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STUDIES OF SPECIFIC VIRUSES FROM GUTHRIE CARDS AND PROGNOSTIC MARKERS IN BONE MARROW SAMPLES FROM CHILDREN DIAGNOSED WITH LEUKEMIA

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Cover: A picture of p53 overexpression in a bone marrow sample taken four months post HSCT, from a child with AML, who relapsed.

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” Nog finns det mål och mening i vår färd - men det är vägen, som är mödan värd.”

Karin Boye

To my wonderful family

ABSTRACT

Aims: The aim of this thesis was to increase understanding of how molecular processes influence the development and risk assessment of childhood leukemia. *Studies I and II* investigates whether a specific virus infection *in utero* could be involved in a "first hit" in leukemogenesis. *Studies III and IV* examine whether alterations in protein expression from cell cycle regulating genes may predict a relapse in children with myeloid malignancies undergoing hematopoietic stem cell transplantation (HSCT).

Background: Genetic alterations, analyzed at time of diagnosis in children who develop leukemia, have been traced back to neonatal dried blood spots (DBS). This suggests that the majority of chromosome translocations occur *in utero* during fetal hematopoiesis, generating a "first hit". A "second hit" is then required to generate a leukemic clone. Today, experiments in vitro, animal models, and clinical observations have revealed that several viruses are oncogenic and capable of initiating a genetic alteration. Smith M postulated the theory that an *in utero* infection might be the "first hit", causing genetic aberrations that could later lead to the development of the leukemic clone, which is supported by the early age of onset and space-time clustering data, based on time, place of birth, and diagnosis.

Leukemia develops as a result of hematopoietic or lymphoid tissue with uncontrolled cell division. Normally cell division is controlled by the cell cycle, the network of which is complex with numerous regulating proteins both up and down stream, but also containing several feedback loops. The important regulators of this process are tumor suppressor genes, essential for normal cell proliferation and differentiation as well as for controlling DNA integrity. Errors in these genes or their protein expression affect the ability of the cell to check for DNA damage, thus tumors may occur. Proteins from these genes could serve as prognostic markers and predict relapse.

Methods: In *studies I and II* we investigated neonatal DBS by PCR for the presence of adenovirus DNA (243 samples) and the three newly discovered polyomaviruses (50 samples) from children who later developed leukemia but also from controls (486 and 100 samples respectively). In *studies III and IV* we explored the expression of one (p53) respectively four (p53, p21, p16 and PTEN) cell cycle regulating proteins in bone marrow at diagnosis as well as pre and post HSCT in myeloid malignancies in children. We retrospectively collected clinical data and bone marrow samples from 33 children diagnosed with chronic myeloid malignancies (MDS, JMML and CML), 34 children diagnosed with AML as well as 55 controls. The samples were prepared by tissue micro array (TMA) as well as immunohistochemistry and examined for protein expression in a light microscope.

Results: In *study I* we detected adenovirus DNA in only two patients who later developed leukemia, but in none of the controls. In *study II* all the samples were negative for KIPyV, WUPyV and MCPyV DNA in both patients and controls. In *study III* we found an overexpression of p53 protein at diagnosis that significantly predicted relapse after HSCT in children with rare chronic myeloid malignancies. In *study IV* a significantly higher p53 expression was found in the relapse compared to the non-relapse group at six months post HSCT in children with AML, suggesting that p53 may be used as prognostic markers for predicting a relapse. In addition, the calculated cut off level for p53 at diagnosis (*study III*) and at six months (*study IV*) post HSCT was approximately 20%, which indicates that a p53 expression over 20% may predict relapse in children with myeloid malignancies.

Conclusion: Although we did not find an association between adenoviruses or the three newly discovered polyomaviruses and the development of childhood leukemia, a virus could still be involved in this process; the virus may have escaped detection, other new viruses could be involved or a virus could precipitate the "second hit".

We suggest that evaluation of p53 protein expression may be used as a supplement to regular prognostic markers both pre and post HSCT. To further evaluate this, a prospective multicenter study has been started.

LIST OF PUBLICATIONS

I. **Honkaniemi E*,** Talekar G*, Huang W, Bogdanovic B, Forestier E, von Doblen E, Engvall M, Ornelles DA, Gooding L and Gustafsson B.
Adenovirus DNA in Guthrie cards from children who develop acute lymphoblastic leukaemia (ALL). Br J Cancer, 2010 Mar 2;102(5):796-8

II. Gustafsson B*, **Honkaniemi E***, Goh S, Giraud G, Forestier E, von Döbeln U, Allander T, Dalianis T, Bogdanovic G.
KI, WU and Merkel Cell polyomavirus DNA was not detected in Guthrie cards of children, who later developed acute lymphoblastic leukemia (ALL). Journal of Pediatric Hematology-Oncology. 2012 Jul;34(5):364-7.

III. **Emma Honkaniemi***, MD, Kristin Mattsson*, Gisela Barbany MD, PhD, Birgitta M. Sander MD, PhD, Britt M. Gustafsson MD, PhD.
Elevated p53 protein expression; a predictor of relapse in rare chronic myeloid malignancies in children? Pediatric Hematology-Oncology, 2014 May;31(4):327-39.

IV. Kristin Mattson*, **Emma Honkaniemi***, MD, Gisela Barbany MD, PhD, Britt M. Gustafsson MD, PhD.
Increased p53 protein expression as a potential predictor of early relapse after hematopoietic stem cell transplantation in children with acute myelogenous leukemia. Submitted.

*Shared first authorship

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LIST OF ABBREVIATIONS

DBS	Dried blood spots
ALL	Acute lymphoblastic leukemia
AML	Acute myelogenous leukemia
HSCT	Hematopoietic stem cell transplantation
MRD	Minimal residual disease
MDS	Myelodysplastic syndrome
JMML	Juvenile myelomonocytic leukemia
Ph+	Philadelphia chromosome-positive
CML	Chronic myeloid leukemia
MLL	Mixed lineage leukemia
IGH	Immunoglobulin heavy chain
TCR	T-cell receptor
SNP	Single nucleotide polymorphism
CNVs	Copy number variations
EBV	Epstein Barr virus
HHV	Human herpes virus
HTLV1	T-cell lymphotropic virus 1
PyV	Polyomavirus
JCV	JC-virus
BKV	BK-virus
pRB	Retinoblastoma protein
MPyV	Murine polyomavirus
SV40	Simian virus 40
KIPyV	Karolinska Institutet polyomavirus
WUPyV	Washington University polyomavirus
MCPyV	Merkel cell polyomavirus
HPyV	Human polyomavirus
MCC	Merkel cell carcinoma
TSPyV	Trichodysplasia spinulosa-associated polyomavirus
MWPyV	Malawi polyomavirus
MXPyV	Mexico polyomavirus
STLPyV	Saint Luis polyomavirus
NJPyV	New Jersey polyomavirus
NCCR	Non-coding control region
mRNA	Messenger RNA

LT	Large T
ST	Small T
PP2A	Protein phosphatase 2 A
MAPK	Mitogen-activated protein kinas
PI3K	Phosphatidylinositol 3-kinase
LP1	Functional agnoprotein
PML	Progressive multifocal leukoencephalopathy
iAMP21	Intrachromosomal AM1 amplification
FLT3	FMS-like tyrosine kinase 3
NPM1	Nucleophosmin 1
CDK	Cyklin-dependent kinas
M	Mitosis
G	Gap
S	Synthesis
CKIs	CDK inhibitors
INK4	Inhibitor of kinas 4
HSC	hematopoietic stem cell
MDM2	Murine double minute protein-2
HPV	Human papillomavirus
FAMM	Familial multiple mole/melanoma
PTEN	Phosphatase with tensin homology
PIP2/3	Phosphatidylinositol biphosphate/triphosphate
NOPHO	Nordic Society of Pediatric Hematology and Oncology
TBI	Total body irradiation
HLA	Human leucocyte antigen
MEM	Minimal essential medium
ALB	Albumin gene
TMA	Tissue micro array
Ct	Threshold cycle
OR	Odds ratio

1 GENERAL INTRODUCTION

Cancer, both solid tumors and hematological malignancies, is believed to occur as a result of genetic and epigenetic alterations in stem or precursor cells, where hematological malignancies develop in hematopoietic or lymphoid tissue with uncontrolled cell division. Alterations in tumor suppressor genes and oncogenes are two causes involved in this process, their protein function being essential for cell proliferation and differentiation [1,2]. In many cases the time point and etiology of the genetic changes that lead to leukemia are still unknown, making preventive actions more difficult. However, identified leukemic alterations can be traced back in neonatal dried blood spots (DBS) (also known as Guthrie cards) or cord bloods, indicating an early event, maybe even *in utero* [3]. Furthermore, in developed countries the early childhood peak of acute lymphoblastic leukemia (ALL) at 2-5 years of age also points to a primary event *in utero* and a second event in early childhood [4]. Several etiological factors have been suggested as possible triggers of a "first hit" *in utero*, including oncogenic viruses that can cause cancer both in vivo and in vitro [5-7].

In Sweden, the annual incidence of childhood cancer is 16/100.000 (children <15 years), of which approximately 30% involves a diagnosis of leukemia [8]. Improved chemotherapy protocols, better supportive care, the prevention of infectious disease, as well as a stricter classification into different risk groups are factors that result in a better survival rate. Before 1948, the survival in pediatric blood malignancies was practically zero. Today the overall survival is 90 % for ALL and 70 % for acute myelogenous leukemia (AML) [9-12]. However, the outcome remains poor for relapsed patients in all childhood leukemic groups [13]. Prognostic markers are important tools for dividing leukemia into different risk groups with their own treatment protocols, but also for following the patient during and after treatment in order to prevent relapse. However, new prognostic markers are needed to further improve survival.

1.1 CHILDHOOD LEUKEMIA

1.1.1 Acute lymphoblastic leukemia

ALL, the most common type of childhood leukemia, originates in the lymphoid precursor cell and represents 75-80 % of all pediatric leukemias, with an incidence in developed countries of three to four cases per 100.000 children and an incidence peak at 2-5 years of age, where infant ALL accounts for 2.5-5% [8,11,14-16]. ALL is divided into B-cell lineage (80-85%), T-cell lineage (15-20 %), and few numbers of non-lineage ALL. Forty-six percent of those diagnosed are female and 54 % are male [16]. Today, 80-90% of children with ALL survive, compared to the situation prior to 1948, when the survival rate was extremely low [9-11]. The difference was due to the revolutionary discovery of chemotherapy, which was groundbreaking for oncology, where most of the drugs were developed before 1970 [11]. Since then, complementary therapies such as intrathecal chemotherapy, radiation and hematopoietic stem cell transplantation (HSCT), coupled with enhanced supportive care, have continued to increase the survival rate [11,17]. Moreover, survival was further improved by better tools for identifying prognostic markers such as biological subtypes and response to treatment (minimal residual disease, MRD), in addition to distinctive treatment protocols enabling customized treatment for different risk groups [9,11]. Essential elements of diagnosis are morphological identification of lymphoblasts by microscopy as well as immunophenotypic evaluation of lineage commitment and stage by flow cytometry, complemented by chromosomal and genetic analysis. This is followed by assignment to different risk groups (standard, intermediate and high risk) by prognostic factors such as age, leukocyte count at diagnosis, T- or B-cell immunophenotype, genetic alterations, and response to initial therapy [11,17]. The standard treatment of ALL typically takes 2-2.5 years, including induction of remission, consolidation, and maintenance. All patients are initially treated with cytostatic drugs, but HSCT is required during the first remission if the child is diagnosed with specific unfavorable prognostic markers or has a persistent disease with high MRD levels after induction therapy [11].

1.1.2 Acute myelogenous leukemia

AML, the second most common type of childhood leukemia, occurs in the myeloid cell precursor and accounts for 15-20% of all childhood blood malignancies with the highest

incidence peak at two years of age, followed by a decrease and a new peak at nine years of age [16,18,19]. Forty-four percent of those diagnosed are female and 56% male [16]. The incidence of childhood AML in the Nordic countries is 0.7 cases in 100.000 children [20]. The overall remission rate for all forms of AML is 92%, with an overall survival of 70 % [12]. There are many AML subtypes with a different prognosis and sensitivity to treatment due to the variety of myeloid precursors as well as the diversity of genetic events that can create the leukemic clone [16]. Morphological identification of blasts from the myeloid cell lineage by both microscopy and immunophenotypic evaluation, complemented by chromosomal and genetic analysis, are essential for diagnosis, followed by assignment to different risk groups [12,21,22]. Although the prognostic significance of clinical and cell biological factors are interpreted differently by various treatment protocols, important prognostic factors include cytogenetic and molecular abnormalities in addition to initial treatment response, where post induction MRD seems to represent the new era of treatment stratification in the AML group. Chemotherapy is the standard treatment for AML, whereas the indications for HSCT have been debated. Around ten years ago, all children in Sweden with AML were transplanted if an HLA-identical donor was available. Today, candidates for HSCT in first remission are patients diagnosed with cytogenetic or molecular genotyped unfavorable prognostic markers, or those with blasts >15% after first induction [12].

1.1.3 Rare clonal myeloid malignancies

1.1.3.1 Myelodysblastic syndrome

Myelodysblastic syndrome (MDS) is a clonal myeloid malignancy, accounting for <5% of leukemia in children, with an incidence of 1.8 per 10^6 children [23]. The median age is 6.8 years and the gender distribution is equal [24]. Historically, myeloid leukemia in Down's syndrome and juvenile myelomonocytic leukemia (JMML) were included in MDS, but are separated nowadays due to better diagnostic tools [23]. In contrast to AML, the bone marrow is not dominated by blast cells, as the malignant cells retain some differentiation potential and have a tendency to undergo apoptosis [16,23,24]. However, diagnosis is often complicated, as MDS with a high number of blasts is difficult to distinguish from AML, where the threshold for distinguishing between them is 20% of blasts [23,24]. Furthermore, MDS with a low blast count is hard to differentiate from nonclonal bone marrow disorders, such as aplastic anemia, where the risk of children

with aplastic anemia developing MDS is 10-15% [23,24]. The diagnosis of MDS is based on morphological and cytogenetic abnormalities and not always directly due to severe separable differential diagnoses. The treatment of choice is HSCT without prior heavy chemotherapy, which results in a five year survival of 60% [24,25].

1.1.3.2 Juvenile myelomonocytic leukemia

Another clonal myeloid malignancy is JMML, accounting for 2-3 % of childhood leukemia, with an incidence of 1.2 per 10^6 children [16,23]. The onset of JMML occurs in infancy or early childhood at a median age of 1.7 years and the gender distribution is 67% male and 33% female [16,26]. The characteristics of JMML are high white blood count, monocytosis, elevated hemoglobin F, blasts in peripheral blood, and monocytic cell infiltration of organs [27,28]. The bone marrow contains < 20% blasts, which by itself is not diagnostic and must be negative for the Philadelphia positive chromosome (Ph+) [26,27]. Neurofibromatosis type1 is present in 14% of children with JMML and may strengthen the diagnosis [27]. An increased frequency of JMML has also been observed in children with Noonan´s syndrome [16]. The treatment of choice is HSCT, which results in a five year survival rate of 50 % [27-29].

1.1.3.3 Chronic myeloid leukemia

A third clonal myeloid malignancy is chronic myeloid leukemia (CML), accounting for <2 % of childhood leukemias, with an incidence of 1.0 in 10^6 [30,31]. The onset of CML often occurs later in childhood, at a median age of 12.5 years. Sixty percent of the children affected are male and 40 % female [30]. More than 95% express the Ph+, which results in an oncogenic BCR-ABL gene fusion. This gene encodes to BCR-ABL1 tyrosine kinase, a dysfunctional membrane-associated protein, which is an important medical target [31]. The development from chronic phase to blast crisis is usually related to the appearance of additional chromosomal aberrations. The diagnosis is based on clinical characteristics such as hepatosplenomegali, extramedullary disease (infiltrated in skin or lymph nodes), Ph+, myelocytosis, and increased blast count in bone marrow (not exceeding 20% in the chronic phase) [31]. The treatment of choice is HSCT with a five year survival rate of 60-90%, but the introduction of specific BCR-ABL1 inhibitors may change this trend [31,32].

