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VIRAL INFECTIONS IN CHILDREN WITH CANCER

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The cover picture is painted by Henri Lindblom 2008.
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ABSTRACT

Viral infections are the most common cause of infection morbidity in children. Nevertheless, few studies have been devoted to exploring the viral panorama in children receiving chemotherapy for cancer. The present thesis aims to study viral infections in children with cancer with special focus on Parvovirus B19 (B19) and viral infections during episodes of neutropenic fever.

B19 was for many years, believed to be a lytic virus cleared by the humoral response. However, subsequent studies in our laboratory led to the discovery of a B19-specific $CD8^+$ T-cell response persisting for up to two years indicating persistence of antigen. To learn more about the kinetics of B19 viral load and with the aim of developing a more sensitive method, we applied a Taqman-based quantitative PCR with a sensitivity of 2 geq/mL. With this assay we reassessed consecutive collected samples from five acutely infected patients and found a persistent viral load of 10^3 – 10^5geq/mL, not detected with the less sensitive qualitative PCR. This challenges our current understanding about the virus pathogenesis and suggests that B19 frequently causes persistent infection.

As long as twenty years ago, complications from B19 infection were found in children undergoing anti cancer treatment. To further study the pathogenesis of B19 in a group of children with proven or suspected malignant disorders, bone marrow aspirates obtained were supplemented with testing for parvovirus B19 DNA. Here we could confirm results from other studies in which severe cytopenia led to multiple transfusions, withdrawal or postponed chemotherapy and B19 infection mimicking a relapse. Moreover, for the first time, we were also able to include a control group of B19-negative patients and compare them with the B19 DNA-positive patients during maintenance therapy for acute lymphoblastic leukemia. Particularly striking was the number of treatment days lost in the B19 DNA-positive group, strongly indicating the potential of this factor to affect treatment outcome.

Furthermore, we did a one year prospective study on children presenting with neutropenic fever at two pediatric oncology units in Stockholm, Sweden and Sydney, Australia, focusing on viral infections. Little is known about the etiology behind neutropenic fever and the microbial detection in 15-30% of the cases corresponds mostly to bacterial findings. In this study we showed that respiratory virus RNA/DNA are a common finding in nasopharyngeal aspirates from children presenting with neutropenic fever. Moreover, the addition of PCR-based viral diagnostic tools to the routinely performed bacterial blood culture increased the detection rate of a possible etiological agent to two thirds of the cases.

In conclusion, this thesis provides support for viral infections as a common cause of morbidity also in children receiving treatment for cancer. Neglect of viral diagnostic efforts may lead to inappropriate clinical management or suboptimal therapy intensity.

LIST OF PUBLICATIONS

I. **Anna Lindblom**, Adiba Isa, Oscar Norbeck, Susanne Wolf, Bo Johansson, Kristina Broliden, Thomas Tolfvenstam. Slow clearance of human parvovirus B19 viremia following acute infection. Clin Infect Dis. 2005 Oct 15;41(8):1201-3.

II. **Anna Lindblom**, Mats Heyman, Igge Gustafsson, Oscar Norbeck, Tove Kaldensjö, Åsa Vernby, Jan-Inge Henter, Thomas Tolfvenstam, Kristina Broliden. Parvovirus B19 infection in children with acute lymphoblastic leukemia is associated with cytopenia resulting in prolonged interruptions of chemotherapy. Clin Infect Dis. 2008 Feb 15;46(4):528-36

III. Igge Gustafsson, Tove Kaldensjö, **Anna Lindblom**, Oscar Norbeck, Jan-Inge Henter, Thomas Tolfvenstam, Kristina Broliden. Detection of Parvovirus B19 infection in children with malignant or hematological disorders. Submitted.

IV **Anna Lindblom,** Vivek Bhadri, Stefan Söderhäll, Lars Öhrmalm, Michelle Wong, Cecilia Lindau, Maria Rotzén-Östlund, Tobias Allander, Daniel Catchpoole, Luciano Dalla-Pozza, Kristina Broliden, Thomas Tolfvenstam. Respiratory viral infections are the most common findings employing PCR-based diagnostics in children with neutropenia. In manuscript.

CONTENTS

LIST OF ABBREVIATIONS

AdV	Adenovirus
B19	Human parvovirus B19
CMV	Cytomegalovirus
CoV	Coronavirus
CPE	Cytopathic effects
CRP	C-reactive protein
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
EI	Erythema infectiosum
ELISpot	Enzyme linked immunospot assay
EV	Enterovirus
FACS	Fluorescence-activated cell sorting
Flu	Influenza
FUO	Fever of unknown origin
HBoV	Human boca virus
HHV6	Human herpes virus 6
HIV	Human immunodeficiency virus
hMPV	Human metapneumo virus
HSCT	Hematological stem cell transplantation
HSV	Herpes simplex virus
IFN	Interferon
KIV	KI-polyoma virus
LRTI	Lower respiratory tract infection
MBL	Mannose binding lectin
MSC	Mesenchymal stem cells
NCLR	Nordic childhood leukaemia registry
NF	Neutropenic fever
NPA	Nasopharyngeal aspirates
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PIV	Parainfluenza virus
RNA	Ribonucleic acid
RSV	Respiratory syncytial virus
RV	Rhinovirus
TAC	Transient aplastic crisis
URTI	Upper respiratory tract infection
URTS	Upper respiratory tract symptoms
VZIG	Varicella Zoster Immune Globulin
VZV	Varicella zoster virus
WUV	WU-polyoma virus

1 BACKGROUND

1.1 CANCER IN CHILDREN

Childhood cancer is rare and accounts for less than 1% of all incidences of cancer in industrialized countries. Moreover, cancers in children differ in many ways from those in adults. In pediatric patients, malignancies can arise at multiple primary sites and have great histological diversity. Whereas the most common cancers in adults are carcinomas of the breast, lung, large bowel and prostate, these forms are extremely rare in children. Instead, many of the childhood cancers develop in embryonal precursor cells.[1,2] The two dominant cancer types in children are leukemia, accounting for one-third, and brain and spinal tumors, constituting about a quarter of all malignant tumors of childhood. About 10% are lymphomas and 6-7% each consists of soft tissue sarcomas, neuroblastomas and Wilms tumors. The remaining types are retinoblastomas, bone sarcomas, germ cell tumors and epithelial tumors.[1]

1.2 CANCER TREATMENT

Today, the treatment of children with cancer is a success story, considering that three-fourths of these children in the world can expect a survival time of at least five years.[3] This success can be attributed mostly to powerful combination of drug and radiation therapies as well as advances in surgical procedures. However, these new strategies have also led to an increased risk of complications. Managing this group of severely sick children has relied greatly on improvements in intensive care units and knowledge about infections. The results have significantly contributed to high survival rates.[1] However, major challenges remain. Children with certain brain tumors and disseminated diseases still have poor prognoses, and some types of cancer have reached a survival plateau.[2] Additionally, more than 60% of children with cancer have little or no access to therapy and, consequently, a small chance of enduring.[4]

1.3 ALTERATIONS IN THE HOST DEFENSE CAUSED BY TREATMENT

The radiation and chemotherapy given to these patients are not only toxic to the hematopoietic cells but can also destroy the skin and mucosal barrier so that deterioration of the host defense often follows.[5] Indwelling catheters necessary for such treatments also contribute to the risk of infection. An overview of therapy-induced alterations in the host defense of this pediatric population is outlined in Figure 1. Therapy-induced or disease-induced neutropenia is the major risk factor for bacterial infections in these children. Moreover, neutropenia is the most common cause of chemotherapy dose reduction and delay, which may compromise disease-free survival.[6] But the decreased quantity of neutrophils is not the only adverse effect; several. studies of neutrophils taken from patients under treatment with chemotherapy have shown depressed or absent functional activity. Other phagocytotic cells are also affected, but since few studies exist, the clinical importance

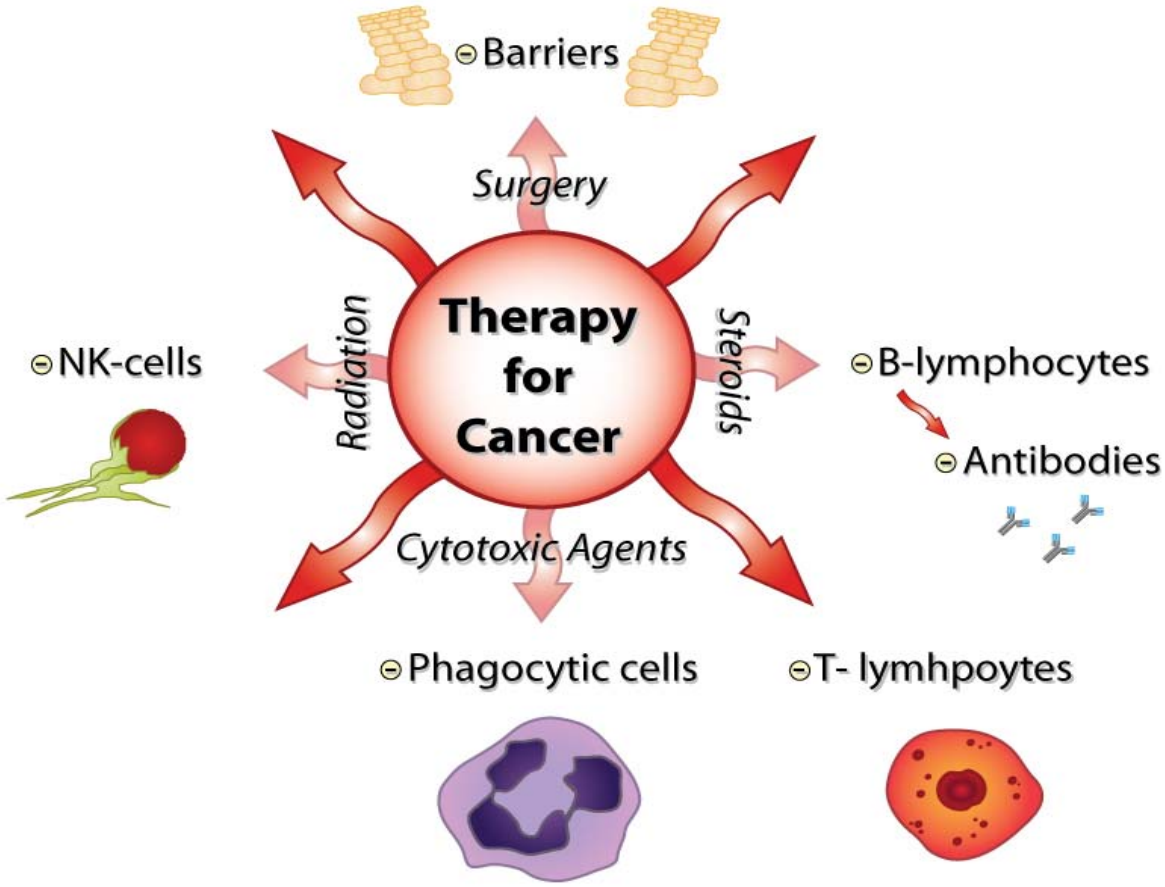

Figure 1. Therapy induced toxicity on the immune defense

of this issue is not known.[7] The specific cellular and humoral immunity are also affected, with an increased risk of viral and fungal infections. These defects can remain even after therapy ends.[8,9] One such example is the loss of humoral immunity to viral antigens present in vaccines.[10,11] Another side effect is the complete loss of natural killer (NK) cells, which has been correlated with disseminated viral and bacterial infections.[7]

