000. In paper I, 25 patients treated according to the Swedish national ALL protocol from 1986 were also included. Compared to the 1992 protocol, the 2000 protocol had the same framework with minor changes. As shown in Table 1, patients stratified into standard or intermediate risk/intensity schedules at diagnosis using the
**MATERIAL AND METHODS**

1992 and 2000 protocols were grouped together and classified as low-risk (LR). In these studies, patients stratified into high- or very high-risk (1986, 1992) or intensive, very intensive, or extra intensive schedules were classified as high-risk (HR).

The studies were approved by the regional ethical review board in Umeå also on behalf of patients in Uppsala and Stockholm.

**Methods**

**Preparation and selection of bone marrow samples**

BM biopsies obtained in Umeå were fixed in a solution consisting of 68 g methanol, 8 g 4% formaldehyde, and 4 g concentrated acetic acid. Decalcification was performed in buffered formic acid. In Uppsala, BM biopsies were fixed in buffered 4% formaldehyde and decalcified in Parengy (Bie & Berntsen A-S, Rødovre, Denmark). In Stockholm, Susa solution was used for fixation and decalcification (165). All biopsies were embedded in paraffin. BM sections of 5 µm were obtained.

The only criterion for inclusion in the studies was sufficient technical quality of the diagnostic BM biopsy. Samples were excluded if they could not be stained with antibodies against blood vessels or when they were too crushed or too small, as described in paper II. The biopsy frequency and success rate differed between the three centers (see paper II) and the reasons for this could be several. However, in all centers the success rate of obtaining BM biopsies of proper quality has improved considerably since a BM biopsy needle with an acquisition system was introduced during the 21st century.

**Staining of vessels**

In the 5 µm BM sections, vessels were identified with an antibody to von Willebrand factor (rabbit anti-human von Willebrand polyclonal antibody, code no. A0082; Dako, Glostrup, Denmark) (anti-vWF). After deparaffinization, slides were pretreated with protease I and immunostained with a Ventana ES automated stainer (Ventana Systems, Tucson, AZ) using buffers, detection reagents, and protocols supplied by the manufacturer. Tissue sections were incubated with the primary antibody (anti-vWF) for 32 min at a working dilution of 1:12,000 in BM sections from Umeå and 1:2,000 in sections from Uppsala and Stockholm. The aminoethyl carboxale detection kit (Ventana Systems) was used for visualization of antigen; sections were counterstained with hematoxylin. The different dilutions of anti-vWF were chosen after much effort in optimizing the stainings. The MVD result in total did not differ between the three centers, which was interpreted as an indication that the proper dilutions of the vWF antibody had been chosen.

**Microvessel density (MVD)**

Microvessel hot-spot density (MVD) was measured as described previously (108,166). Briefly, each biopsy sample was first scanned at 100× magnification, and at least three independent areas with abundant microvessels were chosen and defined as hot-spots. At 400× magnification in each hot-spot, a square lattice representing 0.06mm² was adjusted until the maximum number of vessels for that hot-spot was
within the square. Vessels were then counted. MVD of a BM specimen was calculated from the mean value of the three hot-spots with the highest MVD. For further details, see paper III. A diagnostic ALL BM section with vessels stained with anti-vWF is shown in Figure 5.

Figure 5. Example of anti-vWF-staining of vessels in the BM at diagnosis of childhood ALL.

**Blast-congested vessel fraction (BCVF)**

The blast-congested vessel fraction (BCVF) was defined as the number of vessels packed with leukemia cells per total number of evaluated vessels; i.e. the percentage of vessels filled with leukemia cells. At 400× magnification in 10 fields within the marrow area, a square lattice representing 0.06mm² was adjusted until a maximum number of vessels with lumina were within the square. Only vessels with an evaluable lumen were counted. Vessels were considered filled with leukemia cells if more than half of the lumen was occupied with cells. More details are given in paper III.

**Reticulin fiber density (RFD)**

BM sections were stained with silver impregnation for visualization of reticulin fiber content (167). We wanted the evaluation of the degree of fibrosis to result in a continuous variable for statistical reasons, and the method to be objective and reproducible. Thus, a simple stereological method was chosen. Reticulin fiber volume density (RFD) – the volume of reticulin fibers / the volume of reference tissue, i.e. the percentage of bone marrow tissue occupied by reticulin fibers – was assessed in all samples. Using a 121-point eyepiece graticule
MATERIAL AND METHODS

Figure 6. Example of reticulin-staining of BM at diagnosis of childhood ALL.

representing 0.06 mm² at 400× magnification in 10 randomly selected fields within the marrow area, the number of graticule-crossing points (hits) overlaying reticulin fibers and hits over reference space (remaining BM area) was counted. The BM fields were selected randomly regarding the degree of fibrosis. For details, see paper II. An image of a diagnostic ALL BM section with reticulin fiber staining is shown in Figure 6.