1.2 ETIOLOGY

During recent decades many theories about the cause of childhood leukemia have been discussed. Some are still relevant while others have been ruled out, although the etiology remains unknown in more than 95% of ALL and 80-90% of AML cases [14,15,33]. Specific constitutional and inherited syndromes as well as exposure to ionizing radiation or chemotherapeutic agents are some of the known causes of childhood leukemia [7,34-41].

Several **constitutional syndromes** are associated with an elevated risk of malignancies. For example, in patients with Down´s syndrome, the risk of developing ALL or AML is 10-20 times higher and in the case of megakryoblastic leukemia 600 times higher [40-42]. Other examples of constitutional diseases with an increased risk of childhood leukemia are **inherited disorders** such as Bloom's syndrome, congenital neutropenia, neurofibromatosis, Dyskeratosis congenital, Shwachman syndrome, Noonan syndrome, Ataxia-telangiectasia, Fanconis aplastic anemia, Kostmann syndrome, familial monosmy 7, and Li Fraumenis syndrome [43-53].

As a result of the atomic bomb dropped on Hiroshima in 1945, leading to a radiation level of over 200 mSv, we have learned that **ionizing radiation** can cause leukemia, as the incident rate of leukemia in Japan increased after exposure to the radiation [36]. However, there is no consistent proof that the Chernobyl reactor failure in 1986 increased the incidence of childhood leukemia either immediately or over time. Although some studies have considered the matter there is no conclusive evidence. On the other hand, the incidence of thyroid cancer, especially in children, increased dramatically [54]. From a historical perspective, blood malignancies have also been associated with work-related ionizing radiation where early radiologists suffered from leukemia, for example Marie Curie and her daughter [4]. However, even lower dose levels (10 mSv) due to diagnostic exposure of the fetus to X-ray pelvimetry during pregnancy are correlated with childhood leukemia [35]. Background radiation and non-ionizing electromagnetic fields as a cause of leukemia have been a debated extensively, but most epidemiological studies have found no correlation between childhood leukemia and background radiation or electromagnetic fields [7,55]. However, a weak correlation between long term exposure to high doses (above 0.3/0.4 microT) of magnetic fields and pediatric leukemia was

detected in two meta-analyses, although no experimental studies of the mechanism involved or causal link have been able to firmly establish this connection [7,55-57]. Finally, several studies reveled that children who received radiation therapy for various malignant diseases had a slightly elevated risk of leukemia, in particular AML [16].

It has been demonstrated that secondary leukemia, especially AML, can be induced by treatment of earlier malignancies with **cytotoxic drugs** [33]. Chemotherapeutic agents eradicate cancer cells by damaging DNA, but can also cause DNA injuries in normal cells that could later trigger tumor development. The risk of secondary AML is for example, five times higher in a patient previously treated with cytostatic drugs, especially alkylating therapy, compared to the general population [58]. Alkylating drugs such as busulphan, cyclophosphamide, and melphalan are also commonly used as myeloablative induction therapy in HSCT [33]. Moreover, it is well known that leukemia can occur 3-4 years after melphalan treatment of ovarian or breast cancer [58,59]. In addition, anthracylins, such as doxorubicin and topoisomerase II inhibitors, for example etoposide, are also reported to be possible triggers of secondary malignancies [33,58]. However, only a small group of patients treated with cytostatic drugs develop secondary malignancies, suggesting that they could have a genetic predisposition [58].

In addition to the known leukemogenesic triggers discussed in this chapter, several studies have investigated the etiology of pediatric leukemias, both *in utero* (described in chapter 1.2.1) and in childhood, but no obvious triggers were identified. For example, two different Meta-analyses suggest an increased risk of childhood leukemia due to contact with pesticides, both during pregnancy and childhood [60,61]. Other potential triggers investigated include vitamin K supplements, icterus at birth, solvents, industrial facilities, and obesity [16,62-65].

1.2.1 Prenatal origins of leukemia, a “first and second hit”

While there is space-time clustering data based on time, place of birth, and the incidence rate of ALL has an early peak at 2-5 years of age, it has been hypothesized that the development of ALL in children occurs due to “two hits”, where the first may take place at an early stage, maybe *in utero*, leading to a chromosome aberration and a preleukemic clone that is activated in the postnatal period by the “second hit” [4,7]. The **“first hit”**

has been studied by analyzing genetic abnormalities in archived neonatal DBS, also termed Guthrie cards and in cord blood, as well as by performing twin studies [3]. Mutations found at diagnosis have been analyzed in DBS from children with leukemia, including t(4;11), mixed lineage leukemia (MLL), t(12;21), ETV6-RUNX1 (TEL-AML1), t(8;21), RUNX1-ETV, and hyperdiploidy [66-72]. Some other studies indirectly support the prenatal origin of leukemia, although chromosomal aberrations were not found at the time of diagnosis. By analyzing rearrangement in the immunoglobulin heavy chain (IGH) and T-cell receptor (TCR), Taube et al. were able to trace rearrangement of the IGH from the time of diagnosis back to the DBS in 71 % of the leukemia cases [73,74]. In another study that investigated both the TCR and IGH in the same specific way, it was possible to trace back in all four cases [74]. Additional support for the *in utero* origin of some leukemias is the short latency period and high concordance rate (nearly 100%) of infant monozygotic twins with MLL [3,75]. On the other hand, in ETV6-RUNX1 fusion, one of the most frequent genetic lesions in childhood ALL, also found in DBS, the incubation time is longer (2-15 years) and the concordance rate lower (10%) among monozygotic twins, suggesting a postnatal **" second hit"**, e.g. deletion of ETV6 from the other allele [3,7,75]. In addition, ETV6-RUNX1 fusion has also been found in one percent of 567 healthy newborns whose cord blood was analyzed, representing a 100-fold greater risk than the incidence of childhood ALL [76]. This further supports the theory of a "second hit", where the first is necessary but not sufficient for leukemogenesis by itself. However, one recent study of 1417 umbilical cord blood samples could not detect ETV6-RUNX1 fusion gene in any of them [77]. Genome-wide analyses by means of single nucleotide polymorphism (SNP) arrays have recently found copy number variations (CNVs) in ALL and in concordant ALL twins with ETV6-RUNX1 [78,79]. Additionally, the CNVs are matchless among the paired twins, further supporting the presence of a "second step", verified by single cell clonal analyses [80,81]. Recently, the total genome sequencing from leukemic cells of two monozygotic twin pairs was analyzed, showing that shared prenatal coding-region SNP was restricted to assumed initiating lesions, whereas all other unidentical SNP differed between tumors and was thereby assumed to have occurred postnatally [78].

Although many studies have investigated the etiology *in utero*, no definite trigger has yet been identified. However, interestingly, MLL not only occurs in infant leukemias, but also in secondary leukemias induced by a **topoisomerase II inhibitor** [37-39].

Topoisomerase II inhibitor is a common component of many different compounds, for example quinolone antibiotics, flavonoids in food and drinks, catechins, podophyllin resin, benzene metabolites, and estrogens. Hypothetically, if a fetus with an MLL rearrangement is exposed to this substance *in utero*, it could trigger the development of childhood leukemia. Moreover, a correlation has been found between exposure to DNA-damaging drugs in utero and the development of infant leukemia with MLL gene fusion where the infant leukemia might be initiated by transplacental chemical carcinogenesis [4,37,82-85].

Several other possible triggers *in utero* have been evaluated. A slightly increased risk has been seen for maternal age in large epidemiological studies as well as for alcohol consumption during pregnancy [86-89]. Furthermore, in two different Meta-analyses contact with pesticides during pregnancy was suggested as a cancerogenic agent that could cause childhood leukemia [60,61]. Moreover, use of marijuana in the year before or during pregnancy has been found to correlate significantly with AML, but the results could not be replicated [90,91]. Other suggested triggers include smoking, ultrasound, high meat consumption during pregnancy, as well as high birth weight, but due to conflicting or negative results none of these factors were found to have a definite correlation with childhood leukemia [92-97]. Several studies have been conducted in an attempt to find preventive factors, for example folate supplementation, maternal vitamin use during pregnancy, and a healthy diet including fruit, vegetables as well as beans, but with inconclusive results [92,98,99].

1.2.2 Virus and leukemia

In 1879, Gowers suggested infection as a possible etiology of childhood leukemia. This theory was further discussed, based on clinical observations in the early 1900's [100,101]. However, Gowers' theory was ruled out when it became clear that the disease was not contagious [102]. It was later discovered that a specific virus could be oncogenic and cause malignant blood diseases such as Epstein-Barr virus (EBV) that could induce Burkitt`s lymphoma (B-cell lymphoma) and Hodgkin's lymphoma, human herpes virus 8 (HHV8) that could transform lymphoid cells, and T-cell lymphotropic virus 1 (HTLV1) that could induce T-cell lymphoma as well as T-cell-ALL [7,103,104]. To preserve the integrity of the viral genome during viral replication, the virus must control the

machinery of the cell, by for example, suppressing cellular DNA repair and taking command of the cell cycle [105-107]. After the primary infection, some viruses, for example polyomavirus (PyV), adenovirus, and EBV, may remain latent in the lymphocytes or lymphoid tissue for many years, but can be reactivated in the event of immunosuppression, thereby theoretically inducing genomic instability [108,109]. Another theory is the hit and run mechanism described for several viruses, for example adenoviruses [110,111].

The early age of onset, space-time clustering data based on time, place of birth, and diagnosis as well as molecular studies of DBS, cord blood, and twin studies may correspond to a relationship between childhood ALL and early or *in utero* infection [112,113]. Three non-exclusive hypotheses have suggested infections as a trigger of leukoemogenesis. The **first theory presented by Kinlen L** occurred in response to clusters of leukemia in localities associated with rapid population growth, which led Kinlen to propose that childhood leukemia is due to infection in susceptible, previously unexposed individuals [114,115]. According to this **"population mixing"** model, childhood leukemia is a rare response to a common infection [115,116]. In the **second theory**, **Greaves M** proposed a **"delayed infection"** model, in which delays in exposure to common infections evolutionarily programmed to be met early in life, lead to an abnormal immune response, precipitating the "first and/or second hit" required to produce leukemia [104]. Interestingly, similar immunological arguments are presented in the hygienic hypothesis for childhood allergies and some autoimmune diseases [7,117,118]. The delayed infection theory is consistent with the incidence rate, which seems to be higher in richer societies, at least according to the few studies that have been conducted to date in developing countries [7]. To confirm the delayed infection hypothesis, several epidemiological studies have been performed. Some studies have suggested that daycare attendance during the first year of life may protect against childhood ALL, but other studies were unable to verify this theory [7,119]. A number of studies have investigated birth order, use of breastfeeding, and vaccinations but with contradictory and variable results [120]. Furthermore, diagnostic samples of leukemic cells from either peripheral blood or bone marrow were evaluated for the presence of a variety of viruses; JC-virus (JCV) and BK-virus (BKV), HHV-4, 5, 6, 7, and 8, bovine leukemia virus, and the circovirus-like TT virus, but none of these were associated with leukemia [121-124]. Additionally, a small study screened for non-human sequences in

childhood ALL by means of representational difference analysis, but without any positive results [125]. In the **third model, Smith M** proposed that a prenatal infection elicits pre-leukemic changes in the hematopoietic cells without causing overt disease in the fetus [5]. This, **"infection *in utero*"** model is supported by space-time clustering studies from Sweden and the UK, where clusters of childhood ALL are accumulated both at time of birth and diagnosis [112,113,126,127]. According to Smith, the candidate virus requires certain properties to be oncogenic in utero such as; causing genetic instability, having a specific effect on B-lymphocytes, giving mild symptoms at primary onset, ability to cross the placenta, and not causing common malformations. PyV and adenovirus are among the viruses discussed [6]. Smith´s theory has been tested by analyzing DNA by PCR in DBS for the following viruses; HHV-6 and EBV, CMV, human parvovirus B19, JCV, and BKV, from children who later developed ALL and from controls, but none of these viruses could be detected [128-131]. Adenovirus DNA was detected in 13/51 Guthrie cards from the ALL patients, but only from 6/47 healthy controls (p=0.0122) [132]. However, in another study of pediatric ALL patients in California, adenovirus DNA was not found in DBS [133].

1.3 ADENOVIRUS C AS A POSSIBLE PRENATAL ORIGIN OF CHILDHOOD LEUKEMIA

In 1953, a virus was discovered in human adenoids when searching for the agent that caused the common cold [134]. One year later, acute respiratory disease was investigated in military employees and an agent, possibly a virus, was isolated [135]. It was later revealed that these viruses were related and they were given a name derived from initially isolated cell tissue [136].

Adenovirus has been one of the keys to understanding both fundamental virological and cellular process as well as the interaction between them. It has also been important for the development of gene therapy, where a functional gene (DNA) is introduced to a target cell by a vector to replace a dysfunctional gene, and adenovirus is today the most commonly used vector [137].

In humans 57 serotypes have been identified and organized into seven species (A-G). The serotype classification system is based on serology, hemagglutination configurations as

well as biological and oncogenic characteristics [138]. Adenoviruses can cause diverse clinical symptoms, from mild respiratory infections in children to severe multi-organ disease in immunocompromised patients [139].

1.3.1 Structure and genomic organization

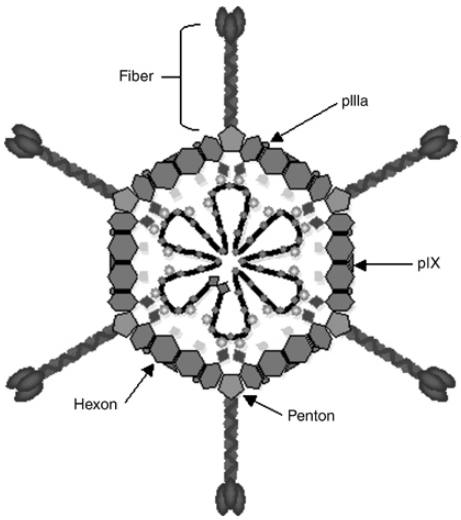

Figure 1: The structure of the viral capsid and the genome of adenovirus

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Adenovirus is a medium-sized (70-100 nm) non-enveloped linear double-stranded DNA virus with an icosahedral nucleocapsid [140]. Hexon is the most frequent protein in the capsid. There are 12 prominent proteins/fibers around the capsid that mediate binding to the target cell [141].

The adenovirus genome (30-40 kbp) contains five early transcription units (E1A, E1B, E2, E3 and E4), two intermediate (IX and IVa2), and five late mRNAs (L1-L5) that encode for over 40 different proteins due to effective organization and alternative splicing of the genome. Moreover, there are two virus-associated RNAs (VA RNA I and II) [142]. The early proteins are expressed before virus DNA replication and their function is to facilitate viral gene expression and disturb host anti-viral mechanisms, whereas the function of the late proteins, which are expressed after viral replication, is to assemble the virions and release them from the cell [142].

1.3.2 Adenovirus in humans

Adenovirus spreads by aerosol and the fecal-oral route of transmission. These infections are typically associated with self-limited respiratory, conjunctival and gastrointestinal

disease in immune competent children and adults [143]. The common endemic species C adenovirus (serotypes 1,2,5, and 6) normally infects young children and is responsible for 5-15% of respiratory tract infections in children under the age of five years, with a seroprevalence of 40-60% in childhood [144,145]. In a seroepidemiologic study of various infections in young men, 98.7% were seropositive to adenovirus, indicating that most of them were infected at a young age [146]. Tonsillitis caused by adenovirus is very common and the peak of children undergoing tonsillectomies with adenovirus DNA detected in their mucosal lymphocytes are aged 2-5 years, which correlates with the peak presentation of childhood ALL [7,145,147]. After a primary infection, adenovirus remains latent in T cells [147]. In immunosuppressed patients, adenovirus infections are related to severe morbidity and mortality as a result of both the primary infection and reactivations [143].

There are still no standard antiviral drugs available for treatment of adenovirus infection, but cidofovir and foscavir are used without a license [148]. Between 1971 and 1996 an oral, live, enteric-coated vaccine to prevent infections was used in the military, with good efficacy [149,150]. However, it has not been used commercially due to lack of prospective, large, randomized controlled trials [151].