1.4 NEUTROPENIC FEVER IN CHILDREN

As mentioned above neutropenia is a common complication in children undergoing chemotherapy for malignancies and is one of the most important risk factors for infections. Infections account for substantial morbidity and mortality in this patient group, and fever is often the only indication of infection since the inflammatory response often is blunted due to the immune suppression.[12,13] Castagnola et al. recently showed in a prospective study that 34% of children given chemotherapy/hematological stem cell transplantation (HSCT) developed fever during neutropenic periods.[14] Recognition of a relationship between the duration of neutropenia and the increased risk of severe infection together with prompt administration of broad-spectrum antimicrobials have improved the management of febrile granulocytopenic patients.

Accordingly, mortality rates due to infection have decreased from over 90% in the 1960s and 60–70% in the early 1970s to less than 5% at present.[6,13]

Neutropenic fever (NF) is defined as oral temperature >38.0 on two occasions 60 minutes apart or >38.5 on one occasion and an absolute neutrophil count <=500 cells/mm^3. As mentioned above, there is a direct relationship between the degree and duration of neutropenia and the risk of death from infection. Neutropenia for 10 days or more and a neutrophil count <=100 cells/mm^3 greatly increase the risk.[6,15] Other risk factors are altered mucosal immunity, indwelling catheters, defective humoral and/or cellular immunity and the underlying disease.[6] Some studies on children treated for cancer suggest that Mannose-Binding-lectin (MBL) and MASP-2 deficiencies could heighten susceptibility to an increased number and severity of neutropenic infections.[16-18] However this conclusion is still controversial, since results from other studies disagree.[19-21]

The etiology of NF is known in only 15-30% of the cases and usually corresponds to bacterial findings.[14,22] Castagnola et al. recently described a group of children undergoing cancer treatment (96%) or receiving allogenic HCST (4%); overall, 79% of these children had fever of unknown origin (FUO), 10% had bacteremia, 3% had a microbiologically detected infection without bacteremia, 6% manifested clinically detectable infection and 2% suffered invasive fungal infection.[14] FUO have been reported to be more common in children than in adults.[14,23]

When a patient with neutropenic fever arrives for treatment and all the relevant cultures are secured, empiric antibiotic therapy should begin immediately. Physical examination should be done and then repeated daily. Antibiotics are to be given either alone or as a combination therapy. A combination of a beta-lactam antibiotics and an aminoglycoside has been used for many years as an empiric therapy in children with NF.[24] For combination therapy with aminoglycoside, the following beta-lactam antibiotics are the most frequently administered: penicillin, cephalosporin and carbapenem.[22,24-28] Additionally, some penicillins, cephalosporins and carbapenems have proven useful as empiric monotherapy.[28-33]

1.5 VIRAL INFECTIONS

Viruses are small microorganisms that, to replicate, must invade and occupy living cells whose reproductive machinery they manipulate. Viruses consist of a genome (deoxyribonucleic acid (DNA) or ribonucleic acid (RNA)) and a protein-containing structure, the capsid. Some viruses also have an envelope that consists of a protein-containing lipid bi-layer. These structural elements form a complete virus particle, called a virion (Figure 2). Some viruses can be subdivided into multiple family members, and certain subfamilies simply include virions with similar structures, genomes or mechanisms for replication.[34]

Viral infections are most commonly transmitted from one acutely infected individual to another through the air, shared food, exposure to blood or physical contact. A vast majority of virus infections are acute and self-limiting leading to lifelong immunity.[35] However, some viruses do not kill the host cells they inhabit and thereby establish a

persistent or latent infection. In the case of a persistent infection, viral replication and release occur without killing host cells. In latent infections, the viral genome is "sleeping" inside the host cell without replicating at all. However, replication can then be reactivated months or years after the primary infection.[34]

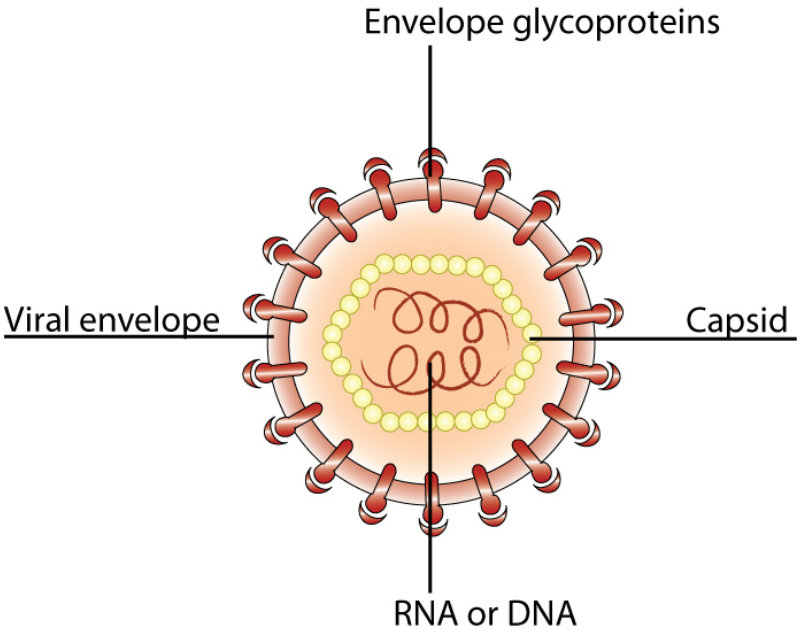

Figure 2. A virion consists of the nucleic acids and the capsid.

Virus infections are diagnosed with many different techniques, and the choice of technique depends on the virus sought and the underlying or overt disease. The most common techniques used in the diagnostic viral laboratory are serology, viral culture, molecular based techniques, antigen enzyme immunoassay detection, immunohistopathology and immunofluoresence. Serology answers questions as to whether the infection is acute or latent and recent or past. However, immune suppressed patients, who may fail to mount a humoral response, exemplify a limitation to such detection. Viral culture is slow and the clinician often receives the test results after the acute infection has resolved, whereas immune fluorescent techniques are quick but not available for identifying all viruses. For the detection of viral RNA/DNA, several polymerase chain reaction (PCR) techniques have been developed. This highly sensitive method facilitates accurate viral detection in many different human biological materials. The two types of PCR methods include "qualitative PCR" and "quantitative PCR" versions; the latter is designed to measure viral load, which is very useful for determining the effectiveness of treatment. The disadvantages of PCR techniques are the risk of contamination when analyzing high loads of viruses during acute infection and the detection of RNA/DNA from remnants of dead viruses rather than an actively replicating virus.

1.6 VIRAL INFECTIONS IN THE IMMUNOCOMPROMISED CHILD

Viral infections are the most common cause of morbidity in children. As described above, the cell-mediated immune defense is affected, which increases the risk of developing a severe viral infection.[7] Nevertheless, few studies have been devoted to exploring the viral panorama in children receiving chemotherapy for cancer.

1.6.1 Respiratory viruses

Respiratory infections are common in patients of all ages and a major cause of morbidity. Upper respiratory tract infections (URTI) are the most frequent infections in children.[36] Most often the URTI is of viral etiology and causes symptoms of the common cold; runny nose, coughing and sore throat.[37] Lower respiratory tract infections (LRTI) are most common during the first year of life and include pneumonia. and bronchiolitis. In children with bronchiolitis, about 90% have detectable viruses.[38,39] The etiology behind pneumonia is difficult to determine, since a representative specimen from the lower respiratory tract is hard to obtain.[40] It is also impossible to distinguish viral and bacterial pneumonias by clinical symptoms.[41] Asymptomatic carriage of some pathogens, especially bacteria, in the respiratory tract further complicates the diagnosis.[42,43] Whether the causative agent is bacterial or viral in persons with pneumonia depends partly on the age group studied, since viral infections dominate in pre-school-age children above 6 months, and children under 6 months and over 6 years of age are more prone to bacterial diseases.[44-48] However, new studies based on PCR techniques have highlighted the role of viruses in school-age children as well.[49]

Only a few studies have focused on the significance of respiratory viruses in children during anticancer treatment. In the 1970s and 1980s, research conducted based on viral cultures, electron microscopy and immunofluorescence recognized viruses as important factors of morbidity in children during anti cancer treatment.[50-52] Respiratory viral infections have also been more frequently reported in children with cancer compared with a control group of otherwise healthy children.[53] Studies conducted in the 1990s and 2000s are outlined in Table 1.[54-58] With different methods used and different pathogens studied, viruses were detected in 9-44% of the respiratory tract samples. Results from the three studies in which the detection of rhinovirus (RV) was feasible revealed that the most common pathogen was RV followed by respiratory syncytial virus (RSV).[54,56,57] In two of those studies, newly developed PCR methods were applied, providing the possibility of detecting such recently discovered and agents not normally detected by culture; human metapneumo virus (hMPV), coronavirus (CoV) and human boca virus (HBoV).[56,57] Their role in immunologically compromised hosts deserves further attention as case reports indicate severe infections in these patients.[59-61] The connection between cytopenia, neutropenia and viral infection was also studied by Koskenvua et al. They concluded that a correlation exists between lymphopenia, respiratory tract symptoms and virus detection but found no association with neutropenia.[57]

