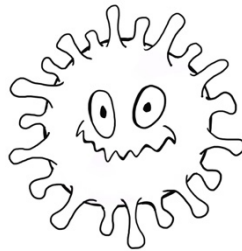


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ETIOLOGY AND RISK FACTORS OF FEBRILE NEUTROPENIA IN CHILDREN DURING CANCER TREATMENT

Martina Wahlund



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ETIOLOGY AND RISK FACTORS OF FEBRILE NEUTROPENIA IN CHILDREN DURING CANCER TREATMENT

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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”Den enda sanna visheten är att veta att du egentligen inte vet någonting”

– Sokrates

ABSTRACT

Side effects due to chemotherapy is still a major issue during cancer treatment. Febrile neutropenia and associated microbiological defined infections (MDIs) are dreaded complications, and still a cause of death during pediatric cancer treatment. In many of the febrile neutropenia episodes the cause of the fever is unknown, and risk factors of developing febrile neutropenia are poorly characterized. To be able to pin-point children at risk of febrile neutropenia and anticipate those at risk of more severe episodes could lead to more individualized treatment. Therefore, the aim of this thesis was two-fold: to investigate the microbiological causes of febrile neutropenia episodes and to investigate risk factors of developing this condition and associated MDIs.

In **papers I and IV**, causes of febrile neutropenia episodes were assessed. In **paper I**, we identified a respiratory virus in 45% of the episodes, which was in line with earlier reports. In addition, follow-up nasopharyngeal sampling showed that only rhinovirus and coronavirus were persistent and all other viruses cleared from the nasal cavity. This indicated that the respiratory virus identified was indeed the cause of the fever. However, causality could not be established. Therefore, **paper IV** investigated the innate immune response during these episodes. By using gene-expression profiling, the aim was to investigate specific innate signatures in blood. Unfortunately, due to the immunosuppression, there was insufficient RNA from ~30% of the samples and a specific innate signature, similar to that of immunocompetent children, could not be identified. Therefore, the feasibility of using gene-expression profiling to correlate the microbiological findings to an active infection and as a diagnostic tool in children treated for cancer remains challenging.

In **papers II and III**, risk factors of developing neutropenia and febrile neutropenia with associated MDI and low end doses of 6-Mercaptopurine (6-MP) during pediatric ALL treatment were addressed. In **paper II**, genetic variants in important enzymes involved in drug metabolism were investigated. Here, TPMT genetic variants were associated with a decreased risk of developing neutropenia and febrile neutropenia during the maintenance II period, and deficiency in ITPA (rs1127345) to a decreased risk of developing febrile neutropenia (unadjusted). In addition, genetic variants in NUDT15 were associated with decreased end doses of 6-MP. From the results from **paper II**, we could still not fully understand the role of TPMT and ITPA and the risk of febrile neutropenia. In addition, NUDT15 seem to play an important role for the 6-MP doses. However, due to the small samples sizes, our results need further investigation in larger cohorts. In **paper III**, the febrile neutropenia episodes were characterized and genetic variants in important innate immune proteins were investigated. Viral infections were the most common detected infection during febrile neutropenia episodes. However, in the majority of the episodes the cause of the fever remained unknown. TLR4 genetic variants increased the risk of viral infections and variants in the IL-1Ra gene decreased the risk of developing bacterial bloodstream infection. There were no association between MBL and the investigated genetic variants. Therefore, TLR4 and IL-1Ra seem to have a role during infectious episodes in children treated for cancer, however, the results needs to be confirmed in future studies.

In conclusion, respiratory virus are common during febrile neutropenia. However, additional more sensitive methods are needed to be able to identify and prove causality between the microbiological findings and the febrile neutropenia episode. Some of the investigated genetic variants seem to play a role in the risk of developing febrile neutropenia and infections. However, some of these need to be further evaluated before any modifications of the management of febrile neutropenia could be recommended.