1.3.3 Adenovirus and cancer

In 1962, adenovirus type 12 was found to induce multiple tumors in newborn hamsters including sarcomas, neuroectodermal tumors, adenocarcinomas, retinoblastomas, and medulloblastomas [152,153]. Since then the oncogenic potential of adenovirus has been of interest to the scientific community. In a recent study by Kosulin, over 500 diagnostic samples from 17 different types of pediatric malignancy including solid tumors, leukemias and lymphoma were tested for adenovirus, with the majority of results being negative [154]. Adenovirus sequences were detected in different pediatric brain tumors, but also in healthy brain tissue. It was unclear whether the adenovirus had persisted from an earlier infection, had a tropism to brain tissue or if the virus was involved in the oncogenesis [154]. Furthermore, bone marrow or peripheral blood samples from 130 pediatric leukemias including pre-B-ALL, T-ALL, AML, and CML were analyzed but only two samples were found positive for adenovirus DNA [154]. It could not be excluded that this finding was only an expression of occasional persistence of the virus in

peripheral blood, thus its role in human oncogenesis is still unclear [154]. Adenovirus has also been studied in adults with different types of tumor but no clear association was observed between the virus and tumor development [155,156].

Adenovirus is unique among human viruses because its genome persists in the infected cell as a linear double-stranded DNA. Furthermore, adenovirus suppresses cellular DNA repair in order to preserve the integrity of its genome during viral replication [105]. Adenovirus has unique potential to be mutagenic and is well known for its ability to transform rodent cells through sustained expression of oncogenes such as E1A and E1B [110]. E1A and E1B are transcription factors for viral and cellular genes interacting with important tumor suppressors, such as Retinoblastoma protein (pRB) and p53 proteins [110,157]. Studies have revealed that mutant adenovirus that does not express E1B protein is only able to replicate and lyse cells with defective p53 expression, but not those with wild type p53. Moreover, it was demonstrated that mice with a mutation of *TP53* had a reduction in tumor size when abnormal adenovirus was inoculated into the tumors, making mutant adenovirus a candidate for the treatment of tumors with aberrant *TP53* [158]. In addition, the E4 region of the virus includes three oncoproteins that cooperate with E1B to transform cells [105]. The viral E4 and E1B genes that block DNA repair have three distinctive tasks. First, the E4orf3 protein of species C adenovirus is able to disturb the MRN complex that controls both the signaling and repair activities of the DNA [159]. Second, cellular proteins, that are important for DNA repair are targets for degradation by a viral ubiquitin ligase created by E1B and E4 proteins [105]. Third, the E4orf6 protein blocks double-stranded DNA-break repair by inactivating other proteins that contribute to both repair and signaling [160]. Finally, studies have concluded that adenovirus is able to transform cells through a "hit and run" mechanism, making adenovirus C a candidate for causing the initial genetic aberration that may lead to malignancy [110,111].

1.4 POLYOMAVIRUS AS A POSSIBLE PRENATAL ORIGIN OF CHILDHOOD LEUKEMIA

In 1953 Ludwik Gross made the remarkable discovery that as a result of contamination by an unknown “agent”, extracts from mouse leukemia cells injected into newborn mice induced the development of salivary gland carcinoma instead of the expected leukemia

[161]. A few years later, Stewart and Eddy demonstrated that this “agent” could induce tumors not only in mice but also in rats and hamsters and they later isolated a small DNA virus [162-165]. This virus was named polyoma, known today as murine polyomavirus (MPyV), and belongs to the Polyomaviridae family [166,167]. Shortly afterwards simian virus 40 (SV40) was isolated from monkey kidney cells and together with MPyV contributed to some of the most important tumor cell biology models [168,169]. In 1971, BKV and JCV were isolated from kidney and brain tissue, respectively [170,171]. In 2007, Karolinska Institute polyomavirus (KIPyV) and Washington University polyomavirus (WUPyV) were discovered in respiratory samples of children with acute respiratory symptoms, and the following year the Merkel cell polyomavirus (MCPyV), the fifth human PyVs (HPyV), was detected in Merkel cell carcinoma (MCC), a rare aggressive skin cancer [172-174]. Since then, ten new HPyVs have been discovered; HPyV-6, HPyV-7, HPyV-9, HPyV-10, HPyV-12, Trichodysplasia spinulosa-associated polyomavirus (TSPyV), Malawi polyomavirus (MWPyV) and Mexico polyomavirus (MXPyV), Saint Luis polyomavirus (STLPyV), New Jersey polyomavirus (NJPyV) [175-183]. The number of viruses belonging in the Polyomaviridae family has increased during the last decade and today (summer 2014) it has 15 human and 17 non-human members.

1.4.1 Structure and genomic organization

PyV is a small (40-45nm) non-enveloped circular double-stranded DNA-virus (5000 bp) with an icosahedral nucleocapsid [184].

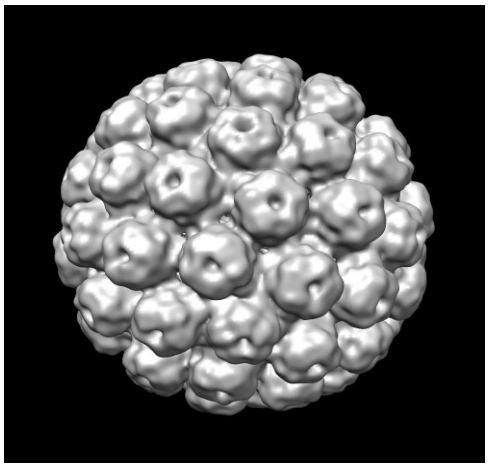

Figure 2: An electron microscopic photograph of JCV capsids
Reprinted by permission of the Protein Data Bank japan: PDBj

The genome of the PyVs family is divided into three regions encoding for early and late proteins in addition to the non-coding control region (NCCR). The early and late proteins are highly conservative, whereas the NCCR is more variable and contains the origin of replication, promoters, enhancers, and binding sites, which are important for replication and transcription [185].

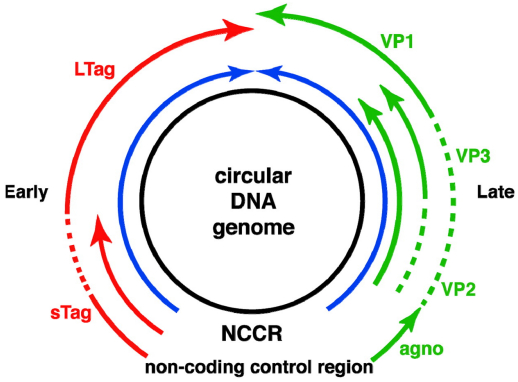

Figure 3: Genomic map of prototype Polyomavirus

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The early region includes one shared pre messenger RNA (mRNA) that generates two early mRNAs by alternative splicing, which are translated into two proteins; large T (LT) and small T (ST) [185,186]. By additional splicing, some types of polyomavirus express supplementary early proteins [187,188].

The LT is a multifunctional protein necessary for both replication of the virus itself and stimulation of the host cell into DNA synthesis. The LT can bind to three specific NCCR sites, thereby regulating both early and late transcription [189-191]. Furthermore, the LT regions can bind to cell cycle regulating proteins (e.g. pRB and p53), blocking their growth suppressor function and forcing the host cell into the S phase. This enables uncontrolled viral replication and is important for the oncogenic potential of polyomavirus [167,185].

Although the ST protein is expressed in all polyomaviruses, its role is less clear. It has been proposed that its main function is to block the protein phosphatase 2 A (PP2A) function, leading to the activation of numerous pathways including mitogen-activated protein kinas (MAPK), and Phosphatidylinositol 3-kinase (PI3K)/AKT, thereby intensifying the oncogenic effect of LT [192].

The late region encodes the three capsid proteins VP1, VP2 and VP3, in addition to multi functional agnoprotein (LP1). These proteins originate from a common mRNA by alternative splicing [185]. VP1 is the most frequently expressed protein (90%) and creates the outer shell of PyV [193]. The VP1 amino acid sequences are highly preserved among HPyV.

1.4.2 Polyomavirus in humans

Known seroprevalence for 9/15 HPyV in adults is 35-99%, as presented in Table 1 [193]. Respiratory and fecal-oral routes for transmission of HPyV have been suggested, while in the case of MCPyV transmission through skin-to-skin contact has also been proposed [108,185,194,195].

Table I

Seroprevalence of HPyVs in adults.

HPyV	Seroprevalence in adults (%)	Country	Method	References
BKV	82–99	USA, Australia, Italy	VLP ELISA and VP1 capsomer based ELISA	Antonsson et al. (2010), Egli et al. (2009), Kean et al. (2009) and Viscidi et al., (2011)
JCV	39–81	USA, Australia, Italy	VLP ELISA and VP1 capsomer based ELISA	Antonsson et al. (2010), Egli et al. (2009), Kean et al. (2009) and Viscidi et al. (2011)
KIPyV	55–90	USA	VLP ELIA and VP1 capsomer based ELISA	Carter et al. (2009) and Kean et al. (2009)
WUPyV	69–98	USA	VP1 capsomer based ELISA Multiplex antibody binding assays	Kean et al. (2009) and Carter et al. (2009)
MCPyV	60–81	Italy	VLP ELISA Multiplex antibody binding assay	Carter et al. (2009) and Viscidi et al. (2011)
HPyV6	69	USA	VLP ELISA	Schowalter et al. (2010)
HPyV7	35	USA	VLP ELISA	Schowalter et al. (2010)
TSV	70	The Netherlands	Multiplex antibody binding assay	van der Meijden et al. (2010)
HPyV9	21–53	France, Germany	VLP ELISA VP1 recombinant protein ELISA	Nicol et al. (2012) and Trusch et al. (2012)
MWPyV/HpyV10	ND*			

* Not done.

Complete references can be found in the published article

In healthy individuals the primary HPyV infection appears to be asymptomatic or with only mild respiratory symptoms and occurs often in childhood [108,196,197]. After the primary infection, the HPyV remains latent and may be reactivated by the occurrence of

immunosuppression [108]. Reactivation of **JCV** is associated with progressive multifocal leukoencephalopaty (PML), characterized by demyelinating plaques and a classic triad of symptoms; cognitive impairment, visual deficits, and motor dysfunction [198]. Reactivation of the **BKV** virus is associated with hemorrhagic cystitis in HSCT patients, the outcome of which is occasionally fatal [199]. In kidney-transplanted patients, reactivated BKV is associated with nephropathy and ureteral stenosis [185].

KIPyV and **WUPyV** have been detected in the upper airways, tonsil tissue, blood, plasma and stool, but as yet there is no confirmed link between the disease and infection [172,173,194,200,201]. In a recent retrospective study, 222 bone marrow transplanted patients were followed up on a weekly basis for one year after HSCT, by monitoring clinical status and nasal aspirates. The results revealed a cumulative incidence of 26% for KIPyV and 8% for WUPyV. The infections were associated with wheezing and sputum production [202]. In another study of 200 patients with respiratory disorders, 89% of whom were immunocompromised, KIPyV was detected in 8% and WUPyV in 1%. In line with the previous study KIPyV was significantly more frequent in HSCT patients than in other immunocompromised individuals (17.8% vs 5.1%) [203].

MCPyV was found in MCC in elderly and immunosuppressed patients, but has also been frequently detected in samples of healthy skin and in extracutaneous locations such as lymph nodes, esophagus, salivary glands, oral mucosa, as well as in breast and vaginal tissue [174,195,204,205].

1.4.3 Polyomavirus and cancer

It is well established that PyVs have oncogenic potential, evidenced by early animal studies [206]. SV40 is known to initiate a tumorigenic mechanism by interaction of the LT antigen with cell cycle regulating proteins such as p53 and pRB at an early stage of the infection process, but also by integration of viral DNA in the host genome. However, it has been postulated that SV40 does not cause tumors in its natural hosts [207,208]. DNA sequences of BKV, JCV and SV40 have been detected in different types of human malignancy, such as colorectal tumors, pancreatic cancer, prostate cancer, mesothelioma, non-UV light associated melanomas, pediatric and adult brain tumors, osteosarcoma, sarcomas and non Hodgkin lymphomas, but the significance of these findings is

controversial [167,193,209,210]. It has recently been shown that hematopoietic stem cells could go through neoplastic alterations when infected with JCV [107]. Despite discordant findings in human and experimental animal studies, the WHO International Agency for Cancer Research Monograph Working Group has recently classified BKV and JCV as "possibly carcinogenic to humans" [211].

A few studies have investigated the role of KIPyV and WUPyV in various malignancies including pediatric brain tumors and non-UV exposed melanomas with negative results [212,213]. However, it has been reported that VP1 sequences from KIPyV were identified in 9/20 lung cancers and sequences coding for the C terminal of the early region were detected in two of these cases [200].

To date, only MCPyV has been strongly linked to human tumors [174]. MCPyV DNA has been detected in the majority of MCCs (80%), in skin cancer as well as in primary gastric MCC [106,197,204,205]. In other types of skin tumor MCPyV DNA was not observed in non-UV light associated primary malignant melanoma, but was detected at low levels in keratoacanthoma and squamous cell carcinoma [213,214]. It is well known that patients with MCC are at risk of developing chronic lymphocytic leukemia and small lymphocytic lymphoma. Recently, Cimino et al. investigated the correlation between MCPyV and these two diseases and detected MCPyV DNA in 13% of T-cells from these patients [204]. Other studies have found no presence of MCPyV in pediatric brain, lung, prostatic, uterine cervix, large bowel, ovary, breast, bone and soft tissue tumors [213,215,216].

1.5 PROGNOSTIC MARKERS OF LEUKEMIA

The long time survival rates have increased dramatically in recent decades and are today 90% in children with ALL and 70% in those with AML [9-12]. The five year survival rate has also improved for MDS (60 %), JMML (50%), and CML (60-90%) [25,29,31,32]. Improved survival is due to more effective chemotherapeutic agents, supportive care and protocols. However, approximately 20% of pediatric ALL patients and 30-40% of pediatric AML patients suffer a relapse and normally undergo a more intensive therapy and/or HSCT [9,10,12,217,218]. Nevertheless, the outcome of relapsed ALL and AML patients is poor, and the long-term outcome remains unsatisfactory with

cure rates of only 30–40% for children with ALL and 20-60% for those with AML [12,219-221]. Children who relapse, those with specific high-risk markers at diagnosis, and almost all children with MDS, JMML, and CML are candidates for HSCT [12,32,222]. Nevertheless, the relapse rate after HSCT is 30-60 % for AML and associated with a poor prognosis [223,224]. In order to decrease the relapse rate after HSCT, treatment must be more personalized, with better risk-group stratification as well as earlier identification of the risk of relapse [11].

Essential prognostic markers for **ALL** are age, leukocyte count at diagnosis, immunophenotype, chromosomal aberrations, and response to initial therapy (MRD) [16]. Examples of favorable chromosomal abnormalities in pre B-ALL are; high hyperdiploidy (>50 chromosomes), hyperdiploidi (47-50 chromosomes), and ETV6-RUNX1. Examples of unfavorable chromosomal abnormalities in pre B-ALL are; Ph+, hypodiploidy (<45 chromosomes), intrachromosomal AM1 amplification (iAMP21), t(1;19) (E2A-PBX1), dic(9;20) (PAX5/various), and MLL rearrangements [11,17,225]. In addition, submicroscopic genetic alterations seem to contribute to leukemogenesis, where high-resolution microarray is used to analyze distinct gene expression profiles such as micro deletion or duplications, allowing new prognostic markers and therapeutic targets [11,17]. Due to the diversity of its precursors **AML** is a multifaceted disease that includes a spectrum of genetic changes [16]. The risk group assessment is mainly based on cytogenetics and response to treatment, where the new era seems to be MRD [12,226]. Examples of favorable chromosomal abnormalities in AML are; t(9;11), t(8;21), and inv (16). Examples of unfavorable chromosomal abnormalities in AML are; MLL rearrangements other than t(9;11), a complex karyotype, monosmy 5, del (5q), and monosomy 7 [12,226]. In addition, a genetic FMS-like tyrosine kinase 3 (FLT3) internal tandem duplication without a nucleophosmin 1(NPM1) mutation has a very poor prognosis [12]. In children with **MDS,** clinical parameters found at diagnosis associated with unfavorable prognosis are; high blast count in bone marrow, elevated hemoglobin F (>10%), and thrombocytopenia [222,227]. To date, no chromosomal aberration found in childhood MDS is correlated with unfavorable prognosis with the exception of monosomy 7 and 5 [222,228]. In **JMML**, clinical parameters associated with poor prognosis are; age >2 years, thrombocytopenia, elevated bone marrow blast count, and hemoglobin F [228]. Prognostic factors in **CML** are; sex, age, spleen size at diagnosis, platelet count, number of myeloblasts, as well as eosinophil and basophil counts [16].