Table 1. Viruses detected in samples from the respiratory tract

	Arola et al 1995	Uys et al 2000	Stubkjaer-Christensen et al 2005	Tager et al 2006	Koskenvuo et al 2008
Cases	75 non-/neutropenic	22 neutropenic	250 non-/neutropenic	44 neutropenic	138 non.-/neutropenic
IF/culture/ ELISA	RSV, Flu A/B, PIV 1-3, AdV, RV	RSV, Flu, PIV, AdV, HSV, mumps, measles, EV, CMV	N/A	Flu A/B, PIV 1-3, RSV, AdV	RSV, AdV, PIV1-3, Flu A/B, RV, EV, HSV, CoV
PCR	N/A	N/A	PIV3, RV, hMPV, RSV, Flu A/B, EV, VZV, HSV	N/A	EV, RV, CoV, hMPV, HBoV
Viruses detected	13 RV, 6 RSV, 5 PIV3, 4 AdV, 3 Flu A, 1 Flu B	4 HSV, 2 AdV, 1 CMV	4 RSV, 1 Flu B, 7 RV, 1 hMPV, 1 PIV3, 2 HSV, 1 EV, 2 VZV	3 Flu, 5 PIV, 3 RSV, 2 AdV	31 RV, 15 RSV, 7 HBoV, 5 Flu A, 3 PIV3, 3 AdV, 3 PIV2, 2 EV, 2 Flu B, 1 CoV, 1 HSV
Incidence*	28/75 (37%)	7/22 (32%)	17/198† (9%)	11/44 (25%)	61/138 (44%)

* Co-infections not included.

† Calculated on respiratory tract samples collected.

Two respiratory viruses have been studied more than others, RSV and influenza. Of these, RSV is more severe with a higher mortality rate and prolonged viral shedding in immune suppressed children compared to healthy subjects.[62] However, the symptoms of RSV infection in children with cancer mirror those in the immunologically competent group: tachypnea, fever, runny nose and cough.[63-65] The progression of RSV infection to LRTI in immune suppressed patients has been reported in 30-70% of the cases.[65-67] Furthermore, the risk of developing LRTI, correlated with the presence of lymphopenia and an age of < 2 years.[65,67] The importance of the number of neutrophils and their function as an independent risk factor is still uncertain. One study showed that patients with myelosuppression more likely developed LRTI compared with non-myelosuppressed patients[68], yet results from other studies linked lymphopenia but not myelosuppression with LRTI.[63,65,67]
Elsewhere, influenza was associated with severe respiratory complications, prolonged hospital stays, postponement of chemotherapy as well as co-infections with bacteria.[69,70] Another report indicated a higher incidence of influenza in an unimmunized group of children with cancer compared with healthy matched controls

and siblings.[71] Lymphopenia has been correlated with severe influenza infection in patients after HSCT.[72] Tasian et al. also found that lymphopenia in children during cancer treatment coincided with influenza infection. Only about one-third of all such patients with influenza were neutropenic, but almost all of them were lymphopenic.[70]

1.6.2 Herpesviruses

The herpesvirus family consists of eight known strains that incite a broad spectrum of clinical diseases. Among the most prominent of these strains are Epstein-Barr virus (EBV), cytomegalovirus (CMV), herpes simplex virus (HSV), varicella zoster virus (VZV) and human herpes virus 6 (HHV6). All herpesviruses can enter a latent state following primary infection and periodically undergo reactivation.[34] Many herpes viruses are known to cause high morbidity and mortality in HSCT patients, but their role in infections of children being treated for cancer is less well-known.

EBV-induced hemophagocytic lymphohystiocytosis and lymphoproliferative disease and CMV-induced enterocolitis and interstitial pneumonia are rare but feared conditions in children during cancer therapy.[73] Although incidences of reactivated or primary EBV and CMV infections in children during cancer treatment are as high as 30%, no data on severity have been reported.[74]

HSV has been correlated with mucositis and detected in 50% of oral swabs from children with severe stomatitis after chemotherapy. Pediatric cancer patients with severe HSV-associated pneumonia have also been reported.[75-77]

VZV infection can cause severe disease for children in cancer treatment programs, and varicella pneumonia is the most common complication. Other severe complications are fulminant hepatic failure and varicella encephalitis.[34] The mortality rate in VZV infection was reported to be 7% in these children before effective treatment was an option.[78] Because the visceral disease can develop without any skin manifestations of the disease, the diagnosis is difficult.[79]

HHV6 infection, if dormant, commonly reactivates during anti cancer treatment. Severe symptoms such as hepatopathy, pneumonitis, encephalitis and bone marrow aplasia have been reported in 6% of these individuals.[80]

1.6.3 Antiviral drugs

The number of antiviral medication available for treating severe viral infections is limited. Aciclovir can be used for HSV and VZV infections. Varicella Zoster Immune Globulin (VZIG) has also been used for severe VZV infection, but its efficacy is debatable. Ganciclovir is the drug of choice when CMV infection is present, and rituxmab can be administered for EBV-induced lymphoprolerative disease. Oseltamivir and zanamivir can be prescribed for patients with influenza, but unfortunately they have not been approved for use in infants. RSV can be treated with ribavirin.[73]

1.7 HUMAN PARVOVIRUS B19

Parvovirus B19 (B19) is a single stranded, non-enveloped DNA virus. It was discovered in 1975 by Cossart and colleagues when evaluating assays for hepatitis B virus surface antigens.[81] B19 belongs to the Parvoviridae family, which can infect both vertebrates and invertebrate cells. The family is further subdivided into Densovirinae and Parvovirinae. The latter contains five genera:
Dependovirus, Parvovirus, Amdovirus, Bocavirus and Erythrovirus. B19 belongs to the latter subgroup together with two other genotypes whose clinical roles are still uncertain.[82,83] Until recently no other viruses from the Parvoviridae were known to cause disease in humans. By molecular screening of respiratory tract samples from children with LRTI symptoms, Allander et al. (2005) found a virus named HBoV. As the name indicates, it belongs to the Bocavirus genera. The virus has now been correlated with respiratory tract disease.[84,85] The other newly discovered parvovirus, named parovovirus 4, is believed to be transmitted by blood contact but its clinical importance has not yet been established. This species has been shown to cluster independently of the three known groups of vertebrate parvoviruses.[86] B19 consists of two capsid proteins, VP1, VP2 and a nonstructural protein NS1 (see Fig 3). B19 is genetically stable with low variability in either of the capsid proteins or the NS1.[87-89]

1.7.1 Infection and tropism

B19 shows a pronounced tropism for and replication in erythroid precursors and thereby inhibits erythropoesis.[90,91] The cellular receptor for B19 is the P antigen expressed on the latter two cell types.

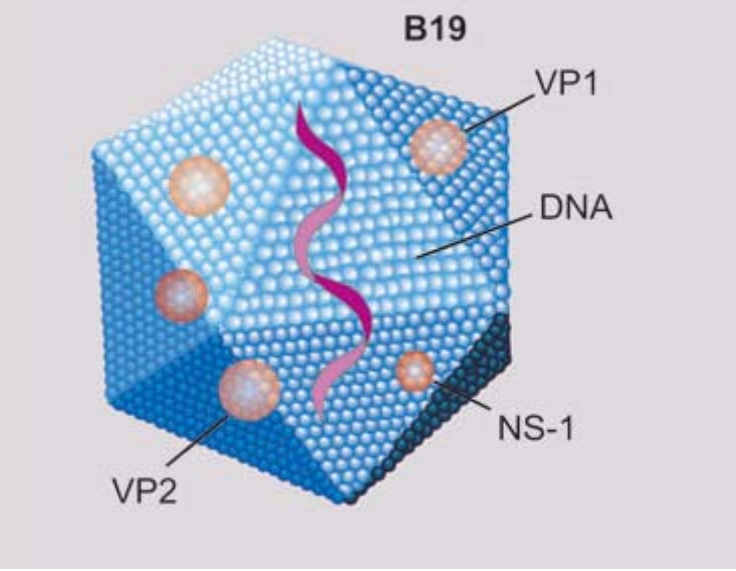

Figure 3. The viral genome of B19 encodes three proteins, the non-structural protein NS-1 and the two capsid proteins VP1 and VP2.[92]

Recently, two co-receptors have been described, a 5b1 integrin (fibronectin) and the Ku80 autoantigen. They are believed to be involved in cell adhesion and entry.[93-95] Other than residence on erythroid precursors, the P antigen is expressed on a variety of cells including megakaryocytes, endothelial cells, foetal myocytes, placental trophoblasts, tissue from kidney, heart, lungs and synovium.[96-99] The virus has not been shown to replicate in the latter cells in vivo. However, studies of infected

megakaryocytes indicate that B19 genome expression could be toxic and inhibits the infected cells' replication. This finding could be the explanation for B19 induced thrombocytopenia.[91] The P antigen was recently shown to be expressed in mesenchymal stem cells (MSC), which are used for experimental treatment of graft versus host disease in patients after HSCT. B19 was further found to persist in MSCs and to transmit B19 to bone marrow cells in vitro.[100] B19 is most commonly spread by the respiratory route but can also be spread vertically from mother to fetus, by HSCT or solid transplants and by blood transfusion.[101-103]

1.7.2 Cellular and humoral immune response.

Humoral and cell-mediated immune responses are both thought to be important in the host defense against viral infection. The humoral response is believed to dominate in the control of acute resolving infections, whereas the T cell-mediated response is more important in eradicating persistent intracellular viruses. B19 has the characteristics of being both an acute resolving and persistent virus, and both the cellular and humoral responses are involved in the host defense.