LIST OF SCIENTIFIC PAPERS

- I. **Martina Söderman***, Samuel Rhedin, Thomas Tolfvenstam, Maria Rotzén-Östlund, Jan Albert, Kristina Broliden, Anna Lindblom**. **Frequent respiratory viral infections in children with febrile neutropenia – a prospective follow-up study.** PLoS One. 2016 Jun 16;11(6):e0157398

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- II. **Martina Wahlund**, Anna Nilsson, Anna Zimdahl Kahlin, Kristina Broliden, Ida Hed Myrberg, Malin Lindqvist Appell*, Anna Berggren*. **The role of TPMT, ITPA, and NUDT15 variants during mercaptopurine treatment of Swedish pediatric patients with acute lymphoblastic leukemia.** The Journal of Pediatrics. 2020 Jan;216:150-157.e1

*Shared senior authorship

- III. **Martina Wahlund**, Malin Lindqvist-Appell, Ida Hed-Myrberg, Anna Berggren, Anna Nilsson. **Genetic sequence variants in TLR4 increase the risk for viral associated febrile neutropenia in children with ALL.** *Submitted.*

- IV. **Martina Wahlund**, Indranil Sinha, Kristina Broliden, Shanie Saghafian-Hedengren, Anna Nilsson*, Anna Berggren*. **The feasibility of host transcriptome profiling as a diagnostic tool for microbial etiology in childhood cancer patients with febrile neutropenia.** International Journal of Molecular Sciences 2020, 21(15), 5305

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LIST OF ABBREVIATIONS

| | |
|--------|--|
| 6-MP | 6-mercaptopurin |
| ALL | Acute lymphoblastic leukemia |
| ANC | Absolute neutrophil count |
| B19V | Parvovirus B 19 |
| BM | Bone marrow |
| BSI | Blood stream infection |
| CMV | Cytomegalovirus |
| CRP | C-reactive protein |
| Ct | Cycle threshold |
| DNA | Deoxyribonucleic acid |
| EORTC | European Organization for Research and Treatment of Cancer |
| EV | Enterovirus |
| FOU | Fever of unknown origin |
| HAdV | Human adenovirus |
| HBoV | Human bocavirus |
| HCoV | Human coronavirus |
| HHV6 | Human herpes virus 6 |
| HMPV | Human metapneumovirus |
| HR | High risk |
| HSCT | Hematopoietic stem cell transplantation |
| IFD | Invasive fungal disease |
| IFN | Interferon |
| Ig | Immunoglobulin |
| IL | Interleukin |
| IL-1Ra | Interleukin 1 receptor antagonist |
| IPA | Ingenuity Pathway Analysis |
| IR | Intermediate risk |
| ITPA | Inosine triphosphate pyrophosphatase |
| LPS | Lipopolysaccharide |
| MBL | Mannose-binding lectin |
| MDI | Microbiological defined infections |
| meMP | Methylated mercaptopurine |
| meTIMP | Methylated thioinosine monophosphate |
| MRD | Minimal residual disease |
| mRNA | Messenger ribonucleic acid |
| MSG | Mycoses Study Group |

| | |
|--------|---|
| MTX | Methotrexate |
| NGS | Next-generation sequencing |
| NK | Natural killer |
| NLR | NOD-like receptor |
| NOD | Nucleotide-binding oligomerization domain |
| NOPHO | Nordic Society for Paediatric Hematology and Oncology |
| NUDT15 | Nudix hydrolase 15 |
| PCR | Polymerase chain reaction |
| PIV | Parainfluenza virus |
| PRR | Pattern recognition receptor |
| RLR | Retinoic acid-inducible gene-I-like receptor |
| RNA | Ribonucleic acid |
| RSV | Respiratory syncytial virus |
| RV | Rhinovirus |
| SARS | Severe Acute Respiratory Syndrome |
| SNP | Single nucleotide polymorphism |
| SR | Standard risk |
| TdGMP | Deoxy thioguanosine monophosphate |
| TdGTP | Deoxy thioguanosine triphosphate |
| TGDP | Thioguanosine diphosphate |
| TGMP | Thioguanosine monophosphate |
| TGN | Thioguanine nucleotides |
| TGTP | Thioguanosine triphosphate |
| TIMP | Thioinosine monophosphate |
| TITP | Thioinosine triphosphate |
| TLR | Toll-like receptor |
| TNF | Tumor necrosis factor |
| TPMT | Thiopurine methyltransferase |
| WBC | White blood cell |

1 BACKGROUND

1.1 PEDIATRIC CANCER

Cancer in children is relatively rare in Sweden, with approximately 350 cases annually (1, 2). Pediatric cancers span over diverse clinical and biological tumour entities, including leukaemia and lymphoma, bone and soft tissue sarcoma, central nervous system tumours, retinoblastoma, neuroblastoma, liver and kidney tumours, germ cell tumours and additional rare cancers. There is an emerging concept suggesting that epigenetic dysregulation is central to many forms of childhood cancer and that many pediatric tumours arise from stem- or progenitor cells during particular developmental time frames (3, 4). In recent decades, the survival rate has increased considerably and the 5-year survival is currently 80–85% (2, 5, 6). Leukemia, is the most common cancer diagnosed in children, accounting for ~30% of the cases, which mainly includes acute lymphoblastic leukemia (ALL) (~80%) and Acute myeloid leukemia (~20%), followed by brain tumors (30%) and lymphomas (10%) (6).