In paper I, sections stained for reticulin fibers as part of the routine at the time of diagnosis were used for evaluation of RFD. The reticulin content varied widely, and some patients scored zero. When patients diagnosed in Stockholm and Uppsala were later analyzed and freshly cut BM sections from these two centers were stained for reticulin fibers at the same time, it was obvious that the RFD was significantly higher in these patients, and no patient scored zero. This made us suspect that the sections cut and stained at diagnosis in Umeå had faded over time, and all Umeå patients were re-analyzed using new sections and stainings. The new RFD values correlated well with the old (r: 0.371, p = 0.001), but the new values were generally higher, as illustrated in Figure 7. This resulted in altered RFD values for all Umeå patients diagnosed from 1986 through 2001; these were comparable to the results from patients diagnosed in Uppsala and Stockholm. RFD values evaluated in recently cut sections from Umeå patients, stained at the same time point, were used in papers II–IV. These circumstances were discovered in 2006 when paper I had already been published.
MATERIAL AND METHODS

**Figure 7.** RFD evaluated in BM sections stained at time of diagnosis versus RFD evaluated in freshly cut and stained sections, in pts diagnosed in Umeå from 1986 through 2001.

*Detection of minimal residual disease by flow cytometry (MRD-flow)*

For some of the patients evaluated for RFD, MRD flow cytometry data were available. The MRD analyses by flow cytometry were, as previously mentioned, performed as part of an ongoing Swedish study, at the three laboratories (Umeå, Uppsala, and Stockholm) using the same protocol. The details of these procedures are described in part in paper II, and have been previously published in full (169).

*Detection of t(12;21)(ETV6-RUNX1) in paraffin embedded material*

Total RNA was extracted from BM trephine biopsies paraffin sections, from patients diagnosed in Umeå without FISH result for t(12;21) at diagnosis. The extraction was performed as described by Bock et al. (170,171), with the following modification: The digestion solution contained 4.2 mol/L guanidinium isothiocyanate, 30 mmol/L TrisHCL (pH 7.6), 2% sarcosyl, 70 mmol/L β-mercaptoethanol and 5 mg Proteinase K. In our hands the yield of extracted total RNA varied between 1.6–7.7 µg (median 4.8). RQ-RT-PCR for detection of *ETV6-RUNX1* was performed as described in paper III. Four out of 14 patients diagnosed before the t(12;21) era were found to be positive for t(12;21). Four FISH positive control patients were confirmed to be carriers of *ETV6-RUNX1* rearrangement.

*Flourometric microculture cytotoxicity assay (FMCA)*

FMCA measurement of cellular *in vitro* drug resistance is an ongoing NOPHO study. All diagnostic centers in the five Nordic countries send diagnostic samples for
this analysis to the laboratory in Uppsala. For a description of the method, see paper IV.

**Statistics**

The intraclass correlation coefficient was used to analyze reproducibility of the RFD, MVD, and BCVF methods. Differences in RFD, MVD, and BCVF between males and females, risk groups, BCP- and T-immunophenotypes and cytogenetic subgroups were analyzed with the Mann-Whitney U-test. Spearman’s correlation test was used for all correlation analyses except reproducibility of methods, as mentioned above. When illustrated in scatter plots, WBC values and flow cytometric MRD values were logarithmically transformed. Survival curves were constructed using the Kaplan-Meier method, and differences in outcome between subgroups were tested with the log-rank test. In paper I, resistant disease (RD) and relapse of the disease were considered adverse events, and patients who died in complete continuous remission (CCR) were censored at the time of death. In papers II–IV, the only event considered was relapse of the disease. In all papers, the time to an event was defined as the interval in years between diagnosis and the event. In Kaplan-Meier and Cox’s regression analyses, children remaining in CCR were censored on June 30, 2004 in paper I and at the last known time of follow-up in papers II–IV. Patients with resistant disease or death in remission were excluded in papers II–IV. For further details, see the individual papers. The level of statistical significance was defined as $p < 0.05$ (two-sided). All data was analyzed using SPSS software for Windows, version 11.0.1 (paper I) or version 15.0 (papers II–IV) (SPSS, Chicago, IL).
RESULTS AND DISCUSSION

The prerequisite for the studies in this thesis was all the BM biopsies obtained at diagnosis of childhood ALL since 1986 in Umeå, and later on also in Uppsala and Stockholm. To our knowledge this material is unique and – together with the diagnostic and follow-up data for all patients in the NOPHO registry – represents an exclusive opportunity to elucidate morphological features of childhood ALL. In the first paper, where patients diagnosed in Umeå were evaluated, we wanted to establish the MVD and RFD methods, to evaluate RFD and MVD in all patients, and consider these parameters in relation to other known diagnostic features and outcome. In papers II–IV, the material was expanded to include patients diagnosed in Uppsala and Stockholm, which made it possible to investigate subgroups of childhood ALL. All studies were retrospective, making the subgroup analyses especially important regarding outcome, since it was very likely that patients in a specific subgroup had received similar treatment. In papers II–IV, patients diagnosed in Umeå before 1992 were excluded for the same reason. As mentioned previously, they were treated with a previous Swedish protocol, and to optimize the chances of correct interpretation of the results regarding outcome and response to treatment, we wanted the patients investigated to have been treated as uniformly as possible.

Papers I and II

RFD and immunophenotype (paper II)

In paper II, RFD was found to be significantly higher in BCP-ALL patients than in T-ALL patients. No difference in RFD was found between LR and HR BCP patients. In paper I, RFD was higher in LR than in HR patients. In this paper BCP- and T-ALL patients were, however, analyzed together, which explained the difference found between LR and HR patients, since all T-ALL patients, characterized by low RFD, were HR. When the T-ALL patients were excluded no significant difference between LR and HR patients were found in paper I. Others have previously found that T-ALL patients have less fibrotic marrow than BCP patients (160), but few patients were included and stereological methods were not used. The reason for this phenotypic difference, now confirmed by us, is unknown, but it emphasizes the fact that these two entities should be investigated and should perhaps also be treated separately.