1.7.2.1 The humoral response

IgM antibodies are known to be directed against VP1, VP2 and NS1 of B19 viruses, and the two former subunits, the capsid proteins, are used for the diagnosis of acute B19 infection. IgM can be detected about 10-12 days after infection begins and persists for up to five months or occasionally longer.[104,105] The conversion to IgG occurs shortly after the advent of IgM and as the clinical symptoms resolve. Decreasing titers of these IgG antibodies, which are directed against both VP1 and VP2, are believed to persist lifelong in hosts infected, but their concentration is occasionally boosted by B19 exposure.[106]

1.7.2.2 The cellular response

For many years, researchers believed that B19 infections were controlled by the humoral response alone. This view radically changed in 2001 when Tolfvenstam et al. discovered CD8+ T-lymphocyte responses to B19.[107] These findings were then followed by studies of previously healthy, acutely infected patients whose blood was sampled for two consecutive years. The results confirmed not only the presence of B19 specific CD8+ T-lymphocytes but also indicate a persistence of viral antigen for up to two years after the resolution of clinical disease.[108,109]

1.7.3 Epidemiology

B19 infection occurs worldwide with an increased incidence during winter and spring in temperate countries. Epidemics are noted with 3-4 year intervals.[110] Most of these infections occur in children between the ages of 5 and 15 years, and seronegative parents and daycare workers are therefore at highest risk for developing an infection in adulthood.[111-113] Seroconversion increases with age, and it is calculated that 15% of preschool children, 50% of younger adults and 80% of the elderly have seroconverted.[82,114,115]

1.7.4 Diagnostic tools and persistence

B19 infection can be diagnosed by either serology or PCR but is not easily cultivated. An additional diagnostic tool is the typical finding of B19 giant pronormoblasts in bone marrow smears and immunohistochemistry.[92] As mentioned above, IgM can be detected in sera from patients 10-12 days after infection and indicates acute infection. However, serology is not a reliable diagnostic tool in immune suppressed and pregnant patients due to their immunological status.[82] The diagnostic tool of choice in these cases is PCR. Amniotic fluid, cord blood, serum and bone marrow are all examples of biological materials that can be analyzed by PCR.[92] B19 DNA is not a common finding in blood or bone marrow from otherwise healthy individuals. In blood donors, the incidence of viremia has been estimated at 1 : 167 – 1 : 35 000.[101,114,116-118] Heegard et al. did a study of healthy individuals and found B19 DNA in the bone marrow of 2%. B19 infection and related genotypes can also persist in synovial tissue and skin.[119,120] In a small group of patients, such symptoms as fatigue, arthralgia and anemia have been shown to persist along with positive B19 DNA in the bone marrow.[121]

1.7.5 Clinical manifestations

The B19 infection has a biphasic course; 8-9 days after primary infection, the symptoms of fever, malaise, myalgia and pruritus arise as the viral load peaks. The bone marrow completely loses erythroid precursors, and reticulocyte counts decrease in the peripheral blood. After about 20 days, the second phase starts with a typical maculopapular rash and sometimes arthralgia.[105,122]

B19 infection is asymptomatic in about 25% of its victims.[123] For the remainder in whom a clinical disease develops, the syndrome is called erythema infectiosum (EI) or "fifth disease." Interestingly, the disease presents with different symptoms in children and adults. The first phase of the disease in both groups involves fever, malaise, myalgia and pruritus. However, the second phase diverges so that children more often develop erythema than do adults. The classical erythema starts on facial cheeks, and some days later appears on the trunk and extensor side of the limbs.[123,124] In contrast, joint manifestations are more common in adults than in children; the incidence is about 30-60% and 10%, respectively.[125] The metacarpophalangeal joints, knees, wrists or ankles typically evince polyarthritis that can persist for weeks to month. The symptoms of some patients even fulfill the criteria for rheumatoid arthritis with positive rheumatoid factor and other autoantibodies.[123,126,127] Both the erythema and the joint manifestations are believed to be immunologically mediated. A significant drop in hemoglobin level, platelets, and leucocytes is rare. However, significant anemia has been reported in children with a poor nutritional status.[128]

Patients with heightened levels of red cell turnover rely on high erythrocyte production rates to maintain adequate hemoglobin levels. Therefore, they are especially vulnerable when infected with B19. B19 infection can, in these patients, lead to transient aplastic crisis (TAC) that may be fatal.[129-131] B19 had been recognized as the most common cause of aplastic crises in children with sickle cell anemia. Often, these patients need transfusions but recover after about ten days.[129]

B19 infections can cause complications in a pregnant woman by transmission of the infection to her fetus. The infection is most common asymptomatic and resolve spontaneously.[132] However, B19 have been correlated to non-immune hydrops fetalis in 15-20 % of the cases.[133-135] B19 infection has also been associated to fetal death in second and third trimester.[136,137]

B19 infection has been suggested to play a role in the onset of several autoimmune disorders such as systemic lupus erythematosus and rheumatoid arthritis.[138,139] B19 have also been found in patients with encephalitis, myocarditis and hepatitis as well as several other disorders.[140-142] However, a clear causality has not yet been established.

1.7.5.1 B19 infections in immune compromised patients

The symptoms of a B19 infection in immune compromised patients differ from those of infected but otherwise healthy persons in two major ways; 1.) The former group's immune mediated symptoms like erythema and joint manifestations are rare. 2.) A negative influence on their hematological parameters caused by suppression of bone marrow function is common and can persist.[143] Patients at risk for complications from B19 infection are those with chemotherapy induced immune suppression, with primary or acquired immune deficiencies, with HSCT or organ grafts and with human immunodeficiency virus (HIV).[92] Lundqvist et al. examined bone marrow aspirates from adults with haematological malignancies and found B19 DNA in 5% of the samples.[144] During the first year after HSCT or solid organ transplantation, complications from B19 infection have been reported in 1-2% of the patients.[145]

Several studies have been done on children with leukemia and B19 infection[82,143,146-154] but only a few on children with solid tumors.[155-157] Anemia, thrombocytopenia and even leukopenia are common findings in these patients, although the classical erythema is seldom seen. Since erythema is believed to be mediated by the immune system, the probable explanation is that these children lack an immune response. B19 DNA has been shown to persist for up to about a year in sera from children with hematological malignancies.[155,158]

Therapy-induced cytopenia is a common complication in children receiving anticancer treatments. Therefore, cytpopenia evoked by B19 is virtually impossible to distinguish from that induced by therapy.[82] Similarly, B19 infection can mimic a leukemic relapse. This circumstance leads to frequent hospital admissions, repeated blood sampling, extra bone marrow testing and transfusions of erythrocyte concentrate and platelets. B19 infection that precedes the onset of leukemia has also been suggested as a triggering agent.[159-162]

1.7.6 Treatment

B19 infection can be treated symptomatically by blood transfusions or by the intravenous administration of immunoglobulin. These infusions have been successful in resolving B19-induced symptoms and sometimes even in clearing the viremia,[143,150-152] although other reports show no such effect.[146] Because the symptoms of B19 can recur, the immunoglobulin injections may require repeated administration.

2 AIM OF THE THESIS

The overall aim of this thesis was to investigate viral infections in children undergoing cancer treatment based on PCR techniques. In particular, the goals were:

- To develop a sensitive quantitative PCR technique for the detection of parvovirus B19 DNA and to study the kinetics of B19 viral load
- To study parvovirus B19 infections in children during anti cancer treatment
- To study viral infections during episodes of febrile neutropenia in children during anti cancer treatment

3 MATERIALS AND METHODS

3.1 STUDY SUBJECTS

3.1.1 B19 viral load in acutely infected patients: development and administration of a sensitive PCR for B19 DNA detection (paper I)

Five previously healthy persons with acute B19 infection were prospectively identified at the Division of Clinical Virology, Karolinska University Hospital, Sweden. All these patients had samples referred for B19 analyses by their general practitioner. During the 128 weeks after inclusion of the first individual, sera and peripheral blood mononuclear cells (PBMC) samples were collected at regular intervals from all these patients, after they signed appropriate consent forms. Medical histories were collected from the participants during the second visit for sample donation, and continuous follow-ups to assess clinical symptoms were conducted throughout the study period.

3.1.2 B19 infection in children with suspected or confirmed malignancies (papers II & III)

During a period of 5.5 years, routine examinations of bone marrow for proven or suspected malignant disorders at the Pediatric Oncology Unit, Karolinska Hospital, Stockholm, Sweden, were supplemented with testing for parvovirus B19 DNA except for samples excluded on technical or logistic grounds. Clinical and laboratory factors at diagnosis as well as follow-up data were retrospectively collected from the Nordic childhood leukaemia registry (NCLR) and from medical records.

3.1.3 Viral infections in children with neutropenic fever (paper IV)

One-year prospective studies were conducted in Pediatric Oncology Units at the Children's Hospital at Westmead, Sydney, Australia, and Astrid Lindgren Children's Hospital at the Karolinska University Hospital, Stockholm, Sweden. All children with an episode of neutropenic fever (axillary temp >38.0^{o}C on two occasions 60 minutes apart or ≥38.5^{o}C on one occasion and an absolute neutrophil count ≤500 cells/mm^{3}) were asked to participate. Paired nasopharyngeal aspirates (NPA) and peripheral blood samples were collected in addition to samples for routine laboratory assessments. Data on the patients' oncological diagnoses, age, sex, presence of upper respiratory tract symptoms (URTS) and C-reactive protein (CRP)-concentration were recorded.

3.2 STUDY CONTROLS

In paper I, 15 B19 IgG positive and IgM negative healthy laboratory workers, with no recollection of parvovirus-related symptoms, were included as controls.

3.3 ETHICS

Approval for paper I was obtained from the local ethics committee at the Karolinska University Hospital (Stockholm, Sweden). Studies II and III were approved by the ethical committee at Karolinska Institutet (Stockholm, Sweden). Study IV was

approved by the regional ethics comittees in Stockholm and at the Childrens Hospital in Weastmead.