Carcinogenesis is a multi-step event that occurs in stem or precursor cells, where genes that regulate cell growth, apoptosis, DNA repair, and other processes are altered and lose their function, or their protein is inactivated due to other mechanisms [1,2]. **Tumor suppressor genes** and **oncogenes** are required for normal cell proliferation as well as differentiation. Variation in protein expression may be present as new prognostic markers [1,2].

1.5.1 The cell cycle and its regulating proteins

In 1855 Virchow discovered the ability of the cell to divide itself in order to create new cell copies, after which scientists studied this skill carefully, but without understanding the underlying mechanism [229]. In the late 1970s and 80s many fundamental discoveries concerning the cell cycle and its mechanisms were made and in 2001 Hartwell, Hunt, and Nurse won the Nobel Prize in Medicine and Physiology for their discovery of the central cell cycle regulating genes and molecules such as cyclins and cyclin-dependent kinases (CDKs), which is the basis of today´s knowledge [230-232].

The Cell cycle is essential for the cell fate and responsible for renewing and growing the cell population. Furthermore, it is also responsible for control and repair of damaged cells. It consists of two main phases, mitosis (M phase) and interphase (divided into gap 1 (G_1), the DNA synthesis (S), gap 2 (G_2) and gap 0 (G_0)) [233,234]. The **M phase** is the first step in the cell cycle and includes both the karyokinesis (division of the nucleus into two daughter nuclei) and the cytokinesis (split of the cells into two daughter cells). The next step is the **G_1 phase**, which is characterized by an increase in cell size as well as the pRB pathway acting as a DNA checkpoint, before the S phase [234,235]. The G_1 phase can last from hours to years. In a non-dividing cell it could persist for a "lifetime", in which case it is called the **G_0 phase**. Cells in G_0 arrest are often differentiated, for example post mitotic nerve and skeletal muscle cells, but even stem cells are mainly inactive and non dividing in the G_0 phase. They may be induced to re-enter the cell cycle in response to the natural need for cell renewal, but also as a reaction to injury of the cell population [234,236]. The pRB and the p53 proteins carefully monitor the step from the G1 to the **S phase**. The pRB pathway is a negative regulator of the E2F family, which is required for entering the S phase. Phosphorylation of the pRB family proteins by CDKs

during the G_1 phase leads to separation from the E2Fs and thereby transcription of genes necessary for entering the S phase [237]. The S phase replicates the DNA of the cell and new chromatids are formed, preparing the cell for the next M phase. The last phase before entering a new M phase is the **G_2 phase**, where the cell continues to grow and no DNA is synthesized. The G_2 phase is also the last checkpoint for DNA control before the start of a new cycle [233,234].

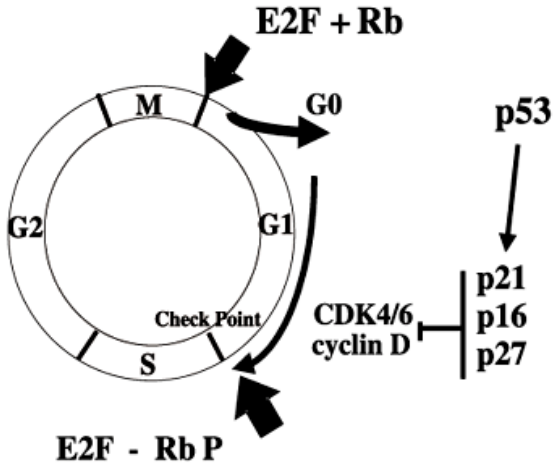

Figure 4: In the cell cycle the pRB pathway strictly controls DNA, preventing damage and possible tumor development.

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The movement through the cell cycle is strongly regulated and controlled by the two key classes of regulatory molecules; cyclins and CDKs [238]. Progression through the cell cycle is complex, where cyklins and CDKs are highly dependent on each other, acting as a complex in a heterodimer, in which the cyclins form the regulatory and CDKs the catalytic subunits. CDKs activated by a cyklin, activate/inactivate target proteins, thus regulating the cell cycle through phosphorylation. Different combinations of cyclin-CDK heterodimers regulate distinctive downstream proteins [233,234].

Negative regulation of CDKs by CDK inhibitors (CKIs) is necessary to prevent uninhibited cell growth. For example, CKIs can arrest the progression from the G_1 to the S phase by binding to the cyklin/CDK complex in response to various stimuli such as growth factors, DNA damage, cellular stress, differentiation, and senescence [234]. There are two CKIs families; the inhibitor of kinase 4 (INK4) family (p15, p16, p18, and p19), which blocks the activity of cyklin-D-CDK4/6 responsible for activation of pRB, and the

p21 (also named CIP/KIP) family (p21, p27, and p57), the members of which are less specific and can inhibit several cyklin /CDK heterodimers [234,239-242].

1.5.2 p53 and its role in tumor genesis

TP53 is located on chromosome 17p13 and was discovered in 1979 by Lane, Crawford, Levine and Linzer as a host protein for the LT antigen from SV40 [243,244]. A decade later its property as a tumor suppressor gene was revealed [245]. Since then, more properties have been identified and today it is known that p53 protein can initiate cell cycle arrest, DNA repair, apoptosis and senescence through different signaling pathways (Figure 5) in response to cellular stress, such as DNA damage, hypoxia, and oncogene activation [246]. For this reason it is also called the "guardian of the genome". However, it is now clear that p53 has a broader role in the cell organism, and today there is evidence that p53 protein is involved in different mechanisms including; regulation of cell senescence, survival, invasion, motility, glycolysis, autophagy, oxidative stress, angiogenesis, differentiation, and bone remodeling [247]. Furthermore, the p53 protein is also a key factor for steady-state in normal hematopoiesis, regulating the regeneration, quiescence, and degradation of the hematopoietic stem cell (HSC), critical for preserving the lifelong pool [246,248].

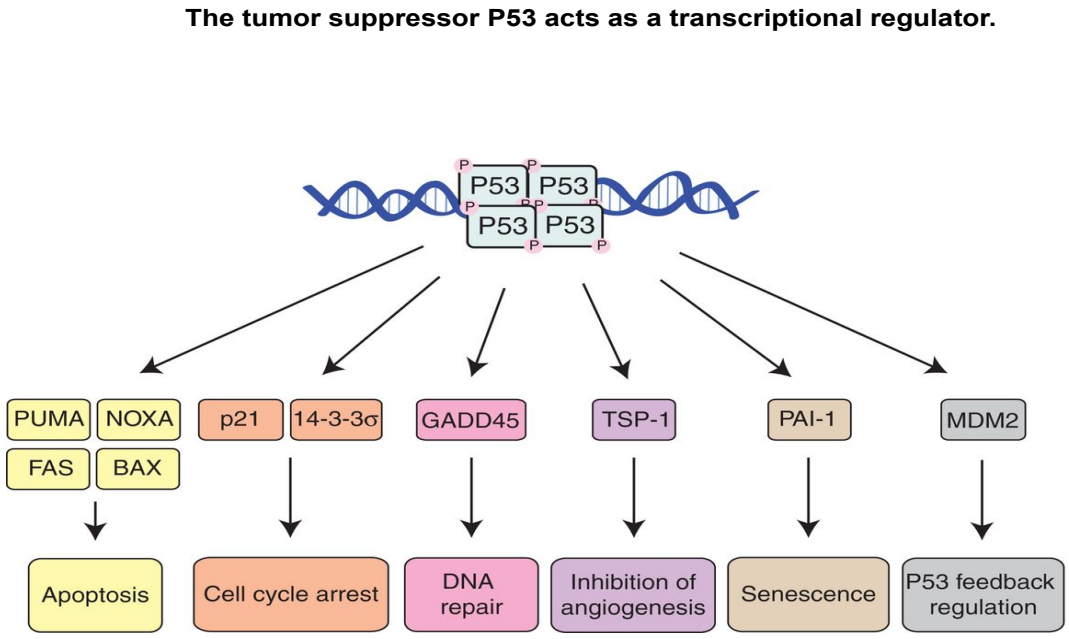

Figure 5: The tumor suppressor P53 acts as a transcriptional regulator. It has the capacity to activate diverse cellular processes. Stimulus and cell type-specific effects determine which particular effector pathway(s) will dominate.

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1.5.2.1 Protein expression of p53

Normally the half-life of the wild-type p53 protein is short (20 minutes) and results in the low level of p53 protein in the nucleus under ordinary physiological conditions [249,250]. In steady-state the p53 protein is regulated by several E3 ubiquitin ligases (enzymes responsible for the stabilization of p53) in a feedback-loop, where *murine double minute protein-2 (MDM2)* is a key regulator [251]. Activation of *TP53* is normally a consequence of cellular stress or oncogene activation [247,252,253]. However, mutations in *TP53* may lead to a defective p53 protein with an extended half-life that accumulates in the cell nucleus [254,255], and it has been suggested that immunohistochemistry of p53 protein expression can be used to find mutations of the *TP53* gene [256,257]. However, accumulation of p53 can also be found without detected gene amplification and in non-malignant diseases, for instance other mechanisms that involve the wild-type p53, such as cellular stress during inflammation or hypoxia [247,257-260]. Finally, overexpression of wild-type p53 protein can also be initiated by abnormal functionality of the proteins responsible for deregulation of p53 protein, such as *MDM2* [259,261].

Inactivation of p53 leading to the loss of function is one of the most common events found in human tumors [262]. More than 90% of alterations in *TP53* leads to ineffective p53 protein and reduced function as a transcription factor [263]. Furthermore, inactivation could also be a result of accumulation of p53 protein in the cytoplasm [264,265]. In addition, inactivation can be caused by inhibiting proteins like MDM2 and by tumor viruses such as adenoviruses, SV40 and human papillomaviruses (HPV) [106,107,110,157,251,266].

1.5.2.2 p53 and cancer

Alterations of *TP53* leading to dysfunctional protein occur in more than 50% of human solid tumors in adults, where p53 mutations are most frequent in ovarian, esophageal, colorectal, head, neck, larynx, and lung cancers [267,268]. These mutations are generally inactivated by single-base replacement and/or loss of alleles, initiated by viral or cellular proteins [268,269]. Missense mutation is the most common alteration of *TP53* and causes single amino-acid changes at many different positions, making it possible to recognize various mutation patterns related to the type of malignancy and etiology. Moreover,

tumors with *TP53* alterations are generally associated with a more aggressive disease and poor prognosis [268].

Early onset of cancer often occurs in conditions caused by hereditary *TP53* mutations, where Li-Fraumeni's syndrome is the best known [43]. In these families the *TP53* alteration is inherited in one allele, meaning that only one "hit" is required to inactivate *TP53* [270]. Li-Fraumeni is characterized by multiple cancers in the family including breast cancer, osteosarcoma, and rhabdomyosarcoma but also AML [270,271]. However, germline mutations were also identified in 23% of children with rhabdomyosarcoma and in 3% in children with osteosarcoma, despite the fact that there was no family history of cancer [272-274]. Moreover, it was found that patients with *TP53* germline mutations are more sensitive to alkylators and such treatment has been suggested to increase the risk of inducing AML or MDS [275-277].

1.5.2.3 p53 in hematological malignancies

Alterations in *TP53* are less common in hematological malignancies than in solid tumors, with a reported frequency of 10-20% [257,267,278]. *TP53* abnormalities are more frequently detected in adult hematological malignancies than in pediatric patients and have mainly been correlated with blast crisis in CML, ALL, Burkit´s Lymphoma, and with impaired disease in adult MDS [279-281]. Moreover, adult patients with *TP53* alterations are frequently more resistant to chemotherapy and have a relatively short survival [246,282].

In the pediatric population the prevalence, knowledge, and impact of *TP53* mutations are less known due to the lack of studies. Such mutations have only been found at diagnosis in 2% of children with ALL, but in 11-28% after relapse, suggesting that a *TP53* mutation is associated with an increased risk of superior relapse [283-288]. Furthermore, studies have indicated that *TP53* alterations are more frequent at time of relapse compared to both time of diagnosis and non-relapsed patients as well as being related to an inferior probability of both event-free and overall survival [288,289]. Moreover, in a group of children with low-hypodiploid ALL (32-39 chromosomes), alterations in *TP53* were detected in 91.2%. Interestingly, 43% of those alterations were found in non-tumor hematopoietic cells, which may indicate an inherited origin, but might also signal a take over of the hematological compartment [290].

Even less is known about *TP53* alterations in AML, MDS, JMML, and CML in children as the sample groups are small and the results conflicting. Alterations of *TP53* have been found in 11-95% of pediatric MDS patients [275,291,292]. However, no alterations were found in children with JMML in three minor studies [291-293]. Finally, there are few studies on the prevalence of *TP53* mutations in children with AML and CML.

Our group has previously reported an overexpression of p53 protein in bone marrow samples from different kinds of pediatric leukemia patients undergoing HSCT in comparison to non-malignant bone marrow disorders [294]. Moreover, we found an increased expression of p53 protein in bone marrow cells from pediatric high risk ALL patients treated with HSCT, both at diagnosis and pre HSCT, compared to samples from ALL patients in remission [295].

1.5.3 p21

The p21 protein was discovered as a mediator of p53 tumor suppressor activity and is encoded by the CDKNA1A gene, located at 6p21.2 locus. p21 has many names including WAF1 and CIP1 but is also known as cyklin-dependent kinase inhibitor1 or CDK-interacting protein 1. p21 is a CDK and proliferating cell nuclear antigen (PCNA) inhibitor activated by different pathways including the p53-p21 pathway, but also by pathways that are independent of p53. It regulates the cell cycle progression from G1 to the S phase, creating cell cycle arrest responsible for growth arrest, cellular sentences, differentiation, and DNA damage repair. In addition, p21 protein can interact with other proteins and influence the cellular mechanism independent of CDK and PCNA [296].

The half-life of p21 is short (20-60 minutes) and under normal physiological conditions cellular p21 protein is expressed at very low levels in the cell [296,297]. However, the protein activity is regulated by multiple mechanisms such as transcription factors, ubiquitin ligases, and protein kinases that regulate the transcription, stability, and cellular location of the p21[296]. In the p53-p21 pathway, an activated wild type p53 protein triggers p21 expression, whereas an altered *TP53* may lead to overexpression of a defective p53, unable to increase the expression of p21 [254,255].

1.5.3.1 p21 and cancer

It is believed that p21 does not act as a sole agent but collaborates with other tumor suppressor genes such as p53. Deregulated p21 was found in some tumors, e.g. colorectal, cervical, head and neck, and small-cell lung cancer [296]. Moreover, p21 is described as a potential oncogene due to its ability to block apoptosis and it was also found overexpressed in several types of human cancer such as cervical, breast, and prostate [296,298]. Furthermore, p21 was detected up regulated in AML patients and correlated with poor prognosis [299].

1.5.4 p16

The protein of p16 is encoded by the CDKN2A gene that was discovered in 1993, located at the 9p21 locus [242,300,301]. p16 is a CDK inhibitor, regulating the G_1 phase by inhibiting phosphorylation of the pRB [300,301]. In normal young tissue, p16 is expressed at very low levels and increases with age in mammalian cells [302,303]. p16 is activated and mediates sentences due to cellular stress such as accumulation of DNA damaged cells caused by inactivated *TP53* and seems to play an important roll in ageing. Thereby, it has been suggested that p16 is a stand-by tumor suppressor gene to p53 and was observed as up-regulated in p53 deficient mice [300].