RFD and WBC count (paper II)

There was a negative correlation between RFD and WBC count in BCP patients. The reason for this is not known, but as the mechanisms behind high WBC count in peripheral blood in leukemia patients are not fully understood, this finding invites speculation. Since the fibrotic tissue is known to be dense, one can imagine that leukemic cells within BM with a high degree of fibrosis are more fixed to the surrounding stroma. Another possibility might be that BM fibrosis restricts leukemic blasts from reaching the blood vessels.
RESULTS AND DISCUSSION

Figure 8. Reticulin fiber density at diagnosis and minimal residual disease (MRD), measured at treatment day 29 in BCP ALL patients. MRD values of zero were set to the maximum possible value according to the sensitivity level for each sample. x=high-hyperdiploid leukemia, *=other BCP patients. Modified from Figure 3a in paper II.

RFD and outcome (papers I and II)

As described in the Methods section above, RFD values were repeated from new silver impregnation staining in patients from Umeå in paper II. When recalculated with the accurate RFD values, the results in paper I were altered slightly. The significance of the difference in RFD between relapsed patients and patients in CCR in the HR group disappeared, when the same patients were re-analyzed. However, the tentative prognostic importance of RFD in LR patients found in paper I was confirmed when the patients in paper II were analyzed. Because of the higher number of patients in paper II, subgroup analysis was possible. When the LR patients were subdivided into HeH and non-HeH patients, it was clear that the major prognostic effect of RFD was present in LR HeH patients. Patients with HeH leukemia, no high-risk criteria, and high RFD had an unfavorable outcome (probability of relapse-free survival (pRFS): 49\% \pm 15\%) compared to HeH patients with low RFD (pRFS: 92\% \pm 6\%) (p = 0.002). Most HeH patients today receive low-intensity treatment. Although HeH is considered to be a favorable feature, since many of these patients respond well to treatment, it is becoming obvious that HeH is one of the most common cytogenetic aberrations among relapse cases in childhood ALL (preliminary NOPHO ALL 1992 protocol results). Thus, there is a need for features that identify patients in the HeH group who are at risk of relapse. Our results suggest that RFD may be a candidate stratifying factor for HeH patients. Whether intensification of the treatment in current protocols is sufficient to save...
HeH patients with high RFD from relapse – or whether another form of treatment is needed – must be investigated in prospective studies.

**RFD and MRD measured by flow cytometry (MRD-flow) (paper II)**

In paper II, there was a correlation between RFD at diagnosis and MRD-flow measured at treatment day 29, as analyzed in 44 patients (Figure 8). This finding was true for all BCP patients regardless of risk group, and was still significant when all HeH patients were excluded ($r = 0.444$, $n = 27$, $p = 0.020$). There is accumulating evidence in studies of CML that marrow fibrosis is prognostic and predictive of therapy failure and accelerated phase (164), but to our knowledge, marrow fibrosis in association with treatment response in childhood ALL has not been investigated during the past two decades. Our finding that high RFD at diagnosis was associated with high MRD at day 29 suggests an effect of degree of fibrosis at diagnosis on treatment response – perhaps also in other BCP patients than HeH cases. The mechanism behind this result is not known, but it may be that fibrosis affects the pharmacodynamic properties of the BM, and influences the treatment response in this way. ALL with high RFD could comprise a subtype of leukemia with reduced drug uptake \textit{in vivo}. This hypothesis is supported by the finding in paper IV that there was no correlation between RFD and cellular \textit{in vitro} drug resistance (see below).

**RFD reduction rate during induction treatment (paper II)**

From clinical experience, it is known that when ALL patients present with pathological fibrosis at diagnosis, in most cases it will vanish during initial treatment. The dynamics and importance of this process are, however, unknown. Thus, RFD in BM sections obtained at treatment day 29 was evaluated in patients diagnosed in Umeå with an evaluable biopsy at diagnosis. Since the process of fibrosis is most likely leukemia-driven, we hypothesized that the rate of reduction of fibrosis during induction treatment may indirectly reflect the rate of response to treatment, and thereby affect outcome. We found that for patients with high RFD at diagnosis (defined as RFD above 20.6%, the upper third of RFD values in BCP-ALL patients), the rate of reduction did not have any effect on outcome. However, the two-thirds of BCP patients with low RFD at diagnosis (below 20.6%) and slow reduction rate had an unfavorable outcome compared to similar patients with rapid reduction. This finding is difficult to explain, but it may reflect differences in pharmacokinetics that might lead to dissimilar concentrations of drugs reaching the leukemic cells. Another explanation may be that slow RFD reduction highlights a subtype of BCP-ALL that is more resistant to treatment. It would be interesting to investigate the association between this feature and both MRD and cellular \textit{in vitro} resistance data. Unfortunately, the number of cases with valid MRD data and/or \textit{in vitro} drug resistance data was too small to allow us to perform such analyses.