3.4 PCR

3.4.1 Nested PCR

All samples described in papers II and III were analyzed with a qualitative nested PCR, representing the non-structural NS protein. The PCR have the sensitivity to detect B19 but not genotype II and III.[163]

3.4.2 Quantitative PCR

3.4.2.1 Detection of B19 viral load

All blood samples collected for reporting in papers I and IV and all samples from patients described in papers II and III with at least one positive sample as measured with nested PCR were analyzed with a quantitative PCR. For assessment of B19 DNA levels, a novel parvovirus genotype 1-3 specific TaqMan real-time PCR assay was developed.

Serum samples (200 mL) noted in papers I-III were extracted with the use of an automated MagnaPure extractor (Roche Diagnostics) using the LC Total Nucleic Acid Isolation Kit (Roche). Samples for paper IV were extracted using an ABI Prism 6100 Nuclide Acid PrepStation (Applied Biosystems).

The assay was performed with an ABI 7700 sequence detection system (Applied Biosystems) in a 50-µL reaction mixture containing 25 µL of TaqMan Universal PCR Master Mix (Applied Biosystems), 5 µL of template DNA, 3 µmol/L of each primer, and 1.5 µmol/L probe for 40 cycles consisting of 15 s at 95▫C and 20 s at 60▫C. The following primers were used in the amplification: sense, 5'-ACAAGCCTGGGCAAGTTAGC-3´ and antisense, 5'-GGCCCAGCTTGTAGCTCATT-3' at B19 genomic nucleotide positions 854–873 and 910–928, respectively (numbers refer to GenBank AY083239). Detection was provided by an FAM-TAMRA–labeled probe (Applied Biosystems) with the sequence 5'-CAACTACCCGGTACTAACTATGTTGGGCCTGG-3'at B19 genomic nucleotide positions 877–908. A B19 viremic plasma, determined to contain 1.4 x 10^{11} genome equivalents (geq)/mL, lot BPL9 (kindly provided by Dr. Kerr, Biotrin International) was used as a standard. The sensitivity of the assay was 2 geq/reaction, as determined by repeated testing of serial dilutions of the BPL9 standard. Negative controls were extracted and analyzed between every 5 patient samples throughout the procedure.

3.4.2.2 Identification of the B19 genotype

Samples from paper I that had positive results of quantitative PCR were partially sequenced to assess viral genotype (B19) using a separate assay. Outer primers in this assay were as follows: sense, 5'-GTGGTGAAAGCTCTGAAGAACTCA-3' and antisense 5'-GCCCAGGCTTGTGTAAGTCTTC-3' at B19 genomic nucleotide positions 37–60 and 844–865, respectively. The inner primers were as follows: sense, 5'-CGGGACCAGTTCAGGAGAATCA-3' and antisense, 5'-GGGGTGGTCAGATAACTGTCCATG-3' at B19 genomic nucleotide

positions 137–158 and 757–780, respectively (numbers refer to GenBank AY083237). Amplification was performed in a volume of 50 µL in 1 x buffer II (Applied Biosystems) and 25 mmol/L MgCl and 10 pmol/L primer at an annealing temperature of 55▫C and for 40 cycles. The amplified product was sequenced using the Big Dye Termination Kit (Applied Biosystems) in an ABI 3100 sequencer (Applied Biosystems).

3.4.2.3 Detection of CMV, EBV & AdV viral load

In paper IV, quantitative real-time PCR was used for detection of CMV in total nucleotide extracted peripheral blood, AdV in total nucleotide extracted plasma, and EBV in total nucleotide extracted serum. Samples were extracted using a ABI Prism 6100 Nuclide Acid PrepStation (Applied Biosystems).[164-166]

3.4.2.4 Detection of respiratory viruses

In paper IV, total nucleic acid was extracted from NPA and analyzed using real-time semi-quantitative PCR targeting a panel of respiratory viruses; RSV, Flu A and B, PIV 1-3, EV, AdV, HRV, HBoV, hMPV, CoV (NL63 / OC43 / 229E / HKU1) and KIV/WUV polyomavirus (KIV/WUV).[167,168]

3.5 VIRAL CULTURES

Viral cultures of NPA samples described in paper IV were monitored daily for cytopathic effects (CPE), with confirmatory immunofluoresence performed on cultures with positive CPE using the local accredited protocols. Pathogens detectable with viral culture were HSV 1 and 2, VZV, PIV, EV, RSV, AdV, PIV1-4, Flu A and B and CMV. RV was only detectable by viral culture in the laboratory at the unit in Sydney.

3.6 IFN –γ ELISPOT

In paper I, the ex-vivo cellular immune responses were measured in terms of antigen-specific interferon (IFN-γ) secretion in an Enzyme linked immunospot assay (ELISpot) assay as previously described, except for the use of streptavidin conjugated with alkaline phosphatase and a corresponding substrate (BioRad, Hercules, CA, USA).[169]

3.7 LYMPHOCYTE COUNTS

In paper I, CD4+and CD8+ T lymphocyte counts were determined by direct staining of PBMCs isolated by Ficoll-Paque (Amersham Biosciences) with fluorochrome-labeled monoclonal antibodies (BD), and subsequent analysis was performed by fluorescence-activated cell sorting (FACS).

3.8 SEROLOGY

In paper I, serum samples were analyzed for B19 IgG and IgM using a commercial EIA (Biotrin International).

3.9 STATISTICAL METHODS

For statistical analyzes in paper II the following methods were used: Groups were compared by using the Mann-Whitney U-test for age and days without treatment and by Fisher's exact test for small groups of nominal variables. The effects of parvovirus B19 DNA- positive samples, age at diagnosis, gender, treatment protocol and treatment time on the outcome "number of days without treatment" were analysed with a multivariable linear regression model. The outcomes "extra bone marrow examination y/n" and "transfusion during maintenance y/n" were analysed by Logistic and Poisson regression, using exact estimates due to the small number of patients. The effects of parvovirus B19 DNA positive samples, age at diagnosis, gender, protocol and time in maintenance treatment were taken into account. The variables included in the both the multivariable linear regression model and in the Logistic and Poisson regression models were chosen because of their clinical relevance rather than for statistical reasons. Assumptions of all models were checked and found reasonable. Evaluation of model fit was done and found adequate. To assess over-dispersion in the Poisson regression model, scaled-deviance was calculated. Group comparisons as well as the Multivariable Linear regression were performed in R (Available at http://www.r-project.org). The exact analyses for the Logistic- and Poisson regression were performed in LogXact 7.0 (Cytel, Cambridge, MA, USA). The test for over-dispersion was performed in SAS version 9.1.3 (SAS Institute Inc., Carey, NC, USA). Two-tailed tests were used throughout, and p-values less than 0.05 were used as cut-off to indicate a statistically significant result.

4 RESULTS AND DISCUSSION

4.1 PAPER I, B19 VIRAL LOAD IN ACUTELY INFECTED PATIENTS: DEVELOPMENT AND USE OF A SENSITIVE PCR FOR B19 DNA DETECTION

The cessation of symptoms caused by B19 infection coincides with a rise in IgG titer and concomitant decrease in viral titer. Therefore, B19 was, for many years, believed to rely on the humoral response. However, subsequent studies in our laboratory led in 2001 to the discovery of a B19-specific $CD8^+$ T-cell response, highlighting the cellular immune system's active role in the defense against B19.[107] To investigate this issue further, we prospectively identified five previously healthy persons who now bore acute B19 infections and were presently patients at the Division of Clinical Virology, Karolinska University Hospital, Sweden. All these patients had come to their general practitioner with symptoms of fever, arthralgia, fatigue and/or rash. All their serum samples sent to the viral laboratory were IgM positive confirming acute B19 infection. None of the patients had any previous history of immune deficiencies or immune suppressive treatment. After enrollment in the study, the patients were followed clinically and were consecutively sampled for two years. Their $CD8^+$ T-cell responses, serology, B19 DNA in serum detected by qualitative PCR and PBMC were measured. Analyses of the cellular immune system showed a sustained $CD8^+$ T-cell response that persisted throughout the two years of follow up. The B19 DNA qualitative PCR showed viral clearance from their sera after 17 weeks; however, PBMC from two of the patients retained B19 DNA during the entire follow-up period. The reason for this circulating pool of $CD8^+$ T-cells was not clear, but the most likely explanation was sustained antigen stimulation even though the majority of the patients seemed to clear the viremia.[108,109]

To learn more about the kinetics of B19 viral load and with the aim of developing a more sensitive method, we applied a Taqman-based quantitative PCR with a sensitivity of 2 geq/ml. All the serum samples (qualitative PCR negative after week 17) were reassessed and surprisingly all assessments by this quantitative method showed persistent B19 DNA viremia (see Fig 4).