1.5.4.1 p16 and cancer

The CDKN2A gene is frequently down regulated by promotor methylation, mutated or deleted in a wide variety of tumors such as; melanoma, breast, brain, ovarian, pancreas, osteosarcoma, head and neck, oropharyngeal squamous cell carcinoma, and esophagus cancer. It is used as a prognostic marker for several kinds of tumor [304,305]. Alterations of p16 are also found in atypical familial multiple mole/melanoma (FAMM), an autosomal dominant disease characterized by multiple familial melanoma [305].

As mentioned above, p16 expression increases with age, but in AML, lymphoma, and glioblastoma patients the opposite is the case. Thereby, it is hypothesized that suppression of p16 that acts as a sentinel for DNA damage in aging cells supports cancer development in older individuals [306].

Mutations of p16 are present in about 20% of pediatric pre-B-ALL and in 70% of T-ALL [307-314]. Recently p16 mutations were studied in 73 pediatric pre-B-ALL patients, where the prevalence of homozygous deletion was 24.7% (n=18), hemizygous deletion 6.8% (n=5) and no deletion 68.5% (n=50). The deletion was compared to a standard risk group, a high-risk group and a combined risk group. When comparing the risk groups with/without deletion, the event-free and overall survival were significantly lower when p16 deletion was found, except in the high-risk group. This result may indicate that deletion of p16 is associated with poor prognosis in childhood ALL, which is also confirmed by other studies [307,308,310,311,313-315]. The frequency of mutation in the Ink4-locus, including p16, has been further studied, where the Bi allelic deletions were detected in around 30% of pediatric pre-B- and T-ALL and mono allelic deletions in approximately 10-15%. The bi allelic deletion was associated with worse prognosis compared to mono allelic deletion or normal Ink4-locus expression [315].

1.5.5 PTEN

PTEN (phosphatase with tensin homology), a tumor suppressor gene located on chromosome 10, which is involved in apoptosis, induction of cell cycle arrest, regulation of cell adhesion, differentiation, and migration was identified in the 1990s [316-321]. The protein produced by *PTEN*, a lipid phosphatase, dephosphorylates phosphatidylinositol triphosphate (PIP_3) into phosphatidylinositol biphosphate PIP_2, which is the main negative controller of the PI3K/AKT signaling pathway, responsible for cell proliferation, metabolism, motility, and cell survival [317,318,322,323]. The occurrence of mutations and/or deletions of *PTEN* result in dysfunctional or decreased protein expression, leading to hyper-activation of the PI3K/AKT pathway and facilitating tumor development [317,322].

1.5.5.1 Protein expression of PTEN

The protein from *PTEN* is comparatively stable and found in the nucleus but also in the cytosol, adhered to the membrane [318]. Mutations of *PTEN* lead to a dysfunctional or decreased protein expression [317,322]. However, not only alterations of PTEN affect the protein expression. *PTEN* can also be inactivated by different mechanisms affecting the protein transcription or translation, as well as posttranslational protein modifications,

leading to stabilization and overexpression of the protein, in addition to inhibition of its tumor suppressing function [317,318].

1.5.5.2 PTEN and cancer

Critically, *PTEN* is regularly deactivated by bi allelic alterations (thought to be a two-hit model), but mutations of one allele may also impair tumor suppression. Thereby, *PTEN* is very sensitive and important for cancer development [317,318]. Inactivated or deregulated *PTEN* is frequently found in human cancers such as breast cancer, carcinoma, gastric tumors, melanoma, prostatic carcinoma, and certain hematological malignancies [317]. Mutations of *PTEN* were also found in 80% of patients with Cowden's disease, an inherited autosomal dominant disorder, characterized by multiple hamartomsas and the risk of breast, emdometrial, and thyroid carcinomas, but also other malignancies [318,324,325].

Although alterations of the PI3K/AKT pathway seem to be rare in hematological tumors, the pathway is frequently involved in leukemia and activated in a majority of AML patients, but also in other leukaemias [317,318,326]. Furthermore, activation of the PI3K/AKT pathway in AML patients is linked to tumor cell survival, proliferation and chemo resistance [317,327-329]. Despite the fact that the PI3K/AKT pathway is important and activated in several hematological malignancies, alterations of *PTEN* are not frequently found in adult hematological malignancies and relatively unexplored in childhood leukemias [317]. However, alterations of *PTEN* have been seen in 5-27% of T-ALL and are associated with poor prognosis [317]. A study in a mouse model has reported that *PTEN* depletion could lead to a myeloproliferative disease that can rapidly develop into AML or ALL [330]. Although there are only a few studies on childhood leukemia, it has been found that PTEN protein expression was significantly lower in AML and ALL blast cells from eight children, compared to normal cells [331]. Furthermore, the *PTEN* promoter was inactivated in 20% of pediatric ALL [332]. However, in a recent study PTEN protein was found overexpressed at diagnosis in a group of children with a five year disease-free survival, when compared with a pediatric patient suffering from a non-malignant disease. Though, the relapsed patient presented with a lower expression of PTEN protein compared to non relapsed patients, indicating that PTEN could be a prognostic marker, although the sample group was too small for statistical analyze [333].

1.5.5.3 Interaction between PTEN and p53

PTEN can interact directly with p53 by binding to it, thereby blocking MDM2 from attaching to p53. It has been further demonstrated that *PTEN* can regulate the cellular localization (from nucleus to cytoplasm) of MDM2 by inactivation of PI3K/AKT, thereby indirectly protecting p53 [322,334]. In addition, *PTEN* may influence the promoter activity of MDM2, suggesting that loss of *PTEN* function leads to decreased p53 function [335]. Moreover, overexpression of MDM2 in the absence of *PTEN* expression was associated with resistance to drug induced apoptosis [334]. Finally, p53 can also up regulate PTEN expression through a p53 binding site at the promotor of *PTEN* [336].

2 AIMS OF THE THESIS

The general aim of this thesis has been to increase understanding of how molecular processes influence the development and prognosis of leukemia in children. The thesis comprises two separate projects, where the first analyses molecular events that could influence childhood leukemia *in utero*, and the second investigates possible molecular prognostic factors for childhood leukemia. The first project (*studies I and II*) investigates whether specific oncogenic viruses can be detected in Guthrie cards from children who later develop ALL. The second project (*studies III and IV*) evaluates protein expression from cell regulating genes in bone marrow samples (by immunohistochemistry) from children with leukemia who were transplanted, at time of diagnosis, as well as before and after HSCT as possible prognostic markers for indicating relapse. In summary, the aims of my thesis are:

-to investigate whether adenovirus, KIPyV, MCPyV or WUPyV can be detected in DBS from children who later develop leukemia. Could an *in utero* infection with those viruses be associated with the subsequent development of childhood ALL?

- to investigate how the expression of the cell cycle regulating proteins p53, p21, p16, and PTEN is altered in bone marrow samples from time of diagnosis, as well as before and after HSCT. Could these proteins predict the prognosis and future relapse in children with leukemia before and after HSCT?

3 MATERIAL AND METHODS

3.1 PATIENTS AND SAMPLES

3.1.1 Studies I and II

Since 1974, all infants in Sweden have been screened for several inborn errors of metabolism at the age of 2-5 days, and today 24 metabolic disorders are tested. Four bloodspots (14mm in diameter, each containing approximately 60 μL blood) are collected on filter paper, known as Guthrie cards or dried blood spots (DBS). Since 1981 the DBS have been stored side by side at 4°C with the humidity never exceeding 30% since 1996 and are organized by date and place of birth.

We randomly identified 417 childhood leukemia patients diagnosed between 1992-2006 from the Nordic Society of Pediatric Hematology and Oncology (NOPHO) register and linked this information to the Swedish Medical Birth register. These two registers allowed us to gain access to the personal code numbers of both the mothers and children, which was necessary to identify the DBS. The samples were then depersonalized and received a passkey that was kept by the main supervisor.

The patients were treated at six different oncological units in Sweden. Their clinical data was collected from the NOPHO register. Eight hundred and thirty-four controls were collected anonymously, but since they are stored side by side, the controls were collected two DBS apart from the patients' DBS, in order to prevent contamination but still be able to match for date and place of birth.

Study I included 243 patients and 486 controls. Two hundred and sixteen (88.9 %) children suffered from pre-B-ALL, 25 (10.3 %) from T-ALL and two (0.8) from undifferentiated childhood leukemia. The median age at diagnosis was 4.3 years (67 days –15), where 141 (58%) patients were male and 102 (42%) female. The cytogenetic status at diagnosis revealed that; 80 patients were hyperdiploid, three were hypodiploid, 32 had t(12;21), and 11 had t(4;11) translocation, seven had t(1;19), six were Ph+ positive, four presented with dic (9;20), 36 had other kinds of alteration, 41 had no cytogenetic modifications and in the case of 23 children no data was available.

Study II included 50 patients and 100 controls, randomly selected from the children in study I. Forty-two (84%) of the patients were diagnosed as pre-B-ALL, six (12%) as T-ALL and two (4%) as undifferentiated childhood leukemia. The patient population comprised 26 (52%) girls and 24 (48%) boys with a median age at diagnosis of 4.7 years (0.9- 14.5). The cytogenetic status at diagnosis was as follows; 19 patients were hyperdiploid, six had t(12;21), and three had t(4;11) translocation, two were *Ph+* positive, two presented with dic (9;20), seven had other kinds of alteration and two had no cytogenetic modification. The cytogenetic analyzes of nine children were unavailable due to missing data.

3.1.2 Studies III and IV

Paraffin-embedded bone marrow tissues fixed in formalin or Stieve's solution were obtained retrospectively from all children who underwent a bone marrow transplant at Karolinska University Hospital between 1997 and 2010, due to a malignant bone marrow disease. Bone marrow specimens were collected at time of diagnosis, at HSCT, and from routine check-ups approximately three, six, and 12 months post HSCT. Twenty-three diagnostic samples were collected from Umeå University Hospital. In total, the material comprised 138 children aged between 0 and 18 years with the following diagnosis; pre-B-ALL (n=50), T-ALL (n=17), AML (n=34), MDS (n=19), JMML (n=9), CML (n=5), and lymphoma (n=4). Diagnosis and staging were made according to standard criteria in the NOPHO protocols. Clinical data was collected from patient records.

The control group comprised 55 children; healthy donors (n=2), children diagnosed with amegakaryocytic thrombocytopenia (n=3), aplastic anemia (n=13), chronic granulomatous disease (n=1), Fanconis aplastic anemia (n=7), glycogenosis (n=2), hemolytic anemia (n=1), idiopathic thrombocytopenia (n=8), Kostmann's disease (n=4), Mb Hurler (n=1), neutropenia (n=1) thalassemia (n=4), severe combined immunodeficiency (n=1), Wiscott Aldrich syndrome (n=2), and suspected malignant disease due to pancytopenia, although no malignant disease was discovered (5).

Study III included 33 children, 18 (55%) male and 15 (45%) female, with a median age of 8.4 years (0-16.6) at diagnosis, suffering from rare chronic myeloid malignancies (MDS, JMML, and CML). All the calculations were based on the myeloid malignancies as a

group. The treatment was initially administered at different oncological centers in Sweden, with no or only mild cytostatic drugs. The median age at HSCT was 11 years (0.6-17.1). Prior to HSCT 12 children were conditioned with busulphan and cyclophosphamide, 17 with busulphan, cyclophosphamide and melphalan, three with cyclophosphamide and total body irradiation (TBI), and in one case the data was missing. Twenty-two children received stem cells from matched unrelated donors, ten from human leukocyte antigen (HLA) identical siblings and one from a haploid donor. The stem cell source was peripheral stem cells in one case, cord blood in five cases and bone marrow in 27 cases. Ten (30%) out of 33 patients relapsed at a median of 2.9 (0-9.5) months post HSCT and 13 children died.

Study IV included 34 children with AML, 14 (41%) female and 20 (59%) male, with a median age at diagnosis of 8.6 years (0.3-17.3). The treatment was initially administered at different oncological centers in Sweden, according to the NOPHO protocol. Between 1997 and 2010 two NOPHO protocols were employed, with the more recent AML protocol being used since 2004. It should be noted that the indications for HSCT have changed over time. Initially, all patients diagnosed with AML were transplanted if an HLA identical sibling donator was available, while today, typical candidates for HSCT in first remission are patients diagnosed with cytogenetic or molecular genotyped unfavorable prognostic markers, or those with blasts >15% after first induction [12]. In recent years, MRD has become increasingly important as a marker for engraftment after HSCT, as well as for relapse, even for the AML patients. However, as this was not always the case some MRD data is missing from the first transplanted patients. The median age at HSCT was 9.4 (0.8-17.6) years. Prior to HSCT 26 patients were conditioned with busulphan and cyclophosphamide, while eight received cyclophosphamide and (TBI). Twenty-two children received stem cells from matched unrelated donors and 12 from HLA identical siblings. The stem cell source was peripheral stem cells in four cases, cord blood in three cases and bone marrow in 27 cases. Thirteen (38%) out of 34 patients relapsed at a median of 8.4 (1.4-23.6) months post HSCT and 16 died.

3.2 METHODS

3.2.1 Extraction of DNA

Studies I and II

Four spots, three mm in diameter, were punched out of DBS from 417 patients and 834 controls, containing in total approximately 12 μL of blood, including a minimum of 180.000 leukocytes and 120.000 lymfocytes. To prevent contamination, gloves were changed, the puncher was cleaned with 70% ethanol, and 30 stances were punched in clean filter paper between each sample.

DNA was extracted at the Department of Clinical Micro Biology, Karolinska University Hospital, Stockholm, Sweden, using minimal essential medium (MEM) [131,337]; four spots of DBS were added to a tube with 100 μL MEM buffer. The tubes were incubated, first at 56 °C for 60 minutes, then at 96°C for ten minutes, and thereafter quickly cooled on ice. The tubes were subsequently centrifuged at 6000g for ten minutes and supernatants were rapidly frozen at -70 °C for at least one hour, after which they were stored at -20°C. For *Study II*, the samples were re-extracted using a QIAGEN® kit.

3.2.2 Real time PCR for detection of the human albumin gene

Studies I and II

To ensure the availability of DNA, all samples were tested for the human albumin gene (ALB) at the department of Clinical Micro Biology, Karolinska University Hospital, Stockholm, Sweden, by means of TaqMan real-time quantitative PCR. Five μL of each sample was added to 50 μL of PCR mix (primers presented in Table II). The cycle conditions were 50°C for two minutes followed by denaturation at 95°C for ten minutes, 40 cycles at 95 °C for 15 seconds, and 65°C for one minute by which a product of 119 bp was amplified. The sensitivity of the PCR was ten DNA copies per reaction [338]. Eight samples were excluded from the study due to negative results for ALB.

3.2.3 PCR assays for detection of virus DNA

In study I, the samples were screened for species C adenovirus (serotypes Ad1, Ad2, Ad5, and Ad6) by nested PCR at Linda Gooding's Lab, Emory University Hospital in Atlanta [147]. All the samples were first tested by nested PCR 2 (nPCR-2) for a fragment

of the conserved region of the hexon gene, as described by Garnett et al [147]. In short, five μL of extracted DNA sample was mixed with 45 μL of PCR reaction mix (primers are presented in Table II). The first round consisted of; one minute of denaturation at 94°C followed by 45 cycles of 45 seconds at 94°C, 30 seconds at 58°C, 45 seconds at 72°C and finally seven minutes at 72°C by which a product of 368 bp was amplified. The second round consisted of 30 cycles with the same temperature conditions except for the annealing temperature of 60°C and a product of 310 bp was amplified. As a positive control we used a cloned plasmid of species C adenovirus and as a negative control we included one sample of water per 12 sample run. Thereafter, the PCR product was visualized on ethidium bromid agarose gel. Samples positive for species C adenovirus by nPCR-2 were first retested by the same assay. Afterwards an additional nested PCR assay 1 (n PCR-1) was used for detection of another fragment of the species C adenovirus hexon gene, amplifying a 852 bp product in the first round and a 286 bp product in the second (Garnett et al, primers presented in Table II) [147]. The sensitivities of both nested PCR-1 and -2 for species C adenovirus were five viral copies per reaction of 50 μL. To confirm the serotype, samples positive in both reactions were sequenced at Eurofins MVG Opereon (Huntsville, Al, USA) and compared with species C adenovirus from the Gen Bank.