**RFD and gender differences (paper II)**

No difference between boys and girls regarding RFD at diagnosis was seen when all BCP patients were analyzed; however, within the subgroup of HeH patients, boys were over-represented among patients with high RFD at diagnosis; the boy:girl ratio was 13:4 (3.25) as compared to 15:21 (0.71) for HeH patients with low RFD. Moreover, at treatment day 29 boys had an almost statistically significantly higher
RESULTS AND DISCUSSION

RFD. In light of the results described above, these gender differences regarding RFD may in part explain earlier findings of boys having a slightly worse prognosis in general compared to girls (172-174). Differences in pharmacokinetics between the sexes have been suggested previously, but when investigated they gave conflicting results (172,175,176).

Papers I and III

MVD and immunophenotype (paper III)

MVD was equally distributed within risk groups and no difference in MVD was found between LR and HR patients in the BCP-ALL patients in paper III. However, T-ALL patients had higher MVD than BCP patients. This had not been shown before, but since high angiogenic activity has been associated with poor outcome in other forms of BM disease (177) this indication of an angiogenic phenotype in T ALL may in part explain the need for high-intensity treatment in this subgroup of childhood ALL patients. By the same argument, it was a surprise to find no difference in MVD between LR and HR BCP patients.

MVD and WBC count (papers I and III)

In paper I, a correlation between WBC count and MVD at diagnosis in HR patients was seen when BCP- and T-ALL patients were analyzed together. The difference in MVD between the BCP and T immunophenotypes found in paper III, described above, partly explained this result since T ALL patients are also known to have a higher WBC count. In paper III, however, we could confirm that there was a correlation between MVD and WBC count in HR BCP patients. High WBC count is generally regarded as being synonymous with having a high leukemia cell burden. Angiogenic factors have been shown to act as autocrine growth stimuli in leukemic cells (178). Thus, BCP cases with high MVD and WBC count may be the result of high levels of angiogenic factors promoting both proliferation of the leukemia cells (autocrine effect) and new vessel formation (paracrine effect). However, since MVD was equally high in LR patients (WBC always < 50×10⁹/L), we conclude that in most cases pro-angiogenic signals that result in high MVD do not lead to high WBC count as well, for reasons that are unknown.

MVD and cytogenetic aberrations (paper III)

In paper III, the 12;21 translocation was detected in 4 out of 10 patients diagnosed before the t(12;21) era, by retrospective analysis of RNA prepared from paraffin-embedded biopsies. The method has previously been described for detection of t(9;22) (171), but not for identification of t(12;21). In total, t(12;21) was detected in 21 out of 162 BCP-ALL patients (13%). This frequency was lower than expected, but since the RNA preparation from biopsies was only performed in Umeå, there may be additional unrecognized t(12;21) patients in Uppsala-material obtained prior to the t(12;21) era. The majority of Stockholm patients were tested for t(12;21) at diagnosis. Patients positive for t(12;21) were characterized by lower MVD than HeH patients, and comparison with t(9;22) patients showed the same tendency. As illustrated in Figure 9, HeH patients had higher MVD than the non-HeH BCP patients taken
together, and t(12;21) patients had lower MVD than the non-t(12;21) BCP patients taken as a group. Gene expression array studies performed in cytogenetic subgroups of childhood ALL have shown distinct expression profiles identifying prognostically important subtypes (58). One of the genes involved in the (12;21) translocation, \textit{RUNX1}, has been shown to be overexpressed in t(12;21)-positive leukemia (56). \textit{RUNX1} has also been shown to inhibit the DNA-binding and transcriptional activity of hypoxia-inducible factor-1α (HIF-1α) protein \textit{in vitro}, with reduced expression of HIF-1α-targeted genes such as VEGF as a result (179). The low MVD in t(12;21) patients may thus be related to overexpression of \textit{RUNX1}. All patients can be assumed to have hypoxic conditions in their BM at the time of diagnosis of leukemia (180), but patients positive for t(12;21) may not be able to respond to the hypoxia with increased angiogenesis at the same rate as other BCP patients because of the action of HIF-1α being inhibited by \textit{RUNX1}. In addition, the CD9 gene located 10 cM upstream of the \textit{ETV6} gene on chromosome 12 is expressed in various tumor cells, and one suggested function of this gene is involvement in endothelial cell adhesion and migration during angiogenesis (181). CD9 has been reported to be underexpressed in t(12,21)-positive leukemia (56), which could be another explanation for the low vascular density in these patients.

In paper III, we found differences in MVD scores between subgroups of childhood ALL, suggesting that subgroups of patients with high MVD, such as cases of HeH- and T-ALL, should be the first to consider if anti-angiogenic treatment were to be a first-line option. Because of the wide distribution of MVD within the sub-groups, MVD might serve as a therapy-deciding parameter for such treatment in each individual case.
RESULTS AND DISCUSSION

MVD and outcome (papers I and III)

In paper I, MVD was evaluated in patients diagnosed in from Umeå 1986 through 2001. MVD at diagnosis was lower in HR BCP- and T-ALL patients in CCR than in similar patients who had undergone relapse. This result could not be repeated when we evaluated BCP patients diagnosed in Umeå, Uppsala, and Stockholm from 1992 through 2006. There could be several reasons for this. First, patients evaluated in paper I were treated using three different protocols, which makes outcome analysis hard to interpret. Secondly, HR BCP- and T-ALL cases were analyzed together in paper I, which afterwards – knowing that T-ALL patients have higher MVD – also causes difficulties in interpretation of the results. Thirdly, the number of patients in paper I was small, which means that each relapse in any of the groups would have a noticeable effect on the level of significance.