The quantitative PCR was designed to detect all three erythroviruses, but sequencing showed all of them clustered in the B19 genotype. The viral load curve decreased rapidly at the time point of increasing IgG levels. At this stage, all patients also reported cessation of symptoms. The first sample was collected 5-10 days after presentation of the first symptom. The mean value of the viral load was 1.2×10^7 B19 geq/mL. This correlated with earlier findings,[170] but the peak might have been missed, since titers over 10^{13} have been reported, and our patients were sampled only after a 5-10 day delay.[171] After the peak of IgG development, the curve decreased leaving a prolonged pattern that indicated a persistent viral load of $10^3 – 10^5$geq/mL. Two patients showed an increase in viral load during the follow up. Patient #1 (as referred to in the figure) had a single increase of titer and then became the only patient whose viremia eventually cleared. Subsequently, patient #3 had a twofold increase in titer, but unfortunately, this was the last sample collected. It is interesting to speculate whether this new peak of viral load in patient #1 somehow boosted the immune response and, thereby, eventually

cleared the infection. This patient's cytokine profile was investigated in another study in our laboratory and, surprisingly, manifested an increase in Th1 cytokines that accompanied the peak viral load.[172]

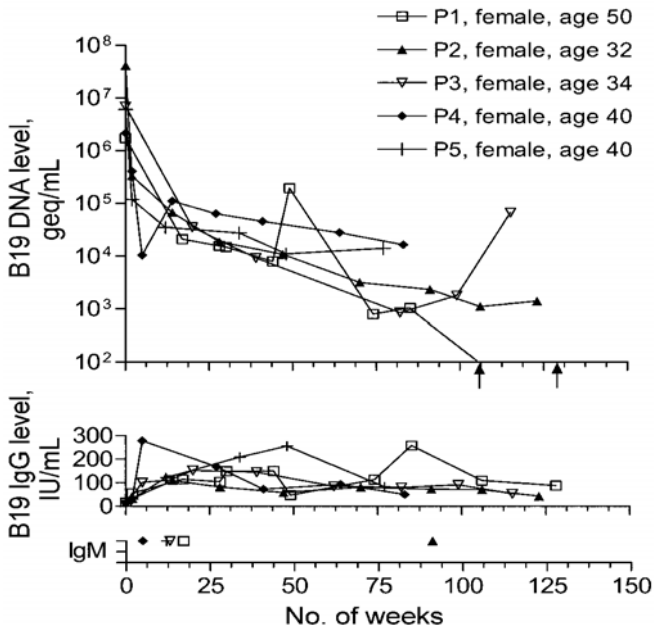

Figure 4.[173] Kinetics of B19 DNA and antibody responses against B19 in serum after acute infection in patients 1–5 (P1–P5, respectively). The lower panel shows the last time point at which each patient tested positive for serum IgM. Arrows indicate negative sample time points for patient 1. The figure refers to the number of weeks after the first sample was taken.

New studies of blood donors and multitransfused patients with hemoglobinopathies have revealed that the B19 virus can persist in levels of 10^1 -10^5 geq/mL for years, which supports our findings.[174-176] With this knowledge of B19's persistence and transmission via blood transfusion, the testing of these viral titers becomes vital. In the literature describing B19 infection transmitted by blood transfusions, one reported a higher prevalence of B19-specific antibodies in groups receiving clotting factors compared to a control group.[176] No exact infectious level of B19 in blood products has been established, and doing so is dependent on identifying the co-presence of B19 IgG in the blood products and in the recipient. Single studies have reported viremia of > 10^7 geq/mL in blood products after transmission. But the same studies concluded that viremia of 10^4 geq/mL did not transmit the infection.[177] Further, B19 viral loads in titers of < 10^4 geq/mL have not been shown to transmit infection in patients with malignances.[178] However, B19 infected clotting factor concentrates have led to seroconversion in recipients with titers of 10^3 geq/mL.[179]

B19 viremia has been reported in only 1% of donated blood.[174,178] Since the seroprevalence of past B19 infection is 50-80% in adults, this low detection rate of B19 viremia indicates that the virus eventually clears. The fact that even our small control group of 15 IgG positive and IgM negative patients was B19 DNA negative further support this supposition. The earlier findings of a sustained circulating pool of $CD8^+$ T-cells together with the persistent B19 viremia found in this study along with its eventual clearance suggest that B19 should no longer be counted as a solely lytic virus.

In this study, we detected persistent B19 DNA in patients with the prior acute infection. Further studies are needed for mapping and establishing the role of B19 as a persistent virus. However, this subject is discussed in two other theses from our group (Norbeck 2005, Isa 2006). Therefore, the present thesis focuses on the development and application of a sensitive, quantitative PCR for B19 detection.

4.2 PAPER II-III, B19 INFECTION IN CHILDREN WITH SUSPECTED OR CONFIRMED MALIGNANCIES

As long as twenty years ago, complications from B19 infection were found in children undergoing anti cancer treatment.[143,156] Afterward, descriptions of B19 infection that adversely affected children with leukemia appeared but only a few other malignancies were similarly troubled.[82,146,147,155-157] The three problems most frequently reported were B19-induced cessation of therapy, the need for transfusion and the requirement for extra bone marrow examinations.[82] B19 has also been known to persist in serum for up to about a year.[155,158]

With the major aims to detect the frequency of B19 DNA in the bone marrow of children during anticancer treatment and to investigate the possible complications of that infection, our team began a five-year study at the Pediatric Oncology Unit, of Astrid Lindgrens Children Hospital. Between the years 1995-2000, bone marrow aspirates were collected from children with proven or suspected malignant disorders and analyzed with a nested, qualitative PCR to detect B19 DNA. The collection details are outlined in Figure 5. Children with ALL and its subgroups are presented in paper II "ALL-B19," and children with none or with other malignancies are described in paper III, "non-ALL B19".

The frequency of B19 DNA-positive bone marrow samples (at least one positive sample) constituted 15% of the ALL group and 7% of the non-ALL group. Heegaard et al. did a retrospective study on 75 patients with ALL to measure serum B19 DNA, IgG and IgM at the start and end of therapy and noted seroconversion with the co-presence of B19 DNA in 8% of the patients.[82] No other follow-up studies were done for the comparison of B19 prevalence. Since B19 has a marked tropism for replicating in the bone marrow, finding a higher number of B19 DNA positives in our ALL patients was not surprising. Since the non-ALL B19 patients were such a heterogeneous group, the majority of whom did not have scheduled or repeated bone marrow aspirates, the lower number of only 7% positivity is readily explained.

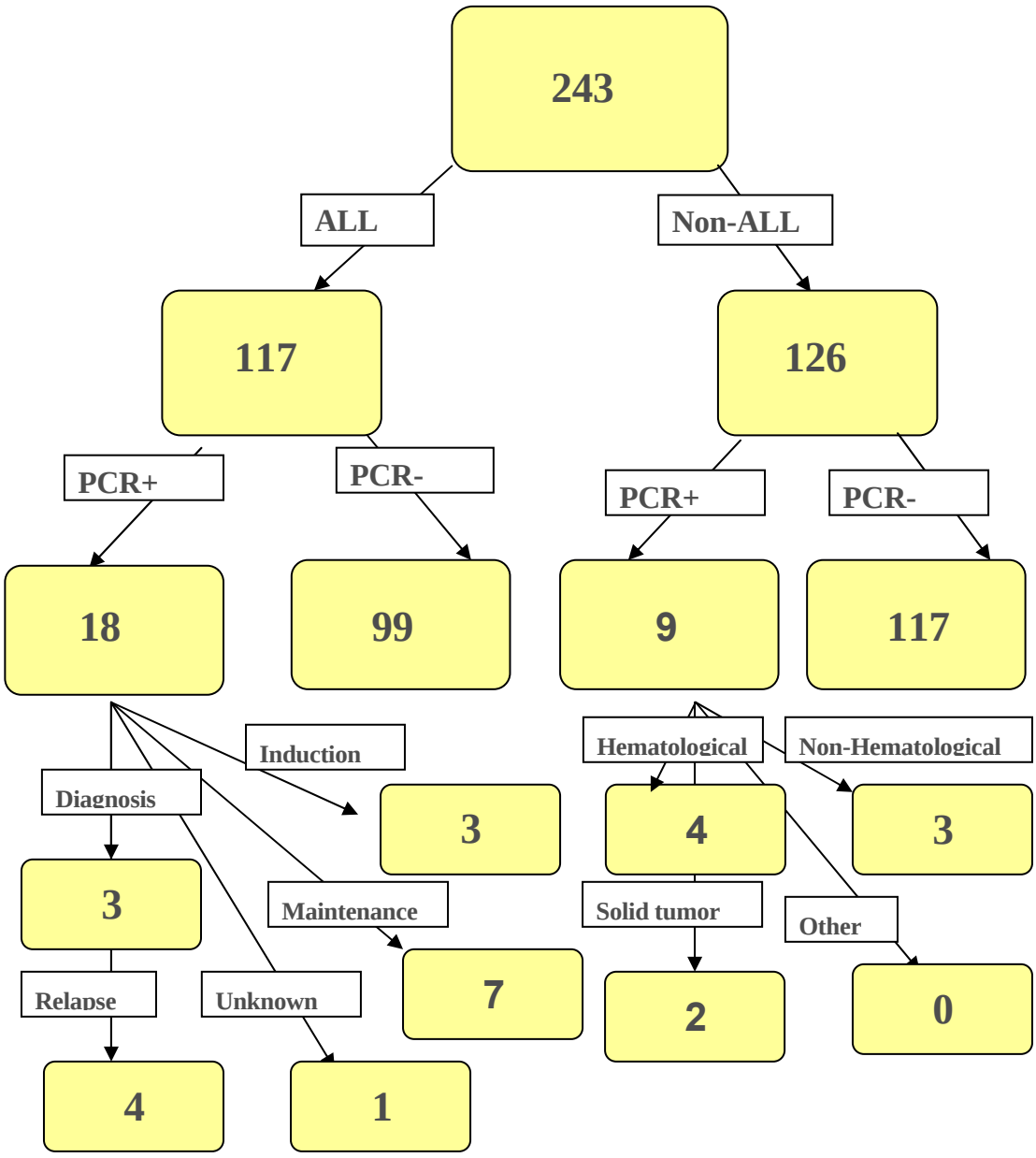

Figure 5.[180] Flowchart over patients included. The eighteen patients diagnosed with ALL and B19 DNA positive are subdivided into time of B19 DNA detection. The nine "non-ALL" patients with detected B19 DNA are subdivided into underlying diagnose.

All samples from patients found positive at least once with the nested PCR were reassessed with our newly developed QPCR. As the nested PCR only can detect B19 and not the other genotypes, we can conclude that the reassessed positive bone marrow samples all belonged to the B19 genera. As a result, some of the patients' samples initially reported as negative with the nested PCR were positive when reassessed with the more sensitive QPCR.

B19 DNA has been reported to persist in serum for up to a year in children with cancer.[155,158] No comparable, follow up studies are available on bone marrow samples. Here, we found evidence that the virus had persisted for two years, and only two patients cleared the B19 DNA completely.