The cornerstones of pediatric cancer treatment are **chemotherapy**, radiation, and surgery while immunotherapy is an emerging treatment modality. The treatment regimens have different acute **side effects**, such as **neutropenia**, that need to be carefully monitored to reduce treatment-related mortality and morbidity. Because **ALL** is the most common diagnosis in children with cancer, most subjects enrolled in the studies included in this thesis were treated for ALL. The protocol for the treatment of ALL used in Sweden during the last decades is the **Nordic Society for Pediatric Hematology and Oncology (NOPHO) ALL treatment protocol**.

1.1.1 Chemotherapy treatment

In pediatric cancers, the malignant cells often exhibit a high proliferation rate and a susceptibility to chemotherapy (7). The initial aim of chemotherapy is to achieve remission and later eradication of the minimal residual disease (MRD). Chemotherapy can be given as a combination of two or more drugs and is administered sequentially to increase the probability of eradicating as many cancer cells as possible (7). The type of chemotherapeutic agent depends on the diagnosis, and currently, standardized treatment protocols are frequently used for the respective cancers in pediatric patients.

There are different classes of chemotherapeutic agents that, depending on the mechanism of action, interfere with cell division or cell death in cancer: (i) anti-metabolite compounds, which lead to interference with the synthesis of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) (*e.g.*, 6-mercaptopurin (6-MP)), (ii) anti-folates, which decrease the synthesis of precursor DNA (*e.g.* methotrexate (MTX)), (iii) anti-microtubular compounds, which disrupt cellular architecture, (iv) L-asparaginase, which depletes plasma and intracellular levels of the amino acid asparaginase, (v) alkylating agents, which alter DNA and its replication by binding alkyl groups, and (vi) anthracyclines which forms free radicals, lipid peroxidation and enzyme interactions (7, 8). Chemotherapy can be administered intravenously, intrathecally, per oral (*e.g.*, 6-MP), or intramuscularly.

1.1.2 Acute side effects of chemotherapy

Because chemotherapy is designed to target highly proliferating cells, it also causes cytotoxicity in non-malignant host cells, such as hair follicles, epithelial cells of the gut and hematopoietic cells. When the gastrointestinal mucosa is affected, inflammation and a disrupted epithelial barrier that increases the risk of infection may occur (9). However, one of the most common side effects is bone marrow (BM) suppression, resulting in anemia (low red blood cell count), thrombocytopenia (low platelets), and neutropenia (low levels of neutrophils). Importantly, chemotherapy affects the production of new blood cells and the maintenance of immune memory cells (B and T-cells), thus resulting in impaired protection against previously acquired infections (10, 11).

1.1.2.1 Neutropenia

Myelopoiesis describes the maturation of different blood cells derived from the hematopoietic stem cell, leading to the formation of red blood cells, platelets, mast cells, lymphocytes, dendritic cells, monocytes/macrophages, and granulocytes (Figure 1). Granulocytes can be grouped into three different cell types: basophils, eosinophils, and neutrophils (12) (Figure 1). Neutrophils are the most abundant white blood cell (WBC) in the blood, with a concentration of $\sim 2-8 \times 10^9/\text{L}$ and are one of the first cell types to respond to infections (12). Neutrophils recognize and ingest microorganisms for intracellular killing, a process called phagocytosis. In response to infection, additional immune cells (*e.g.*, T cells, macrophages, endothelial cells, and fibroblasts) secrete both interleukins (ILs) and granulocyte colony-stimulating factor that act on the BM niche to release mature neutrophils stored in the BM and stimulate the proliferation of granulocyte precursors cells (13-15). Therefore, the concentration of neutrophils in the blood can increase rapidly upon infection (12). However, neutrophils are short lived in the blood (6–9 hours), so it is essential that the BM continuously produce new neutrophils (12). Upon chemotherapy, both the storage and new production of neutrophils are suppressed. Neutropenia (an absolute neutrophil count (ANC) $\leq 0.5 \times 10^9/\text{L}$) is common during cancer treatment and is a major risk factor of infections. Neutropenia is also a common reason for delayed chemotherapy treatment (16, 17). The lowest neutrophil count usually occurs $\sim 7-10$ days after previous chemotherapy treatment. Therefore, careful monitoring of the child's WBC count is performed during intensive cancer treatment to detect neutropenia.