The survival curve in HR patients in paper I, comparing patients subdivided by the upper quartile MVD value, showed an unfavorable outcome for patients with high MVD. The latter, again, was a result of analyzing BCP- and T-ALL patients together. When we performed the same analysis in paper III, subdividing the HR BCP patients by the median MVD value, the result showed a similar tendency, although it was not statistically significant.

Since high WBC count is a risk criterion in childhood ALL, and high MVD is known to be predictive of poor outcome in solid tumors (96,182) and BM disease (102,183), we investigated a possible interaction (in additive scale) between WBC count and MVD regarding the outcome in BCP-ALL patients. For details, see paper III. Patients with WBC $\geq 50 \times 10^9/L$ and MVD $\geq 12.3$ (median) had an excess risk of relapse compared to the added separate relative risks (RRs) for patients identified as having high WBC count or high MVD, respectively (interaction analysis, $p = 0.007$). This result is interesting, but since the number of patients in this particular group was low, it should be interpreted with caution and should be verified in larger prospective series.

Interaction between MVD and RFD with regard to outcome (paper III)

There was no correlation between MVD and RFD when all BCP patients aged 1–17 years were analyzed. However, we also investigated a possible interaction (in additive scale) between MVD and RFD regarding the outcome for BCP patients, because of indications to that effect in paper I. A combined variable was constructed and analyzed by Cox’s regression. Patients with low RFD ($< 20.6\%$) and low MVD ($< 12.3$ (median), group 1) were compared with patients with low RFD count and high MVD (group 2), patients with high RFD and low MVD (group 3), and patients with high RFD and high MVD (group 4). In BCP patients, MVD and RFD interacted synergistically with regard to outcome, i.e. patients with RFD $\geq 20.6\%$ and MVD $\geq 12.3$ had an excess risk of relapse compared to the added separate RRs for high RFD and high MVD, respectively (interaction analysis, $p = 0.012$). As illustrated in Figure 10, patients with high RFD and high MVD also had an unfavorable outcome compared to all other BCP patients (groups 1–3 above).

The reason for this finding remains unknown. It can be speculated, however, that this effect reflects a synergy between different mechanisms. We have found a correlation between high RFD and high MRD, but also no correlation between RFD
and in vitro cellular drug resistance (see below). From these results, we can hypothesize that a high degree of fibrosis reduces drug uptake. Given this hypothesis, when a highly fibrotic leukemic stroma also is highly vascularized (which may be a sign of aggressive behavior under certain conditions), it is likely that leukemic cells within that stroma will be especially difficult to kill.

![Figure 10. Relapse free survival for all BCP ALL patients in paper III. Patients with RFD>20.6% and MVD>12.3 versus the rest of patients.](image)

All survival analyses taken together – concerning MVD in BCP patients (HR-patients, interaction with WBC count, and interaction with RFD in all patients) – indicate that high MVD may be regarded as a risk factor for relapse only in HR BCP patients and for BCP patients with a high RFD. For patients with low RFD, the outcome was not affected by MVD, regardless of risk group.

**BCVF (paper III)**

While evaluating MVD it was noticed that in some cases the lumina of blood vessels were packed with leukemic blasts, so we decided to count the blast-congested vessel fraction (BCVF). We hypothesized that vessels filled with blasts were either poorly functioning because of the tumor infiltration or functioning, with a high number of blasts exiting the BM and entering the peripheral blood. BCVF was assessed in 177 out of 185 BM sections. Perhaps not surprisingly, but to our knowledge never reported before, there was a strong correlation between BCVF correlated and WBC count in all BCP- and T-ALL patients. All BCP patients with WBC count of ≥ 50×10^9/L had a BCVF of ≥ 50%. The significance of this observation is uncertain. It could just be a complicated way of measuring WBC count. Another possibility is that a high BCVF was required for a high WBC, but not the other way around, since
39 patients were seen to have WBC counts of < $10^9/L$ (the cut-off for standard risk therapy) and BCVF of $\geq 50\%$. Thus, BCVF and WBC count in combination may also reflect functionality of the vessels, which, at least in theory, could have importance in the response to treatment through reduced drug delivery to a microenvironment with poorly functioning vessels. The numbers of patients and relapses in the subgroup with low WBC count and high BCVF were too small to evaluate the effect on outcome.

**Paper IV**

It is known that in solid tumors, the microenvironment may influence the phenotype of the tumor cells (70,184). In papers I–III we found indications of associations between stroma factors such as vascular density and reticulin density on the one hand and immunophenotypic and cytogenetic features of childhood ALL on the other, but also between stroma factors and outcome and treatment response. From these findings, we hypothesized that MVD and RFD may also be associated with cellular *in vitro* drug resistance.

**RFD and cellular in vitro drug resistance**

There was no correlation between RFD and *in vitro* resistance to any of the drugs tested. This is interesting in the context of one result in paper II, where we found a strong association between MRD at treatment day 29 and RFD at diagnosis. This result contradicts the hypothesis that high RFD constitutes a subtype of leukemia that is more resistant to chemotherapy *in vitro*. Instead, it can be speculated that fibrosis interferes with drug uptake in the BM and in this way limits the treatment response.