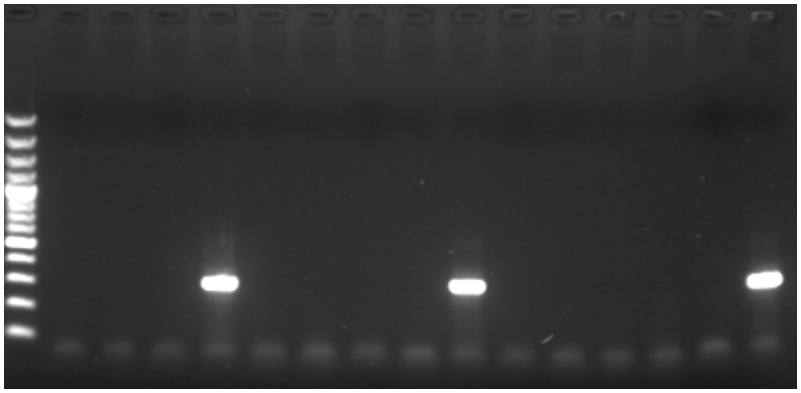

Figure 6: Gel electrophoresis of PCR products from the hexon gene, 310 bp. From left; the loader followed by samples, two of which were positive, and at the end a negative and a positive control.

Emory University, Atlanta, USA. Professor Gooding's laboratory, summer 2008. Professor Linda Gooding and Ganesh Talekar were my excellent teachers.

Table II. Sequence of primers used in PCR for detection of human albumin gene, adenovirus, KIPyV, WUPyV and MCPyV

Primer name	Sequence 5´-3´	DNA	Region
ALB. F	GCTGTCATCTCTTGTGGGCTGT	ALB	Albumin
ALB. R	ACTCATGGGAGCTGCTGGTTC	ALB	Albumin
ALB probe	FAM-CCTGTCATGCCCACACAAATCTCTCCC-TAMRA	ALB	Albumin
Nested outer primers		Adenovirus	
P11 nPCR-2. F	ATGGCTACCCCTTCGATGATGC	Ad2	Hexon
P12 nPCR-2. R	GCGTTGTAGGCAGTGCC	Ad2	Hexon
P7 nPCR-1. F	CATTGTCTTTACGCCA	Ad2	Hexon
P8 nPCR-1. R	TTGGCGTAGAGAAGGTTTT	Ad2	Hexon
Nested inner primers			
P13 nPCR-2. F	GATGATGCCGCAGTGGTCTTA	Ad2	Hexon
P14 nPCR-2. R	GTCCAGCACGCCGCG	Ad2	Hexon
P9 nPCR-1. F	GCCATTACCTTTGACTCTTCTGT	Ad2	Hexon
P10 nPCR-1. R	CCTGCTGATACTCCTTGTATTTAGTACT	Ad2	Hexon
KIPyV2263. F	TTGGATGAAAATGGCATTGG	KIPyV	VP1
		WUPyV	VP1
KIPyV2404. R	TAACCCTTCTTTGTCTAAAATGTAGCC	KIPyV	VP1
		WUPyV	VP1
MCPyV Q-PCR			
LT.1 F	CCACAGCCAGAGCTCTTCCT	MCPyV	LT
LT1. R	TGGTGGTCTCCTCTGCTACTG	MCPyV	LT
LT probe	FAM-TCCTTCTCAGCGTCCCCAGGCTTCA-TAMRA	MCPyV	LT
VP1. F	TGCCTCCCACATCTGCAAT	MCPyV	VP1
VP1. R	GTGTCTCTGCCAATGCTAAATGA	MCPyV	VP1
VP1 probe	FAM-TGTCACAGGTAATATC-MG-BNFG	MCPyV	VP1

Table II: Primers, probes, sequences, type of virus, and genome regions for the PCRs used in our studies.

In study II we analyzed DNA from DBS for the presence of KIPyV and WUPyV at the Tina Dalianis lab, Department of Oncology-Pathology, Karolinska Institutet, Stockholm, Sweden and for MCPyV at the Department of Clinical Micro Biology, Karolinska University Hospital, Stockholm, Sweden. Standard PCR on a fragment of the VP1 region of both KIPyV and WUPyV was performed as described by Giraud et al. [213]. There was a mismatch in the specific primer for KIPyV and WUPyV at base 20, compensated by an extended reverse primer (KIPyV2404.R; the primers are presented in Table II). In short, five uL of the DNA sample was added to 45 μL of PCR reaction mix. The cycle

conditions consisted of denaturation at 94°C for one minute, followed by 40 cycles of 30 seconds at 94°C, three seconds at 53°C, 45 seconds at 72 °C and finally five minutes at 72°C, amplifying a 142 bp product. As a positive control we used a cloned section of KIPyV and as negative controls four samples of water were included per run of 12 samples. Thereafter, the PCR product was visualized by ethidium bromide agarose gel. The sensitivity of PCR for both KIPyV and WUPyV was 10 viral copies per reaction of 50 µL.

To detect MCPyV DNA we used two real-time PCRs for the VP1 and LT regions, as previously described by Goh et al. [339]. Five µL of DNA sample was mixed with 45 µL of PCR reaction mix including two primer pairs and specific hydrolysis probes for either the VP1 or LT region (primers and probes are presented in Table II). The cycle conditions were 50°C for two minutes, denaturation at 95°C for 10 minutes followed by 45 cycles at 95°C for five seconds and 60 °C (LT assay) or 58°C (VP1 assay) for one minute, amplifying a 146 bp product for the LT and 59 bp product for the VP1. As a positive control we used a cloned plasmid of MCPyV amplified from a positive patient. The sensitivity of the real-time PCR assays was two copies of viral DNA per reaction of 50 uL. To investigate for the presence of inhibitors in the reaction, we retested the samples after extra DNA extraction, by both real-time PCRs. Only samples that were positive for both VP1 and LT were considered positive for MCPyV in line with previous studies [174,339].

3.2.4 Tissue micro array (TMA)

Studies III and IV

Bone marrow stances were prepared by the tissue micro array (TMA) method at the Center for Molecular Pathology, Malmö University Hospital, Malmö, Sweden. Two cores of 1 mm in diameter were punched out from each sample by a manual arrayer and collected in a receiving block together with 120 other cores. Thereafter the blocks were cut into 4 um sections and the thin slices placed on a microscope glass, deparaffinized, and stained. This method allows many samples on the same glass, which enables the samples to be treated equally and reduces the risk of different staining. TMA is a tissue

saving and economical method as very little material is required. However, this could also be a disadvantage as smaller samples are more sensitive and permit fewer cells.

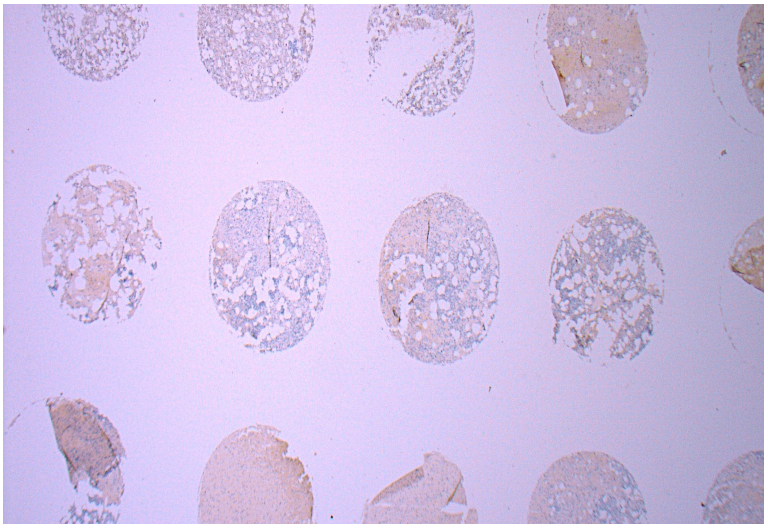

Figure 7: A section of a microscopic slide with a total of 120 bone marrow samples from 60 patients, prepared by TMA. Each dot is one mm in diameter and four µm thick.

3.2.5 Immunohistochemistry

Studies III and IV

Samples were stained by commercially produced antibodies (antibodies are presented in Table III). All staining was routinely performed in a Leica BOND-III machine, according to standardized procedures at the pathology laboratory at Karolinska University Hospital, Stockholm, Sweden. To visualize the antibody, diaminobenzidine-peroxidas reaction weakly counterstained with hematoxylin was used. A positive control on separate glass was included in all staining and negative cell lines in the patient samples were used as negative controls. The differences in quality and staining were controlled on each slide before evaluation of the samples. In addition, the final quality evaluation was made by an experienced hematopathologist.

Table III. Antibodies used in study III and IV, their types, clones and designations

Antibody	Anti-human antibody	Clone	Company
p53◆	monoclonal mouse	DO-7	Leica
p21	monoclonal mouse	EA10	Calbiochem
p16	polyclonal rabbit	ab7962	Abcam
PTEN	monoclonal mouse	6H2.1	Daco
Secondary	multi link Goat°		Leica

◆Reacts with both the wild-type and mutant form of p53; °anti rabbit mouse

The p53, p21, p16, and PTEN protein expression was analyzed together in a microscope at high resolution (40X) by two PhD students who were blinded to the diagnosis as well as relapse status under the guidance of an experienced hematopathologist. A minimum of 100 scored cells per patient was required for inclusion and the median number of calculated cells was 656.

In study III ten samples were excluded due to; lost cores n =5 and poor representativity n=5. The final analysis included 86 samples from patients and 55 controls. *In study IV* 89 samples were excluded due to; lost cores n=40 and poor representativity n=49. The final analysis included 83 (p53), 82 (p21) 79 (p16) and 77 (PTEN) patient samples and 55 control samples.

3.2.6 Mutation analysis by Sanger sequencing

In study III we analyzed 12 samples from 12 patients (7 JMML and 5 MDS, where two had experienced relapse after HSCT), for mutations of *TP53.* Exon 2-11 mutations were analyzed by PCR and DNA Sanger sequencing with M13-tagged primers according to standard protocols at the Department of Molecular Medicine and Surgery, Karolinska Institutet, Sweden.

3.2.7 Statistical analysis

Studies III and IV

All data was statistically calculated using Statsoft Statistica 11 and Microsoft Excel 2011. To investigate whether p53 (*study III*) or p53 p21, p16 and PTEN (*study IV*) protein expression could serve as a predictor of relapse, a nonlinear logistic regression was performed at each time point. In *study III* we tested for both uni- and multivariate models to exclude confounding factors. Based on the non-linear logistic regression analysis, a cut-off level in percentage was calculated at diagnosis in *study III* and at six months in *study IV*.

In *study IV* an independent t-test was used to evaluate the difference in p53, p21, p16, and PTEN expression at all time points between patients who relapsed and those who did not.

A time to event curve was constructed to analyze the number of relapses in patients with p53 expression over and under the median of 13.6% at diagnosis (in *study III*), and under and over the cut off level of 21.3% at six months (in *study IV*). To visualize possible statistical differences between the groups a Log-Rank test was preformed.

Finally, in *study III* a Spearman's rank-order correlation test was performed to analyze possible correlation between p53 and p21 expression.

The results were considered significant if the p-value was <0,05.

4 RESULTS

4.1 STUDY I

The extraction of DNA was successful in 243/243 patients and in 484/486 controls. Two controls who were negative for the human albumin gene were excluded from the study. All samples were analyzed in duplicate by nPCR-2 and 9/727 samples revealed at least one positive result and therefore reanalyzed by nPCR-2 as well as nPCR-1. Samples were considered positive if the species C adenovirus hexon gene was detected in at least one of the two samples in each reaction (nPCR-2 and nPCR-1). Only two of these nine children were positive for the hexon gene in both reactions. However, these two positive samples were derived from the ALL patients and were sequenced and confirmed to encompass species C adenovirus when compared to a Gen Bank product.

4.2 STUDY II

The extraction of DNA was successful in all samples (50 patients and 100 controls). All samples yielded negative results when analyzed for the VP1 region of KIPyV and WUPyV by nested PCR. Twenty-three (12 ALL patients and 11 controls) samples had high threshold cycle (Ct) values when tested by real time PCR for the MCPyV VP1 region. In 19/23 samples the Ct values were equivalent to two copies per reaction (the limit of detection) and 4/23 samples had Ct values corresponding to 4.45 copies per reaction. However, all the samples were negative for the MCPyV LT region and combined with very high Ct values for the VP1 region, the reactions were considered negative by the algorithm, where only samples positive for both VP1 and LT were deemed positive for the MCPyV [174,339]. No change observed in the VP1 results after retesting the samples following additional DNA extraction.

4.3 STUDY III

At diagnosis, the Odds ratio (OR) 1.19 (CI 1.02-1.4) for p53 protein expression was a significant ($p = 0.028$) predictor of relapse. The significance remained in a multivariate analysis adjusted for type of disease (MDS, JMML or CML), sex, cytogenetics, age and platelet count at diagnosis. The cut off level at diagnosis (based on nonlinear regression)

predicting a relapse (probability >50%), yielded a protein expression of 19% (modeled agreement of 75%).

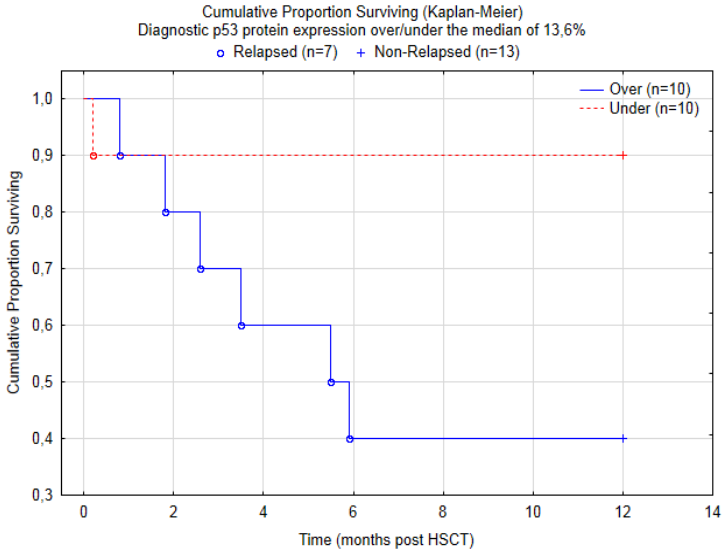

Figure 8: Time to event curve based on diagnostic p53 protein expression at time of diagnosis, over (n=10) or under (n=10) the median of 13.6% p53 positive cells.

Honkaniemi et al, Pediatric Hematology-Oncology, Elevated p53 protein expression; a predictor of relapse in rare chronic myeloid malignancies in children? 2014 May;31(4):327-39, copyright © 2014, Informa Healthcare. Reproduced with permission of Informa Healthcare

A time to event curve (Figure 8) demonstrated that six relapses occurred in the children with p53 expression over the median 13.6% at diagnosis and one relapse occurred in the group with p53 expression under the median where a Log Rank test indicated significance (p =0.033).

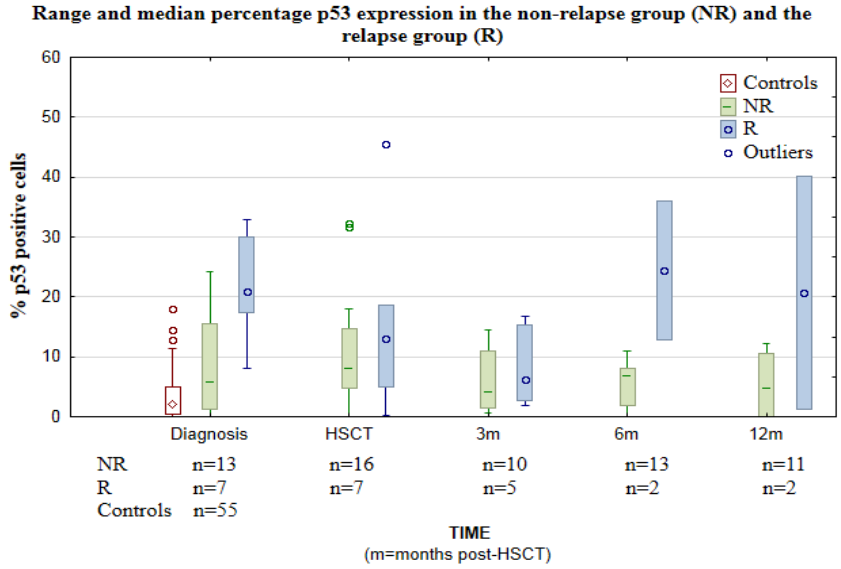

Figure 9: Range and median percentage of p53 protein expression in non-relapsed and relapsed patients at each time point investigated.