1.1.3 Acute lymphoblastic leukemia

In the vast majority of cases, the cause of the ALL is unknown although a few risk factors are associated to ALL, such as Down syndrome and radiation exposure (18-20). ALL arises from genetic changes that affect the precursor-stage of B or T lymphoid cells, which further block lymphoid differentiation and drives abnormal cell proliferation and monoclonal expansion of immature lymphoid cells, leading to a leukemic clone (Figure 1) (21). At diagnosis, the clonal expansion of leukemic cells gives rise to BM suppression, leading to pancytopenia. The typical clinical presentation of ALL includes pallor, fatigue, fever, bruises/bleeding, enlarged liver and/or spleen, swollen lymph nodes, and skeletal pain (7).

Childhood ALL includes a number of subtypes defined by cell lineage (B- or T- cell), differentiation status and genetic alterations (22). These biological characteristics differ by age distribution and

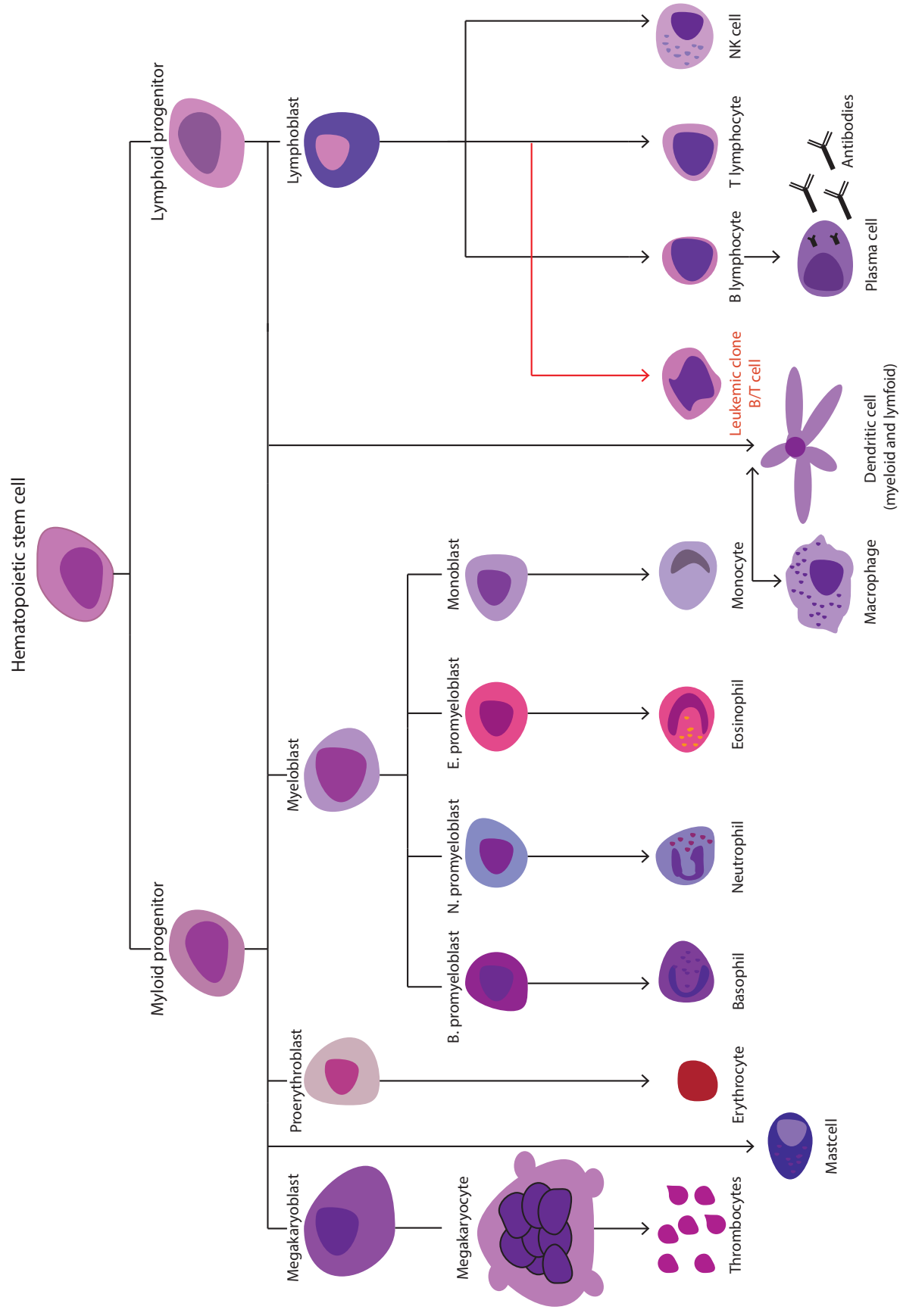


Figure 1: Hematopoiesis and the development of a leukemic clone from B and T cell lymphoid precursors.