<table>
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<th>Patient groups</th>
<th>Ara-C 2.5 µg/ml</th>
<th>Doxo 0.5 µg/ml</th>
<th>Ida 0.5 µg/ml</th>
<th>Dexza 7.1 µg/ml</th>
<th>Pred 50 µg/ml</th>
<th>Mitox 0.5 µg/ml</th>
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</table>

MVD and cellular in vitro drug resistance

In contrast to RFD, there was a negative correlation between MVD and in vitro resistance to several of the drugs tested, in all BCP patients and/or sub-groups (Table 3). Thus, low MVD was associated with high in vitro drug resistance. Frost et al. have previously shown a negative correlation between WBC count and cellular in vitro resistance (185). In paper III, we found a correlation between MVD and WBC count in high-risk BCP patients. To rule out WBC count as a confounding factor for the negative correlation between MVD and in vitro drug resistance, the correlation analysis was done in the subgroup of BCP patients with WBC count of < 10×10⁹/L. These patients were all LR cases and the correlations between MVD and the various drugs were even more pronounced than in all patients taken together, as shown in Table 3.

In solid tumors, one reason behind development of drug resistance is hypoxia, and we speculate that this may also be the case in ALL; this may explain the correlation between low vessel density and high in vitro drug resistance. Hypoxia normally kills cells by apoptosis, and hypoxia therefore selects for cells upregulating anti-apoptotic mechanisms (186). Hypoxia is also known to upregulate genes encoding proteins involved in drug resistance, including P-glycoprotein (187,188). In childhood ALL, differential expression of apoptosis-related genes in microarray studies has been found to be correlated to in vitro cellular drug resistance (189), but hypoxia as a mechanism behind selection of resistant cells has not been studied extensively. Even so, it is widely accepted that both BM disease and solid tumors are dependent on interactions with their surrounding stroma for growth and survival (70). In addition, hypoxic culture conditions were shown to induce resistance to fentetinide (a synthetic retinoid) in childhood ALL cells (190). Hypoxia has been shown to induce resistance to doxorubicin in vitro (191), but to our knowledge similar studies on cytarabine or dexametasone have not been published. Further studies are therefore warranted to explore whether low MVD is coupled to particularly severe hypoxia in the BM, and whether this could be a mechanism that selects for multiresistant cells.

Score combinations for MVD and in vitro drug resistance

We hypothesized that low MVD may lead to conditions that promote selection of resistant leukemic cells with a specific resistance pattern. The median survival indices (SIs: percentage surviving cells compared to controls) for cytarabine (2.5 μg/ml), dexametasone (7.1 μg/ml), and doxorubicin (0.5 μg/ml) were calculated from the Nordic in vitro resistance data for all BCP patients. These drugs were found to be correlated most strongly with MVD, and the in vitro data for these three were combined. For each of the drugs cytarabine (AraC), dexametasone, and doxorubicin, patients with SI below the median were given a count of 1, and those with SI above the median were given a count of 2. For each patient, a score (the AraC-Dexa-Doxo (ADD) score) was calculated by adding up these counts, which could range from 3 (sensitive to all drugs) to 6 (resistant to all drugs). An increasing ADD score corresponded to a gradual decrease in MVD. BCP patients with an ADD score of 6 had a significantly lower MVD than patients with an ADD score of 3–5. The 12 patients with an ADD score of 6 were all LR patients. Similar scoring has been used previously (185).
RESULTS AND DISCUSSION

*In vitro drug resistance score combinations and outcome*

Next, the ADD score was investigated in survival analysis of BCP patients evaluated for MVD. Patients with an ADD score of 6 patients tended to have a worse outcome than patients with an ADD score of 3-5 patients, but the difference was not statistically significant. This analysis was, however, based on a low number of patients; thus, a similar survival analysis was carried out on all Nordic BCP patients with valid *in vitro* tests. When all risk groups were analyzed together, no disadvantage in outcome for an ADD score of 6 was found (p = 0.144). For LR patients, an ADD score of 6 tended to be associated with an unfavorable outcome (p = 0.083). In LR HeH patients, however, the pRFD was clearly unfavorable for patients with an ADD score of 6 (p = 0.008). The reason behind the prognostic importance of an ADD score of 6 in low-risk patients only may be that cellular resistance (and perhaps limited drug delivery *in vivo* due to low vascular density) is overcome by more intensive treatment for patients in the HR groups, but not for patients in LR treatment regimes. Another reason may be that in HR patients, mechanisms other than *in vitro* resistance – with stronger impact on outcome – may be operating.

The findings in paper IV indicate that patients stratified to LR regimes, with a combination of low MVD and high *in vitro* resistance, may benefit from intensive treatment.
CONCLUSIONS

Children diagnosed with ALL, BCP and T immunophenotype

- Higher MVD in BCP- compared to T-ALL (n: 185)
- Higher RFD in T- compared to BCP-ALL (n: 166).