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A box plot (Figure 9) was performed for the relapse and for the non-relapse group. A trend of higher p53 expression was found at each time point in the relapsed group compared to the non-relapsed group. The p53 protein was significantly over expressed in diagnostic ALL samples, compared to the nonmalignant group. However, the Sanger sequencing was negative for any pathogenic mutation and a positive correlation was detected at diagnosis between p53 and p21 expression indicating a functional protein.

4.4 STUDY IV

For p53 protein the mean percentage of positive cells was higher in the relapse group compared with the non-relapse group at all time points investigated. At six months post HSCT p53 was significantly overexpressed in the relapse (25%) compared to the non-relapse group (4.6%) ($p=0.01$). A distinctly lower percentage of cells positive for p53 protein was found at diagnosis in the non-malignant control group (3.5%) compared to the leukemic groups (32,5 %), ($p<0.05$). T-tests were also calculated for p21, p16, and PTEN expression at all time points, where a statistical significance was found for p16 in the relapse (4.6%) compared to the non-relapse group (0.6%) at six months post HSCT ($p=0.03$). At 12 months post HSCT a bone marrow sample was only available from one patient in the relapse group, therefore the analysis will not be discussed in the present study

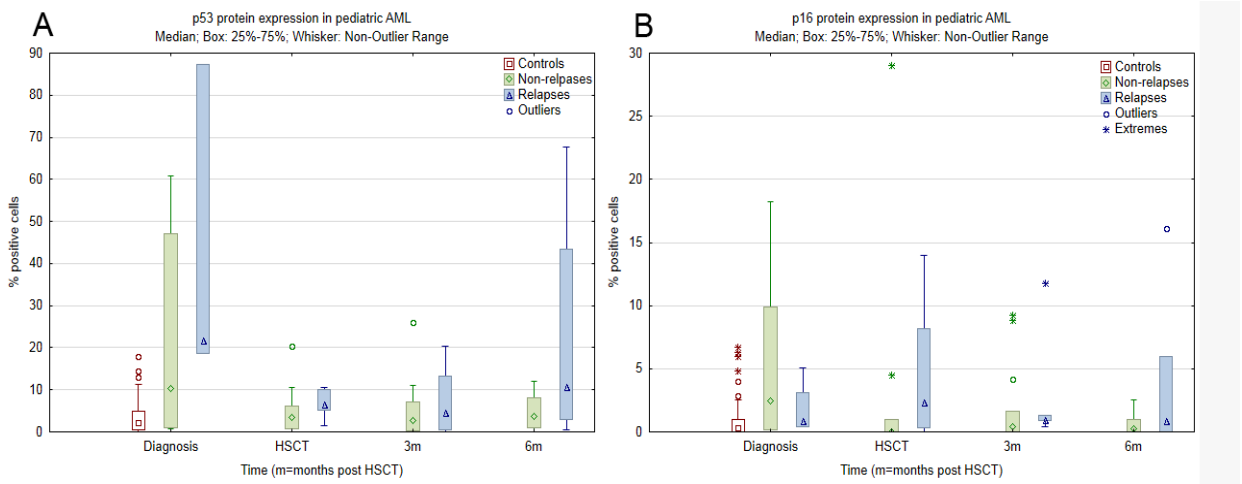

Figure 10: Range and median percentage of p53 and p16 protein expression in non-relapsed and relapsed patients at each time point investigated.

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A boxplot of median and range of percentage positive cells at each time point was produced for p53 as well as for p16 protein expression. The group of patients who relapsed was compared to the group of patients who did not relapse and the control group was included at diagnosis (Figure 10). A slight trend of overexpression of p53 protein was found in the relapsed patients compared to the non-relapsed patients. This trend was not as obvious for p16, thus outliers and extremes may have skewed the results in this group, especially at six months post HSCT.

The Non-linear logistic regression for p53, p21, p16, and PTEN protein expression did not predict relapse at any of the time points. The cut off level for p53 protein expression at six months predicting a relapse (probability >50%), yielded 21.3% (modeled agreement of 85.7%). Based on the cut off level at six months post HSCT, a time-to-event curve was created where patients were divided into two groups; p53 protein over or under 21.3% (Figure 11). Two patients had an expression over the cut off level and both suffered a relapse within six months post HSCT. Three out of 19 patients in the group with expression under the cut off level relapsed within eight to 12 months. A statistical difference was found between the two groups based on the cut off value (p=0.005).

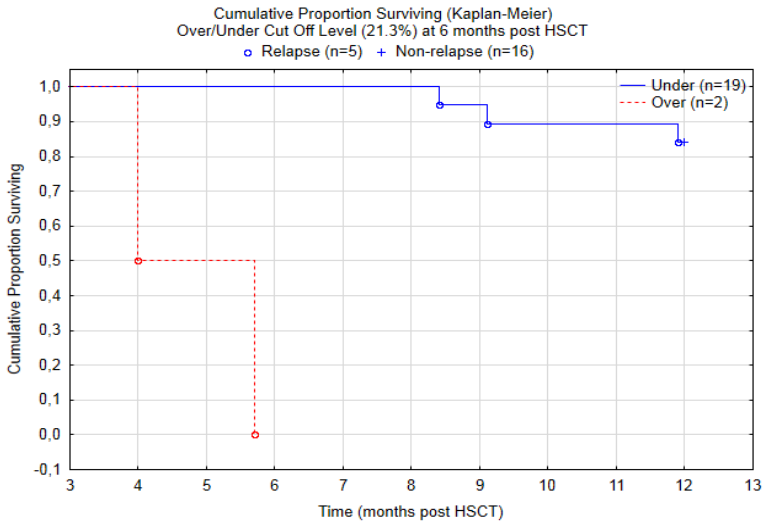

Figure 11: Time to event curve over (n=2) or under (n=19) the cut off level calculated at 6 months post HSCT of 21.3% p53 positive cells. Log rank test p=0,005

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5 DISCUSSION

5.1 A POSSIBLE INFECTION IN UTERO AND CHILDHOOD LEUKEMIA

Studies I and II

One of the earliest theories of the etiology of leukemia was that it might be caused by a common infection, possibly a virus, but that was disregarded when it become clear that leukemia was not contagious [100,102]. Today, several viruses are known to be oncogenic as a result of experiments in vitro, animal models, and clinical observations [5-7,103,104]. In 1997, Smith postulated the theory of an *in utero* infection as a first step in a genetic alteration that develops into leukemia later in life [6]. Such genetic changes are found at birth both in DBS and cord bloods and are more frequent in those children who later develop leukemia [3,70]. According to Smith, a possible virus initiating this "first hit" *in utero* must be able to produce mild symptoms, cross the placenta, and infect lymphocytes without inducing distinct fetal abnormalities. Several viruses are candidates for such in utero infection, including both adenovirus and polyomavirus [6]. In a pilot study, our group found a significantly higher frequency of adenovirus in DBS from children who later developed ALL compared to controls [132]. Our group has previously analyzed DBS for BKV and JCV DNA with negative results. However, new polyomaviruses have since been discovered that are also of interest [131].

Certain virus infections *in utero* could be lethal, result in malformation or be asymptomatic. Reddy et al. demonstrated that 13.5% of fetuses with normal karyotype in amniotic fluid at the second trimester were positive for a range of viruses. Furthermore, 24% of cases with an abnormal ultrasound of the fetus were positive for different viruses compared to 8% of normal pregnancies. Adenovirus, enterovirus, CMV and parvovirus B19 were the most frequent and could be correlated with malformations, intra-uterine growth restriction, and hydrops, but also with mild infections [340].

The negative results for virus in Guthrie cards do not exclude a virus infection *in utero* initiating the "first hit". The primary infection or reactivation may have occurred early in pregnancy and the viremia could have disappeared. However, in cases of maternal reactivation of CMV or HSV infection during pregnancy, it has been reported that viral DNA was detected in DBS [341,342]. Furthermore, the viral infection could be latent in

cell tissues other than peripheral blood, making it more difficult to detect in DBS. However, adenovirus remains latent in T cells and polyomavirus has been found in tonsil tissue, blood, plasma, and could also persist in lymphocytes [147,201,343]. Finally, the virus may have lost or integrated only a part of its DNA in the cell genome, which complicates the choice of detection method [105,207,343,344].

5.2 USE OF GUTHRIE CARDS

Studies I and II

The use of Guthrie cards is an established method for diagnosis of metabolic diseases, genetic alterations and viral infections such as CMV, hepatitis E, hepatitis A, measles, rubella and HIV [3,337,342,345-348]. DBS from children who later developed leukemia have previously been used to screen for HHV-6, EBV, CMV, human parvovirus B19, JCV and BKV and adenovirus [128-133].

An important question is whether the sensitivity and specificity could be influenced by degradation of nucleic acid during a long time period of storage [349]. However, CMV DNA has been detected in up to 17 year old DBS and a recent study revealed that even RNA is conserved for up to 20 years in DBS [349,350]. A possible deterioration due to time and storage conditions cannot be excluded and may have influenced the amount of DNA at the time of testing [349]. Our Guthrie cards were stored between one and 16 years before testing. Hopefully the excellent storage conditions with stable temperature and humidity preserved the DNA. Another important storage issue is the possible contamination from one DBS to another, as they are stored side by side in a plastic bag [349]. To minimize the risk of contamination, we collected the controls two Guthrie cards apart from the patients. We also took all necessary precautions during the collection process [131].

The choice of extraction method may influence the yield of DNA [349]. To ensure the validity of the method, our group previously tested a diluted JCV plasmid; DBS were spiked with JCV plasmid and tested by nested PCR to the detection limit of ten viral copies/sample. Additionally, blood from one known BKV positive patient was detected on an “artificial DBS” [131]. To ensure the presence of DNA and possibility to amplify

it, all samples were tested for the human albumin gene by TagMan real-time quantitative PCR where 1243/1251 yielded a positive result.

The question concerning the presence of possible inhibitors in the extracted material may be raised. It is well known that the existence of hemoglobin from erythrocytes may inhibit enzymes in the PCR reaction [351]. To investigate possible inhibitors, we extracted DNA and reanalyzed it by means of the MCPyV VP1 assay, with no change in results. Another concern is whether the pink color of the MEM buffer could interact with TagMan real-time PCR reaction. As we tested our samples with real-time PCR for the albumin gene with successful results, we concluded that this was not the issue in our study.

5.3 STUDY I

Aims: to investigate whether adenovirus can be detected in DBS from children who later develop leukemia. Does an in utero adenovirus infection have an association with the subsequent development of leukemia?

Two out of 243 samples were positive for species C adenoviruses DNA detected in DBS from children who later developed childhood leukemia, while all the controls were negative. This is in line with a report from California where neither 89 children who later developed childhood leukemia nor 99/100 controls were positive for species A, C or F adenovirus [133]. However, in a previous pilot study from 2007, our group found a significant difference in species C adenovirus DNA expression in DBS from patients (13/49) compared to controls (3/47) [132]. The same methods for detecting species C adenovirus with a sensitivity of five copies/samples were used in both studies. In the recent study we increased the number of patients and controls in addition to using four instead of three blood spots from DBS, in order to maximize the amount of viral DNA if present. Moreover, 2/10 of the patients who were included in both studies yielded a positive result in the first, which could not be replicated in the present study.

As prenatal adenovirus infection is usually asymptomatic and difficult to identify, its prevalence is unknown [352]. The prevalence of detected adenovirus DNA in amniotic fluids from the second trimester of normal pregnancies was 5-10% [340,353]. This could

be compared to the estimated prenatal leukemic clone in the population (1-5%) [76]. Given the prevalence in the amniotic fluid studies and the genetic analyses of DBS we may underestimate prenatal adenovirus infection, which could still be a candidate for leukemogenesis *in utero*. Furthermore, adenovirus is not normally found in peripheral blood unless the child presents with an acute fulminant infection [354,355]. Instead, adenovirus is found in a majority of lymphocytes derived from adenoids or tonsils after tonsillectomy or adenoectomy in healthy children [145,147]. If that is also the case in an *in utero* infection, we may fail to detect adenovirus by using DBS. Another explanation as to why adenovirus cannot be detected might be a "hit and run" mechanism [110,111]. Even though we failed to find causality between species C adenovirus infection in DBS and childhood leukemia, adenovirus remains a "hot candidate", as it has the capability to disturb the cellular DNA repair machinery.

5.4 STUDY II

Aims: to investigate whether KIPyV, MCPyV or WUPyV can be detected in DBS from children who *later develop leukemia. Does an in utero infection with these viruses have an association with the subsequent development of childhood leukemia?*

We could not detect KIPyV, WUPyV, or MCPyV DNA by nested PCR in DBS from children who later developed childhood ALL or from the controls. Interestingly, for the VP1 MCPyV assay we found that 23 samples (12 patients and 11 controls) yield high Ct values, but all were negative by PCR for LT. However, the results were interpreted as negative based on the algorithm, which states that samples must be positive for both the VP1 and LT regions. This low reactivity may be due to a low viral load in the samples or a nonspecific reaction.

Our methods for extraction and PCR-amplification have been previously used and described in other studies as well as functioning well on DBS samples [128-132]. All extracted DBS in this study were positive for human albumin DNA detected by highly sensitive real time PCR. As the sensitivity of PCR assays were high for both KIPyV/WUPyV (ten copies/sample) and MCPyV (two copies/sample), false negative results should not be an issue. However, the choice of PCR primers could also influence

the results. We used specific primers for the VP1 region for KIPyV and WUPyV detection that have been described as being successful in other studies [213]. The choice of primers for MCPyV has been discussed to a greater extent in the literature [174,356]. In line with several other studies we used specific primers for the VP1 and LT regions [174,213,339,344]. Moreover, we tried to rule out the possibility that the presence of PCR-inhibitors might influence the results. MCPyV VP1 assays were repeated on the re-extracted DNA samples with the same results. Another possibility is that MCPyV could be truncated in the C-terminal without disturbing the transformation ability, but theoretically influencing the binding of the primer in the LT region [344]. Dolei et al. described the same phenomena for VP1 in immunocompetent individuals, where only 33% of those positive for NCCR were also positive for VP1, although VP1 positivity seemed to decrease in line with age [343]. The authors suggested that VP1 may be lost during viral persistency or incorporated in the host cell DNA just within the VP1 region, thereby escaping detection.

KIPyV, WUPyV, and MCPyV were only discovered recently, thus knowledge about symptoms, latency, and reactivation is still limited. Both JCV and BKV have been detected in peripheral blood, both in lymphocytes and monocytes, in immunosuppressed patients, but also at a lower frequency in immunocompetent individuals [343,357-359]. Moreover, JCV and SV40 have been detected in mesenchimal stromal cells from cord bloods in 1/35 and 3/35 respectively [107]. KIPyV and WUPyV have also been detected in peripheral blood from immunosuppressed patients [201]. The prevalence in pregnancy and symptoms of prenatal infections for these recently discovered polyomaviruses are still unknown. However, in a study of healthy pregnant women KIPyV and WUPyV DNA were not detected in urine, blood or respiratory samples during the whole period of pregnancy [360].

Even though we failed to show an association between KIPyV, WUPyV or MCPyV infection in DBS and childhood leukemia, polyomavirus is still an interesting candidate due to its oncogenic properties recognized by the WHO International Agency for Cancer Research Monograph Working [211]. The other new polyomaviruses discovered in recent years may also be worthy of consideration. One interesting candidate is HPyV9, whose genome is closely related to LPV, and has been detected in the blood (2%) of women in the second trimester of pregnancy [193,360].