Furthermore, the patients examined here differed in terms of viral load. That is, comparing the B19-ALL group who had been infected at time of diagnosis/during induction therapy with the patients infected during maintenance revealed a significantly higher viral load in the latter group (median viral load, 2.1 x 10^8 copies/mL and 6.9 x 10^4copies/mL respectively; P = .037 (by Mann-Whitney U test). Additionally, in the non-ALL B19 group of patients, viral loads consisted of up to 8.7x10^7copies/ml. Limited data are available in the literature on persistence and viral load of B19 DNA in the bone marrow of children during anti cancer treatment. Fattet et al presented one child with hematological malignancy with a B19 viral load of 10^8 copies/mL in the bone marrow.[146] Therefore, no conclusion can be drawn about the meaning of a specific viral load. Most likely, the high viral load in the maintenance group corresponds to acute/active B19 infection. The lower viral load in the first group may have been the result of persistent low-level B19 DNA replication from an earlier infection, as the older age in this group could indicate. But a low viral load might also stem from an underlying disease, and chemotherapy's toxic effect on the bone marrow could reduce the number of cells available for the replication of B19.

Anemia is the most common symptom detected in children during cancer treatment.[148-151] Anemia has been described to reoccur but also persists for longer periods in children receiving cancer therapy.[143,146] El-Mahallawy et al investigated 50 patients with anemia during maintenance treatment and compared them with 34 non-anemic children during the same treatment period. They found a statistical significant difference in the presence of B19 DNA in the anemic group compared to the non-anemic group. Other groups have apart from anemia, reported thrombocytopenia and leucopenia.[82,146] Frequently reported symptoms in the present study were cytopenia including anemia, thrombocytopenia and leucopenia; however, the classical symptoms of EI were infrequently reported. In only one of 27 patients was B19 infection suspected. Instead the cytopenias were misinterpreted as relapses in 5 patients, leading to extra bone marrow examinations. The cytopenias were so severe that several patients needed multiple transfusions. Because of the prolonged cytopenias, some patients' maintenance treatment was withdrawn.

As described earlier, chemotherapy induced cytopenia is impossible to distinguish from B19 induced. Therefore the findings of these symptoms in earlier studies and in the present not easily can be addressed to the detected B19 DNA. In our B19-ALL group some of the patients have their B19 DNA detected during maintenance treatment. The maintenance phase is far less toxic than the induction phase and cytpopenia is not expected to be pronounced. Our group of patients found B19 DNA positive during maintenance treatments experienced recurrent cytopenias and as a consequence the maintenance treatment needed to be withdrawn. In fact, the number of days these B19 DNA-positive patients had to be denied maintenance treatment was startling. To determine if this number correlated with B19 DNA positivity, we included a control group of B19-negative ALL patients and compared the two groups. Surprisingly, the group with detectable B19 DNA had a whole month more of cancelled therapy than the control group (median, 59 days; range, 34–105 and median 30 days; range, 0–93 days respectively. P <.05, by Mann-Whitney U test). The numbers of transfusions were also significantly higher in the group with detectable B19 DNA compared to the control

group. Since the days with withdraw treatment was so high in the B19 group we speculated whether this could affect the outcome since the treatment intensity during maintenance is of importance for event free survival.[181] Therefore we also collected data on the outcome and find that 29% of the B19 DNA–positive patients and 15% of the control subjects experienced a relapse of cancer. This was not a significant difference but our study groups are small.

The non-ALL B19 group of patients showed a wide diversity of underlying disease. One of the positive patients was a 9-years old girl with unclear cytopenia doing a bone marrow to exclude hematological malignancy. As no hematological disease cold be confirmed, it is interesting to speculate whether the unclear cytopenia was due to a primary B19 infection.

4.3 PAPER IV, VIRAL INFECTIONS IN CHILDREN WITH NEUTROPENIC FEVER

In paper II and III we studied B19 in children with cancer and found an increased morbidity when compared with a control group. What about other viral infections? As described earlier, neutropenic fever is a common complication to cancer treatment. Little is known about the etiology and the microbial detection in 15-30% of the cases corresponds mostly to bacterial findings.[14,23] URTI is the most common infection in children and the majority of these are of viral origin.[36] It is therefore likely that virus also play a role in episodes of neutropenic fever. As mentioned in the introduction, only a few studies with viral focus have been done and they indicate that virus not is an infrequent finding in children with neutropenic fever.[50-52,54-58] To learn more about the etiology behind neutropenic fever we started a one year prospective study at the Pediatric Oncology Units at Astrid Lindgren Children Hospital Stockholm and the Westmead Children Hospital in Australia (paper IV). Collecting data from the northern and southern hemispheres allowed us to compare the spectra of infections and their epidemiology, thus allowing investigation of differences in etiology of infections. From all included patients a NPA was collected for detection of respiratory viruses and blood for analyzes of AdV, B19, CMV and EBV.

The NPAs were first analyzed with conventional techniques and a virus was detected in 14 % of the episodes. All of the positive samples were detected at the Sydney study site. To increase the sensitivity of the methods, samples with enough material were reassessed with real-time PCR targeting the viruses detectable by culture with the addition of agents not normally detected by culture. PCR was superior to the conventional techniques in the cases of overlapping of the both methods, as the conventional techniques could detect a virus in only 29% of the cases with a virus detected by PCR. The increased sensitivity of PCR compared to conventional techniques is well known and published elsewhere.[182]

With PCR, at least one respiratory tract viruses was detected in 46 % of the analyzed NPAs. 69 % of the episodes had correlated URT symptoms. In earlier studies viruses have been detected in 9- 44 % of cases with non- or neutropenic fever.[54-58] As different viruses are studied and different techniques are used it is difficult to compare the studies. However, one can conclude that respiratory viruses are frequentley detected in

children with cancer. RV was the most common virus detected followed by CoV at both study sites. This is also confirmed in other studies on children with cancer and also the far most common detected pathogens in immune competent individuals in all ages.[183-188] [189] Interestingly, when adding bacterial findings to the viruses detected by PCR the detection rate could be increased to 61 % of the cases with NF.

Children are normally colonized by bacteria, but whether this is the case with viruses is still unknown.[43] One of the drawbacks with the more sensitive PCR methods is the fact that it could detect low amounts of DNA/RNA which not for sure can be correlated to acute infection. RV has been detected in asymptomatic healthy children and also shown to persist for several weeks.[190-193] In one study, 27% of the virus-positive NPAs were obtained from asymptomatic healthy children. RSV, Flu A and AdV were infrequently detected in these asymptomatic patients, whereas the majority of the positive cases constituted of RV.[194] However, RV is a pathogen with several genotypes circulating in the community. Jartti et al. showed in a recent prospective study of infants with an increased risk of developing asthma that the same virus strain was present in only 5 % of consecutively samples collected >2 weeks apart. Rarely was the same virus strain even found during persistent and recurrent infections of these children.[195] Prolonged viral shedding of RSV and influenza A has been reported in children with cancer.[52,62] Of 27 patients studied by Koskenvuo et al. whose follow-up samples were taken 1-11 days after the first one, 23 were negative whereas four patients remained virus-positive (3 HRV, 1 RSV).[57] In our present study, eight individuals had NPA sampled repeatedly during separate neutropenic fever episodes and were assessable to whether the virus had cleared or persisted by real-time PCR. Of these individuals, five demonstrated clearance (after a median of 5.5 weeks, range 2.7-14 weeks), while three had the same virus persistently detectable (after a median of 3.6 weeks, range 3.4-5 weeks). This indicates that viral persistence is not a common finding and strengthens the fact of an acute viral infection.

Recently the occurrences of viral-viral- and viral-bacterial co-infections have been highlighted. In our study, co-presence of respiratory virus was detected in half of the episodes with bacteremia which also corresponds to what Koskenvuo found in a group of children with acute leukemia.[196] A correlation between pneumococcal disease and viral respiratory infections have been shown in otherwise healthy children but the meaning of the results in the present study need to be evaluated in future studies.[197,198]

Our finding of EBV and CMV in only one patient each and none with AdV detected suggest that disseminated infections of these viruses are not a major cause of morbidity during NF episodes in children under treatment for a malignancy. This is probably due to the less affected immune defense in these children compared to patients undergoing HSCT. Parvovirus B19 DNA was detected in blood in two cases (2 %) with a very low titer. This is a low figure compared to the children presented in paper II and III. In the present two cases, no bone marrow aspirates were examined for the presence of B19 DNA. The low incidence of B19 DNA in serum in this cohort indicates that B19 DNA is a more common finding in bone marrow and that bone marrow aspirates are needed for diagnostic of B19 DNA.

5 CONCLUSIONS AND FUTURE PROSPECTS

The overall conclusions from this thesis are twofold: 1.) Viral infection/presence in immunologically suppressed children is common and a neglected cause of morbidity; 2.) Quantitative PCR is a helpful diagnostic tool not only for testing immunosuppressed children but also for investigating the scope of infection in immunologically competent patients.

In paper I-III we studied B19 infection in acutely infected patients and in patients with suspected or confirmed malignancy. In paper I we showed that quantitative PCR recorded a persistent viral load of $10^3 – 10^5$geq/mL in patients after acute B19 infection which indicates that B19 not should be considered a lytic non-persistent virus. Further characterizing of the hosts derived response to B19 is needed in future studies. The presence of persistent virus also needs to be taken under consideration in cases of blood transfusions. Frequent recipients of transfusions are patients with chemotherapy-induced immune suppression. As outlined in paper II and III this population has an increased susceptibility to severe B19 infection and is probably affected by relatively small amounts of virus because of their presumed lack of neutralizing IgG. As illustrated in papers I-III, our newly developed quantitative PCR was a more sensitive method for the detection of B19 DNA than the nested, qualitative PCR in acutely infected patients and in children during anticancer treatment. Recommendations today for screening of blood products differ worldwide and are, in many countries, not a routine procedure. Considering the current knowledge of persisting viral loads after acute infection, blood products may in the future be screened for presence of B19 by quantitative PCR.