All BCP patients:
- Positive correlation between RFD at diagnosis and MRD-flow at treatment day 29 (n: 44)
- Rate of RFD reduction during induction treatment was prognostic in BCP pts (n: 60)
- RFD was higher in boys at treatment day 29 than in girls (n: 62)
- MVD was lower in patients positive for t(12;21) than in all non-t(12;21) pts (n: 162)
- MVD was higher in HeH than in all non-HeH pts (n: 162)
- MVD and WBC count interacted regarding outcome (n: 157)
- Negative correlation between RFD and WBC count (n: 146)
- Positive correlation between BCVF and WBC count (n: 156)
- MVD and RFD interacted regarding outcome (n: 157)
- No correlation between RFD and cellular in vitro resistance to any of the drugs tested
- Negative correlation between MVD and cellular in vitro resistance to several drugs, especially to cytarabine, dexametasone and doxorubicin (the combination of the drugs: ADD, see result section, paper IV, for details)
- MVD was lower in ADD resistant patients (n: 46).

LR BCP pts:
- RFD was prognostic for HeH pts (n: 53)
- Boys were overrepresented in HeH pts with high RFD (n: 53)
- ADD score was prognostic for HeH pts in a Nordic cohort of pts with valid in vitro testing (n: 117).

HR BCP pts:
- Positive correlation between MVD and WBC count (n: 28)
- MVD had a tendency to prognostic impact (n: 26).

Figure 11. Summary of results and conclusions in immunophenotypic, cytogenetic and treatment subgroups. n refers to the total number of patients in the result listed. Results concerning HeH patients are highlighted in red.

BIOLOGICAL INTERPRETATIONS

- In addition to the immunophenotypic and cytogenetic characterization of the leukemic cells, MVD and RFD are characteristics that refine the definition of a specific ALL.

- There was a correlation between RFD and MRD at treatment day 29, but not between RFD and in vitro drug resistance at diagnosis, suggesting that fibrosis reduces drug uptake during induction treatment, i.e. fibrosis...
CONCLUSIONS

influences the pharmacodynamics in vivo – and thereby the response to treatment.

- The rate of reduction of RFD for patients with low RFD at diagnosis was associated with outcome, which suggests that the reduction rate may reflect treatment response. Thus, reduction rate may be a surrogate marker for the concentrations of drugs reached in the BM, i.e. a surrogate marker for the in vivo pharmacokinetics.

- Low blood vessel density in the BM could theoretically result in hypoxia and selection of leukemic cells that are difficult to kill, and that are thus resistant to therapy.

CLINICAL INTERPRETATIONS

- RFD could be a candidate stratifying factor for HeH leukemia patients with no other unfavorable feature. Low RFD may help identify HeH patients with a disease that is so easy to cure that the intensity of treatment might be reduced. High RFD may help identify HeH patients in need of intensified treatment.

- MVD in combination with RFD may be a candidate stratifying factor for all B-cell precursor ALL patients. High MVD and high RFD may help in recognizing LR patients who are in need of intensified treatment.

- MVD varies between subgroups of childhood ALL patients. This suggests that subgroups with high MVD, such as HeH- and T-cell ALL patients, should be the first to consider if anti-angiogenic treatment were to be introduced in first-line treatment of ALL. Because of this variation, MVD might also be a therapy-deciding parameter for each individual child with ALL.

- Low MVD in combination with in vitro resistance to cytarabine, doxorubicin and dexametasone may help identify LR patients in need of intensive treatment.
GENERAL DISCUSSION AND FUTURE DIRECTIONS

As treatment stratification at diagnosis in childhood ALL constantly improves together with the implementation of MRD measurements in treatment protocols, in the future we will hopefully be able to better tailor the intensity of treatment for each patient. The general aims for the two-thirds of patients today who are stratified to low-risk treatment should be [1] to reduce intensity of treatment for the patients who are easy to cure, in order to avoid unnecessary late affects, and [2] to intensify treatment for patients who do not display any traditional unfavorable feature at diagnosis, but who nevertheless show relapse. Refinement of MRD measurements and implementation of additional risk factors, stratifying both for less intensive and intensified treatment, may accomplish this. In contrast, for some of the children who are stratified to the one-third comprising the high-risk childhood ALL patients, optimization of treatment and new treatment strategies will be needed to improve cure rates.

Anti-angiogenic treatment could, at least in theory, be a valuable supplement in first-line treatment of childhood ALL. Theoretically, drugs directly targeting blood vessels are the most interesting, since they exert their effects on genetically stable endothelial cells – thus being unlikely to induce drug resistance. However, considering the result in this thesis, the use of anti-angiogenic drugs could be a double-edged sword – and through hypoxia, could induce drug resistance in leukemic cells instead of leukemia cell death through apoptosis. So any possible future introduction of anti-angiogenic drugs in the treatment of childhood ALL should be considered very carefully and thoroughly. Still, clinical trials with these kinds of drugs in pediatric cancer treatment have only just started, so current traditional chemotherapy – with additional CNS irradiation and BM transplant in selected cases – is the therapy that will dominate treatment of childhood ALL in the near future. Additional drugs directly targeting the effects of cytogenetic aberrations, as in the case of BCR/ABL and Gleevec®, are other possible future supplementary drugs for selected patient groups.