5.5 PROGNOSTIC CELL CYCLE REGULATING PROTEINS, TMA, IMMUNOHISTOCHEMISTRY, AND LIMITATIONS OF STUDIES III-IV

The cell cycle is a complex network of regulating proteins both up and downstream with several feedback loops, and remains incompletely understood. New relationships and proteins are still added to the network. A decreased or inappropriate expression of one protein influences the expression of next protein in the chain. Gene alterations may also lead to different expressions of protein. One example is *PTEN* mutations that could result in a dysfunctional but normal/increased or decreased protein expression, whereas the protein from aberrant *TP53* seems to be overexpressed [254,255,317]. Moreover, accumulation of the wild-type proteins can also be found without detected mutations and in non-malignant diseases, as other mechanisms such as cellular stress during inflammation or hypoxia could be involved [247,257-260]. It is important to consider all these factors when interpreting protein expression.

An essential issue is the time aspect, since chemotherapy protocols and the indications for HSCT have changed over time. The indications for HSCT due to AML have changed during this study. Before 2001, all children with AML and a HLA identical sibling were transplanted. In our study 9/12 patients with HLA identical siblings were transplanted before 2001, 4/21 in the non-relapse group and 5/13 in the relapse group. This could of course influence the results of several studies, as the first transplanted patients could have a “milder disease”.

Another concern in *study III* is the small number of patients included (n=33), of whom 10 relapsed. However, MDS, JMML, and CML are rare diseases in children and to compensate for the small number of samples available between 1997 and 2010 we analyzed MDS, JMML and CML together as a group, based on the common origin of the myeloid lineage. Another limitation of *studies III and IV* that affects the power is that not all samples were available in the bio bank and some were lost during preparation. Especially critical was the time of diagnosis, as many of the patients were diagnosed at other hospitals. To compensate for this, we requisitioned those diagnostic bone marrows from one of the largest oncologic centers for children in Sweden. However, in *study III* ten samples were excluded due to; lost cores (n=5) and poor representativeness (n=5). In

study IV 89 samples were excluded due to; lost cores (n=40) and poor representativeness (n=49).

Another concern is the small size of TMA, which makes the sample vulnerable and may miss the leukemic clone. However, a strength is the tissue saving feature and that all samples were collected on only a few glasses, thus eliminating differences in the sample due to staining and other treatment. Finally, it is very difficult to distinguish between the various shades of staining, e.g. strong or weak, because it is very subjective and the grades are to a large extent determined by the viewer. However, we also analyzed all the shades of staining and the outcome was in line with the results presented (data not shown).

5.6 STUDY III

Aims: to investigate how the expression of the cell cycle regulating protein p53 is altered in bone marrow from diagnosis to before and after HSCT. Could overexpression of p53 predict a future relapse in children with chronic myeloid malignancies before and after HSCT?

We found that elevated p53 protein expression at diagnosis was a significant predictor of relapse, OR 1.19 (CI 1.02-1.4). Furthermore, a time to event curve demonstrated that relapse was more frequent in the group of patients with p53 expression over the median value (13.6%) at time of diagnosis, than in the group with p53 expression below 13.6% (Figure 8). Moreover, a descriptive study visualized a trend of higher p53 expression in the relapse group compared to the non-relapse group at all the time points investigated (Figure 9). In line with previous studies, this indicates that pediatric patients with a more aggressive disease may overexpress p53 protein, predicting a future relapse [290,295].

Since mutation of *TP53* is known to stabilize the p53 protein, some reports have suggested that immunohistochemistry of p53 protein expression maybe used for screening for such mutations [256,257]. Importantly, overexpression of wild-type p53 may also be observed without detectable mutations, for instance increased apoptosis or due to blockades of proteins responsible for deregulation of p53 [257,259]. We did not find any mutations in 11 analyzed samples, although our Sanger sequencing method had

a high specificity (99.99%), but a relatively low sensitivity, meaning that mutations will only be detected if present in 20-25% of the cells. Nevertheless, the leukemic clone may be small, especially in MDS and JMML, thus we cannot exclude the possibility that we missed a potential clone. However, we have sequenced exons two to 11, which provide greater coverage compared to many other studies using exons 4-8 or 5-9 [275,291,292]. Despite several trials we were unable to detect any sequence for exon three and thus could not exclude possible alterations in this exon. The prevalence of *TP53* mutations in pediatric MDS, JMML, and CML is unclear, due to few studies and conflicting results [275,291,292]. However, our findings of a positive correlation between p53 and p21 together with no detectable mutations in the sequencing may indicate a functional p53 protein. We suggest that overexpression of p53 in this study is due to enhanced expression of wild-type p53 in response to increased apoptosis and cell cycle arrest, in an attempt to overcome the emerging leukemic clone.

Further limitations of the study are discussed in 6.4

5.7 STUDY IV

Aims: to investigate how the expression of the cell cycle regulating proteins p53, p21, p16, and PTEN are altered in bone marrow from diagnosis to before and after HSCT in children with AML. Could overexpression of these proteins predict a future relapse in children with AML before and after HSCT?

The non-linear logistic regression was non-significant at all time points for p53, p21, p16, and PTEN. However, in a descriptive analysis we revealed a significant difference in means for both p53 and p16 protein expression at six months post HSCT between the relapsed group and the non-relapsed patients. Moreover, the two children who expressed a very high number of p53 positive cells also had a high p16 expression suggesting that p53 and p16 could be important for early identification of relapse. Notably, p16 is known to be activated due to inactivated *TP53*, but also due to cellular stress such as accumulation of DNA damaged cells. Thereby, p16 is suggested to be a stand-by tumor suppressor gene to p53 [300]. This may indicate a dysfunctional p53 protein in those two patients rather than overexpression of wild type *TP53*.

The significantly higher p53 protein expression in the relapse group compared to the non-relapse group at six months post HSCT (25.0% vs. 4.6%) is in line with the cut off level of 21.3% at six months for separating relapses and non-relapses. It is also consistent with the cut off level at diagnosis of 19%, estimated in *study III* [361]. Furthermore, a time to event curve demonstrated that both patients who had > 21.3% p53 positive cells at four and six months post-HSCT, relapsed shortly after transplantation, while the three who had expressions of < 23.1% relapsed later (Figure 11). The cut off level also differs from the mean value for the non-malignant control group (3.5%).

No significant values were revealed at any time points for t-tests comparing the relapse group to the non-relapse group of AML patients for p21, p16, and PTEN, with the exception of the p16 values at six months.

Finally, it would be interesting to analyze the alteration of *TP53* as a comparison to the protein expression in this study. However, since the study is retrospective we had no opportunities for that. The prevalence and significance of mutations of *TP53* in childhood AML is to our knowledge still unknown and should be investigated prospectively.

Further limitations of the study are discussed in 6.4

5.8 ETHICAL CONSIDERATIONS

The Regional Ethical Review Board, Stockholm, Sweden, approved all the studies in this thesis. Study I was also approved by the Institutional Review Board of Emory University, Atlanta, USA.

Childhood leukemia is an unusual, but serious disease and the etiology is in most cases still unknown. If we could find an origin, such as a viral infection in the fetus, maybe we could prevent it by early screening or vaccinations. However, screening of potential harmful viruses in asymptomatic infants may raise ethical and psychosocial concerns.

All the studies were conducted without permission from the patients or their parents. In *studies I-II* we examined samples that in many cases were collected a long time ago and in *studies III-IV* some patients were treated many years ago while others were still

undergoing treatment. Asking permission from families who had lost a child can also evoke strong feelings. In the second scenario, in which the child recovered and returned to everyday life, it can also affect them by reminding them of the past.

Finally, samples from patients must always be retained for future tracking of diseases or comparison with later samples. After collecting our samples, material must always be left for future use. In *studies I-II* we only used four stances of three mm in diameter of the DBS. In *studies III-IV* we used the TMA method, known as a tissue saving method.

6 CONCLUSION

Studies I and II

In studies *I and II* we could not detect adenovirus, KIPyV, WUPyV, or MCPyV in DBS from newborns that later developed childhood leukemia. However, identified leukemic alterations can be traced back in neonatal DBS from children who later developed leukemia, indicating an early event *in utero*. Despite several studies no clear etiology has been found, thus common infections remain a very interesting candidate. If a virus could be identified as the cause of the genetic changes, it could lead to strategies for prevention such as neonatal screening and vaccination.

Studies III and IV

In *study III* we found that an increased p53 protein expression at diagnosis was associated with risk of relapse in children with rare chronic myeloid malignancies (OR 1.19, 95% CI: 1.02-1.40, p=0.028). In *study IV* we found a significant difference in p53 and p16 expression in the relapse compared to the non-relapse group at six month post HSCT, indicating that p53 and p16 could be used as a prognostic marker at that time. The evaluated cut off level at diagnosis and at six months post HSCT for p53 was around 20%. A p53 expression > 20% may suggest a future relapse in children with chronic myeloid malignancies and AML. We suggest that an elevated p53 protein expression may be a complement to standardized prognostic factors such as chimerism and MRD as an indicator of children at risk of relapse both pre and post HSCT and may signal that these children would benefit from a more intense therapy before transplantation or if increased p53 expression occurs after HSCT, be an indicator to withdrawal of immunosuppression or infusion of T-cells. To evaluate this further, a prospective, multicenter study has been started.

7 POPULÄRVETENSKAPLIG SAMMANFATTNING

Syftet med mina studier är att försöka öka förståelsen för hur specifika molekylärbiologiska processer skulle kunna påverka utvecklingen och prognosen för leukemi hos barn. I *studie I och II* analyserar vi om vissa virusinfektioner under graviditeten, kan överförs till barnet och därmed initiera leukemiutvecklingen, s.k ”first hit”. I s*tudie III och IV* undersöker vi om förändringar i proteinuttrycket från cellcykelreglerade gener kan förutspå ett återfall hos barn som genomgått hematopoietisk stamcellstransplantation (HSCT) på grund av myeloisk leukemi.

Studie I och II: Specifika genetiska förändringar som hittats vid diagnos hos barn med leukemi, har kunnat spåras till PKU-blodprov, men även kunnat påvisas i navelsträngsblod hos barn som aldrig insjuknar i leukemi. Detta tyder på att vissa mutationer, exempelvis translokationer i MLL-genen, ETV6-RUNX1 och hyperdiploidy kan uppstå under fosterlivet, av idag okänd orsak. Eftersom alla barn med genetiska förändringar från födseln inte utvecklar leukemi, krävs ytterligare en händelse ”second hit”, som leder till att en leukemiklon bildas. Etiologin till leukemi har studerats i många vetenskapliga arbeten, där man bland annat analyserat exponeringar av den gravida kvinnan såsom kemiska ämnen, alkohol och strålning vilka kan korreleras till leukemiutveckling, medan rökning, ultraljud och hög köttkonsumtion under graviditeten, samt hög födelsevikt, inte har kunnat påvisas ha ett samband med leukemiutvecklingen. I dag känner vi till flera onkogena virus, d.v.s. virus som har förmågan att förändra cellers DNA. En teori som diskuterats är om en virusinfektion *in utero* skulle kunna vara ”first hit” vid leukemiutvecklingen. Viruset skulle då ha specifika egenskaper såsom; mild symtombild hos modern, kunna passera placenta, kunna påverka lymfocyter och inte leda till missbildningar hos barnet. Ett virus med sådana egenskaper skulle kunna ge upphov till mutationer i fostrets benmärgsceller, som senare kan leda till utvecklingen av akut lymfatisk leukemi (ALL). Polyomavirus är ett av de virus som föreslagits, men dessa beskrivningar passar även in på gruppen adenovirus.

I *studie I och II* har vi retrospektivt samlat in PKU-blodprov och klinisk data från 243 barn med ALL. PKU-blod prov samlades också in från 486 friska kontroller. DNA har

extraherats och kontrollerats med PCR för human albumin gen för att säkerställa att DNA fanns tillgängligt i alla prover. 243 patienter och 486 kontroller har analyserats med PCR för adenovirus samt 50 patienter och 100 kontroller för de tre nyupptäckta polyomavirusen (KIPyV, WUPyV och MCPyV). I *studie I* var två patienter positiva för adenovirus DNA, vilket också bekräftades genom sekvensering. I s*tudie II,* var alla prover negativa för polyomavirus. Även om vi inte kunde påvisa ett samband mellan adenovirus, KIPyV, WUPyV eller MCPyV och barnleukemi, så kan ett virus fortfarande vara ”first hit” i leukemiutvecklingen; viruset kan ha undkommit detektion, viruset kan ligga latent i andra celler än i blod, eller nya virus kan vara involverade. Ett virus kan också vara involverat senare i en s.k ”second hit” av leukemiutvecklingen.

Studie III och IV: Leukemi uppstår ur stamceller som förlorat kontrollen över celldelningen. Normalt regleras celldelningen av cellcykeln som fungerar som ”gas, broms och kontrollstation” för DNA kvalitet. Nätverket i cellcykeln är komplext med många olika reglerande proteiner både upp och nedströms, men även med flera ”feedback” kedjor. Tumörsupressorgener är viktiga ”övervakare” i denna process och kontrollerar att allt är korrekt med cellens DNA, tillväxt, mognad och utveckling. p53 genen som är mest känd kallas för ”the guardian of the genome” och mutationer av denna är kopplat till flera cancertyper och kan påvisas som ett förhöjt proteinuttryck. I dag drabbas 16/100,000 barn varje år av cancer, där ca 30 % får diagnosen leukemi. Fram till 1940 talet var leukemi en dyster diagnos, då barnen i praktiken inte överlevde. Behandlingen av leukemisjukdomen har utvecklats sedan dess och idag överlever ca 80-90% av barnen med ALL och 70 % av dem med akut meyloisk leukemi (AML). Den förbättrade långtidsöverlevnaden är ett resultat av bättre diagnosverktyg som möjlig gör uppdelning i riskgrupper, skräddarsydda intensivare behandlingsprotokoll, tidig infektionsbehandling, nutritionsbehandling, samt bättre omvårdnad. Barn som svarar dåligt på primär behandling, får återfall (recidiverar), de med specifika riskmarkörer vid diagnos samt nästan alla barn med myelodysplastiskt syndrom (MDS), juvenil myelomonocytic leukemi (JMML) och kronisk myeloisk leukemi (KML) genomgår HSCT. Barn med återfall efter HSCT har dålig prognos och därför är det viktigt att kunna identifiera ett recidiv tidigt. De främsta markörerna för att följa barnets prognos efter HSCT är idag chimerism och minimal residual disease (MRD).

I *studie III och IV* har vi insamlat benmärg från 33 barn MDS, JMML och KML samt 34 barn med AML som transplanterades på Karolinska Universitetssjukhuset, Huddinge 1997-2010. Som kontroller har vi insamlat 55 benmärgpreparat från barn som genomgått benmärgsundersökning men ej diagnostiserats med malign blodsjukdom. Benmärgspreparaten och klinisk data insamlades retrospektivt från diagnos, samt före och efter HSCT. Preparaten preparerades med tissue micro array (TMA), som är en vävnadsbesparande metod och färgades sedan in med antikroppar för p53 och p21 (*studie III*) samt p53 p21, p16 och PTEN (*studie IV*). Preparaten analyserades i ljusmikroskop, där examinatorerna var ”blindade” för diagnos och utfall. Därefter jämfördes procent andelen av positiva celler, vid de olika tidpunkterna, för de olika proteinerna mellan två grupper; de med återfall samt de som blivit friska. I *studie III* fann vi att ett ökat uttryck av p53 protein vid diagnos hos barn med kroniska myeloiska sjukdomar predicerade för relaps efter HSCT. I *studie IV* fann vi ett signifikant högre uttryck av p53 hos återfallsgruppen sex månader efter transplantationen. Proteinuttrycket av p53 kan vara ett komplement till de etablerade markörerna för återfall, för att möjliggöra tidigare åtgärder som förhindrar relaps. För att studera sambanden närmare har vi startat en prospektiv multicenter studie.

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