In papers II and III, the viral load was measured consecutively for the first time in bone marrow aspirates from children. Therefore, such limited results make it impossible to draw conclusions about the clinically importance of any specific viral load, and follow-up in other studies is necessary. Still, we could confirm results from other studies in which severe cytopenia led to multiple transfusions, to withdrawal or postponement of chemotherapy and to B19 viral infection mistaken for a relapse. Particularly striking was the number of treatment days lost in the B19 DNA-positive group, strongly indicating the potential of this factor to affect an event-free outcome. On clinical grounds, only one of the totally 27 B19 DNA-positive patients was suspected of having a B19 infection. Supported by these data, we suggest that B19 PCR testing should be introduced as a routine analysis for children with unexplained cytopenia. As a consequence; an important question to address in future studies is which sample material should be used for quantitative PCR testing of children with malignances, serum or bone marrow? Many children need anesthesia for bone marrow aspirates, and even if they do not, bone marrow aspiration is still much more invasive than serum sampling. However, for paper IV, serum samples were prospectively collected from children presenting with febrile neutropenia. B19 was detected in only 2% of the serum samples compared to 15% of the bone marrow samples from B19-ALL patients (paper II) and 7% of those from the non-ALL B19 group (paper III). Also, when a small number of paired samples (serum and BM) were compared,[163] more B19 DNA was found in the bone marrow. Based on these data and until more study results are

available, we recommend that suspected B19 infection should be excluded by examining the bone marrow.

Viral infections in children with neutropenic fever are not well studied. Our results as described in paper IV indicate that respiratory virus RNA/DNA is a common finding in children with cancer. As pointed out in the discussion, an important question to address is if these viral DNA/RNA copies correspond to an acute infection, or whether they are remands of an old infection or viral colonization. To answer this question we will now launch a prospective, longitudinal study on children presenting with NF at the Pediatric Oncology Unit, Astrid Lindgrens Children's Hospital. NPA:s collected will be acutely analyzed and children with detectable respiratory viruses will be sampled for additional NPA samples two and four weeks after the first one. In this way, we can follow the viral load and see if the virus eventually is cleared. To see whether viral DNA/RNA could be detected in children without fever we also plan to include a control group consisting of neutropenic children without fever.

Neutropenia is a well established risk factor for bacterial infections and in cases of NF empirically administrated antibiotics have been shown to be lifesaving. However, to fight viral infections we are dependent on both the cellular and humoral immune defenses. Some studies have shown a relation between lymphopenia and severe RSV and influenza infection in immune suppressed patients. These results were independent of the neutrophil count.[65,67,72] Based on this knowledge we plan to investigate the distribution of lymphocyte populations and also immunological function analyzes in correlation to viral infections.

Infection complications during chemotherapy results in additional admittance to the hospital for the child with a decrease of life quality as a consequence. Additional drawbacks include the increased risk of colonization of bacteria and bacterial resistance devolvement following treatment with broad spectrum antibiotics. One other important side effect is the postponed or withdrawed chemotherapy with a possible effect on an eventfree outcome. Several researchers have tried to identify risk factors to single out the patients at highest risk of lifethreatening infections and in need of hospital care with broad spectrum antibiotics, and patients with lower risk and thereby suitable for less broad and even ambulatory treatment.[199-202] I believe that increased knowledge about viral infections in children with cancer as well as the etiology behind the NF episodes will improve the management of these children and lead to more individually based treatment strategies. The studies included in this thesis indicate that viral infections accounts for a significant amount of morbidity in children with cancer. This knowledge open up for improvements of diagnostic routines and also highlight the need of viral vaccines and antiviral drugs in this patient group. Hopefully in the end, this will lead to an increase in the quality of life for the child in its family.

6 POPULÄRVETENSKAPLIG SAMMANFATTNING

Barncancerbehandlingen har under de senaste årtionderna utvecklats enormt och idag överlever en klar majoritet av de som insjuknar. Detta kan till stor del tillskrivas tuffare cellgiftsbehandlingar och strålning men också en utveckling inom tumörkirurgin. Den tuffare behandlingens baksida är att barnen drabbas av fler komplikationer, ty inte bara tumörceller dör av behandlingen utan även andra för oss viktiga celler i kroppen. I vår benmärg produceras blodkropparna, där de vita blodkropparna är viktiga för att försvara oss mot infektioner, de röda för att transportera syre till vävnader och blodplättarna för att vi ska sluta blöda när vi slår oss. Alla dessa tre påverkas av cellgifterna och ger en blodkroppsbrist, vilket leder till att barnen blöder lätt, blir trötta och riskerar att drabbas av fler och svårare infektioner. Vid samtidig feber och brist på vita blodkroppar måste barnen vårdas på sjukhus för att få antibiotika givet direkt i blodet. Pågående infektion kan leda till att cancerbehandlingen måste skjutas upp eller inte ges alls.

Parvovirus B19 är orsaken till femte sjukan som hos barn ger feber och efter ca 10 dagar uppstår ett klassiskt rött utslag på kinderna och röda uslag på kroppen. Hos vuxna är det senare ovanligt och ledsmärtor är i stället ett vanligare symptom. Hos barn med cancer ser man sällan symtom från parvovirus i form av röda kinder och utslag. Dessa barn drabbas isället av blodkroppsbrist, där både de röda och vita blodkropparna men också blodplättarna påverkas. För att studera följderna av parvovirus infektion hos barn med cancer inkluderade vi under fem år barn på barncanceravdelningen som gjorde en benmärgsundersökning. Parvovirus förökar sig i benmärgen och detta är därför ett utärkt ställe att leta efter viruset. Vi hittade viruset i benmärgen hos 15 % av barnen med akut lymfatisk leukemi och hos 7 % av barnen med andra blodsjukdomar eller tumörer. Så som visat i tidigare studier så var vanliga symptom blodkroppsbrist vilket också är en vanlig biverkan till cellgifterna. Symptomen från parvovirus infektioner var därför vara svåra att skilja från de från cellgifterna.

Behandlingen för akut lymfatisk leukemi består av olika delar där den inledande behandlingen är tuff och syftar till att utplåna leukemicellerna. Sedan följer en mildare behandling vars syfte är att hålla leukemicellerna borta. Under denna behandling förväntar man sig inte att de friska blodkropparna ska bli lika påverkade som under den inledande. Detta är därför en lämplig behanlingsperiod att studera symtomen av parvovirus infektioner. Vi såg då att barnen med parvovirus hade låga antal blodkroppar som ledde till att man var tvungen att göra uppehåll i cellgiftsbehandlingen. Blodkroppsbristen var ibland så uttalad att transfusioner med blod och blodplättar var nödvändiga. För att se om de barn med parvovirus skilde sig från barn utan parvovirus så matchade vi en kontrollgrupp av barn med akut lymfatisk leukemi utan parovovirus med de där viruset hittades. Barnen med parovovirus hade i median 59 dagar med indragen behandling vilket ska jämföras med 30 dagar hos kontrollgruppen. Barnen med parvovirus behövde också signifikant fler transfusioner jämfört med kontrollgruppen. För att undvika återfall av leukemin är det viktigt att behandlingsintensiviteten upprätthålls. Hos bara ett av barnen så misstänkte läkarna att barnet hade parvovirusinfektion och eftersom våra prover analyserades i efterhand så gavs ingen behandling. Parvovirusinfektioner kan behandlas om de upptäcks och man

kan på det sättet undvika onödiga behandlingsuppehåll i cancerbehandlingen. Resultaten från den här studien ledde därför till en rekommendation att rutinmässig provta för parvovirus då barn med cancer uppvisar oväntad blodkroppsbrist.

En stor majoritet av förkylningar hos i övrigt friska barn beror på virusinfektioner. Mot virus hjälper ingen antibiotika och förkylningarna får därför läka av sig självt. Som beskrivet ovan så löper barn under cancerbehandling större risk att drabbas av fler och svårare infektioner och tvingas till sjukhusvård vid feber och samtidig brist på vita blodkroppar. Direkt när barnet kommer till sjukhus tas blod för bakterieodlingar och om det finns tecken på infektion någon annanstans, tex i huden, så tar man också prov därifrån. Så snabbt som möjligt så ger man också bred antibiotika som ska hjälpa mot en eventuell bakteriell infektion som kan vara livshotande hos dessa barn. Snabbt insatt behandling mot bakterier har räddat livet på många barn och dödligheten i infektionskomplikationer är idag låg. Tyvärr så är den liberala användningen av antibiotika inte bara av godo utan kan snabba på den redan fruktade utvecklingen av resistenta bakterier både hos individen och i samhället. Därför behöver läkarna verktyg för att på ett tidigt stadium skilja ut de barn med livshotande infektioner från de med mindre allvarliga. I cirka 15-30% av fallen blir odlingarna positiva. I resten av fallen så hittar man ingen bakterie och orsaken till febern förblir okänd. Eftersom en vanlig orsak till infektioner hos i övrigt friska barn är virus, spekulerade vi i om virus också är en vanlig orsak till feber hos barn med cancer. Rutinmässigt bedrivs inte mycket virusdiagnostik hos dessa barn. Vi bestämde oss därför för att under ett år provta barn med feber och låga antal vita blodkroppar för ett stort batteri av olika virus med stort fokus på de olika förskylninsvirus som vi vet finns i samhället. Studien utförde vi på barncanceravdelningen på Astrid Lindgrens barnsjukhus i Stockholm och på en barncanceravdelning i Sydney. Vi fann förskylningsvirus hos 46 % av barnen. När vi la ihop resultaten från de bakteriella analyserna med de virus vi hittade så kunde vi hitta antingen ett virus eller bakterie i 61 % av fallen. Det ska jämföras med 15-30% som tidigare är känt. Denna studie var ett första steg som visade att virus är vanligt förekommande hos barn med cancer. Fler studier behövs dock för att bekräfta våra fynd och bevisa att virusen som vi hittade faktiskt orsakade febern hos dessa barn.

I arbetena i den här avhandlingen kunde vi visa att virusinfektioner är vanliga hos barn med cancer och ger komplikationer som leder till att cancerbehandlingen måste skjutas upp. Dessa fynd uppmärksammar också behovet av nya diagnostiska verktyg för att detektera virus och att mediciner och vacciner mot virus utvecklas. Förhoppningsvis kommer också användadet av antibiotika att kunna minskas och på så sätt hjälpa till att bromsa resistensutvecklingen. Dessa förbättringar skulle i slutänden leda till färre dagar på sjukhus och ökad livskvalité för barnen och deras familjer.

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