Leukemia is still sometimes described as a “liquid tumor”. This is not an appropriate description. Leukemia should be looked upon as a hematological tumor, originating from and expanding in the BM, with early spread to peripheral blood – which is the only feature related to being “liquid”. This BM tumor has an organized stroma originating from the normal stroma in the BM. The findings in this thesis indicate that there is interplay between the BM/tumor stroma and the leukemic cells, and that this interaction can affect outcome in certain subgroups of ALL. MVD may be a candidate risk stratifying factor for some subgroups of patients, but may also be a possible therapy-deciding parameter for anti-angiogenic treatment. RFD could be a candidate risk-stratifying factor as well, at least for LR HeH patients, but also in combination with MVD in all BCP patients. Thus, it would be of value to investigate MVD and RFD prospectively in order to verify the findings in this thesis, and to analyze other cytogenetic subgroups further in a larger patient material. It would also be of interest to explore other ways of evaluating RFD and MVD that do not
require biopsies. Shih et al. assessed angiogenesis in the spine of AML patients by dynamic contrast-enhanced magnetic resonance imaging (dMRI), and showed an unfavorable outcome for patients with signs of high levels of angiogenesis (113). This kind of method has the advantage of evaluating much larger BM volumes than a BM biopsy, and also of being a non-invasive measurement, although being very demanding in resources. The most convenient way to evaluate the angiogenic status of ALL would be to find factors reflecting MVD and RFD, measurable in peripheral blood or in a BM aspirate. Any prospective studies that are set up to evaluate this further would require introduction of a mandatory BM biopsy at diagnosis and standardized procedures in handling the biopsies in all centers included in the project.
POPULÄRVETENSKAPLIG SAMMANFATTNING

Bakgrund och målsättning: Akut lymfatisk leukemi orsakas av att omogna lymfocyter (ett slags vita blodkroppar) av B- eller T-cellstyp delar sig ohämmat i benmärgen, oftast på grund av olika genetiska förändringar. Varje år insjuknar i Sverige mellan 75 och 85 barn i akut lymfatisk leukemi (ALL), som i dag har en god prognos (drygt 80 % överlever), med tanke på att sjukdomen obehandlad är dödlig till 100%. Fortfarande dör dock 15-20% av barn med ALL av sjukdomen, eller av behandlingsrelaterade orsaker. Bland dessa finns barn som vid diagnos inte upptäckte någon känd riskfaktor för svårbehandlad sjukdom. Behandlingen av barn-ALL är riskfaktoranpassad, vilket innebär att riskfaktorer som är relaterade till barnet eller leukemicellerna avgör vilken sorts behandling som ges (mer eller mindre intensiv cellgiftsbehandling). I denna avhandling har faktorer undersömts i benmärgsstromat, dvs i den lokala vävnaden runt leukemicellerna, där dessa interagerar med den omkringliggande vävnaden. Målsättningen var att relatera dessa stromafaktorer till andra kända faktorer och utfall efter behandling. Detta för att få en bättre förståelse av leukemibiologi och för att kunna identifiera ytterligare riskfaktorer som kan bidra till förbättrad behandling vid ALL.

Metoder: Vi mätte kärltätheten (förekomst av kärl, MVD), andelen kärl fyllda med leukemiceller (BCVF) samt graden av fibros (bindvävsinlagring, RFD) i snitt från benmärgsbiopsier tagna vid diagnos av ALL i Umeå, Uppsala och Stockholm. RFD bedömdes även i bemärgssnitt från behandlingsdag 29.

Resultat: Fibrosgraden hade betydelse för utfallet av given behandling hos barn med en hög-hyperdiploid leukemi (51-61 kromosomer i leukemicellerna, HeH). Dessutom var en snabb reduktion av fibrosgraden under de första fyra veckornas behandling associerat med fördelaktig prognos. Fibrosgraden vid diagnos korrelerade också med fraktionen kvarvarande sjukdom (MRD) mätt med flödescytometri efter fyra veckors behandling hos patienter med ALL av B-cellstyp.

Patienter med ALL av B-cellstyp med både hög fibrosgrad och hög kärlförekomst hade ett sämre utfall efter behandling jämfört med övriga. Dessutom var både kärlförekomst och fibrosgrad associerat med immunfenotyp (B- eller T-cellstyp) och kärlförekomst var associerat med olika specifika genetiska förändringar. Förekomsten av kärl korrelerade dessutom med antalet vita blodkroppar i perifert blod (WBC) vid diagnos, men endast hos patienter med högriskfaktorer. BCVF korrelerade starkt med WBC hos alla patienter med ALL men inte med kärlförekomst eller fibrosgrad.

Förekomsten av kärl korrelerade negativt med cytostatikaresistens i laborativ miljö (in vitro) för flera av drogerna, hos patienter med ALL av B-cellstyp. Dvs låg kärlförekomst var associerat med hög cytostatikaresistens. En drogresistens-score som kombinerade drogerna starkast korrelerade till MVD – cytarabin, doxorubicin och dexametason (ADD score) – hade en prognostisk betydelse hos HeH-patienter utan högriskfaktorer.
Slutsatser: Sammantaget indikerar dessa studier att stromafaktorer, som kärlförekomst och fibrosgrad är relaterade till både egenskaper, men även genetiska förändringar hos leukemicellerna. Stromafaktorer verkar dessutom kunna påverka svaret på den första induktionsbehandlingen, *in vitro* cytostatikaresistens och utfall efter behandling i specifika undergrupper av patienter med barn-ALL. Dessa resultat betonar värdet av benmärgsstromat vid akut leukemi och nödvändigheten av utvidgad användning av benmärgsbiopsi vid diagnos.
ACKNOWLEDGEMENTS

A lot of people have contributed to this work and helped me complete this thesis. My special thanks to:

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