clinical outcome, and are therefore used for tailoring therapy to the predicted risk of relapse. Pre-B ALL is the most common phenotype, accounting for ~85–90% of the ALL diagnoses in children (23). Currently, phenotype, genetic aberrations and response to therapy is used for ALL risk stratification and further treatment (21). The treatment of ALL includes steroids, chemotherapy, and in a few children hematopoietic stem cell transplantation (HSCT) may be an option. The standard treatment in Sweden has been provided through collaboration with the Nordic countries and the establishment of NOPHO ALL treatment protocols (23, 24).

1.1.3.1 NOPHO ALL treatment protocol

The NOPHO ALL 2000 and 2008 protocols were used between 2000–2008 and 2008–2019, respectively. In 2019, a new protocol was implemented through a European collaboration called “ALLTOGETHER” and is currently used in the Nordic countries. In the following section, the NOPHO ALL 2008 protocol and the differences between the 2000 and 2008 protocols will be discussed. In these treatment protocols, chemotherapy dosing was based on body surface area to ensure optimal dosing.

Within the NOPHO 2008 protocol, ALL treatment was divided into the following phases: induction, consolidation, delayed intensification, maintenance, and “block treatment” (only for children in the high-risk (HR) group) (Figure 2). At diagnosis, patients were stratified to either HR induction if T-ALL and/or $WBC > 100 \times 10^9/L$ or low-risk induction if pre-B ALL and $WBC < 100 \times 10^9/L$. After a new BM sample day 29, additional stratification was performed into risk groups based on (i) response to treatment (BM MRD), (ii) leukemic phenotype (pre-B ALL or T cell ALL), (iii) karyotype of the leukemic clone, and (iv) presence of leukemic cells in the spinal fluid. The NOPHO ALL-2008 protocol contains three different risk groups: standard risk (SR), intermediate risk (IR), and HR. Additionally, children with poor response could be allocated to HSCT according to specific criteria. Lastly, a third stratification was performed after measuring MRD in BM at day 79. Particularly during the first 36 weeks of treatment, the HR group differed substantially from that of the SR and IR groups (Figure 2). The entire treatment protocol proceeded over a 2.5-year period.

There were some differences between the NOPHO ALL 2000 and 2008 protocols: (i) children treated within the HR group per the 2008 protocol received more intensive treatment than in the 2000 protocol and were treated for 2.5 years instead of two years, (ii) there were in general slightly higher doses of chemotherapy used per the 2008 as compared to the 2000 protocol. In the 2008 protocol, a randomized study involving the doses of 6-MP was performed, and therefore, some children treated according to the 2008 protocol may have received higher 6-MP doses (Figure 2), (iii), during the maintenance phase, 6-MP/MTX doses were adjusted to a target WBC to $1.5\text{--}3.5 \times 10^9/L$ and $1.5\text{--}3.0 \times 10^9/L$ (2000 and 2008 protocol, respectively).

1.2 INFECTIONS IN CHILDREN

Infections are a common cause of morbidity and mortality in children (25). Infections can be of **viral**, **bacterial**, **fungal**, or parasitic origin and often result in symptoms such as fever, upper respiratory tract symptoms, rash, and fatigue. Respiratory viral infections are one of the most common causes of disease in younger children, causing mild symptoms to severe or even lethal conditions in children, especially those <1 year of age. Several different **laboratory methods** for microbiological agent detection, all with different sensitivity and specificity, can be used.

1.2.1 Viral infections

Viruses are intracellular infectious agents dependent on the host cell's machinery to infect and spread. A virus consists of a small genome, either DNA or RNA, packed in a capsule of proteins called a capsid (12). For some viruses, this capsid is covered by a lipid layer membrane, and the virus is then called an enveloped virus. In general, an enveloped virus is more sensitive to external factors such as temperature, alcohol, pH, and dehydration than a non-enveloped virus. Viral infection can either be acute and cleared by the immune system (*e.g.*, respiratory viruses, gastrointestinal viruses, measles) or incorporated in the host cell leading to persistent or latent infection (*e.g.*, human immunodeficiency

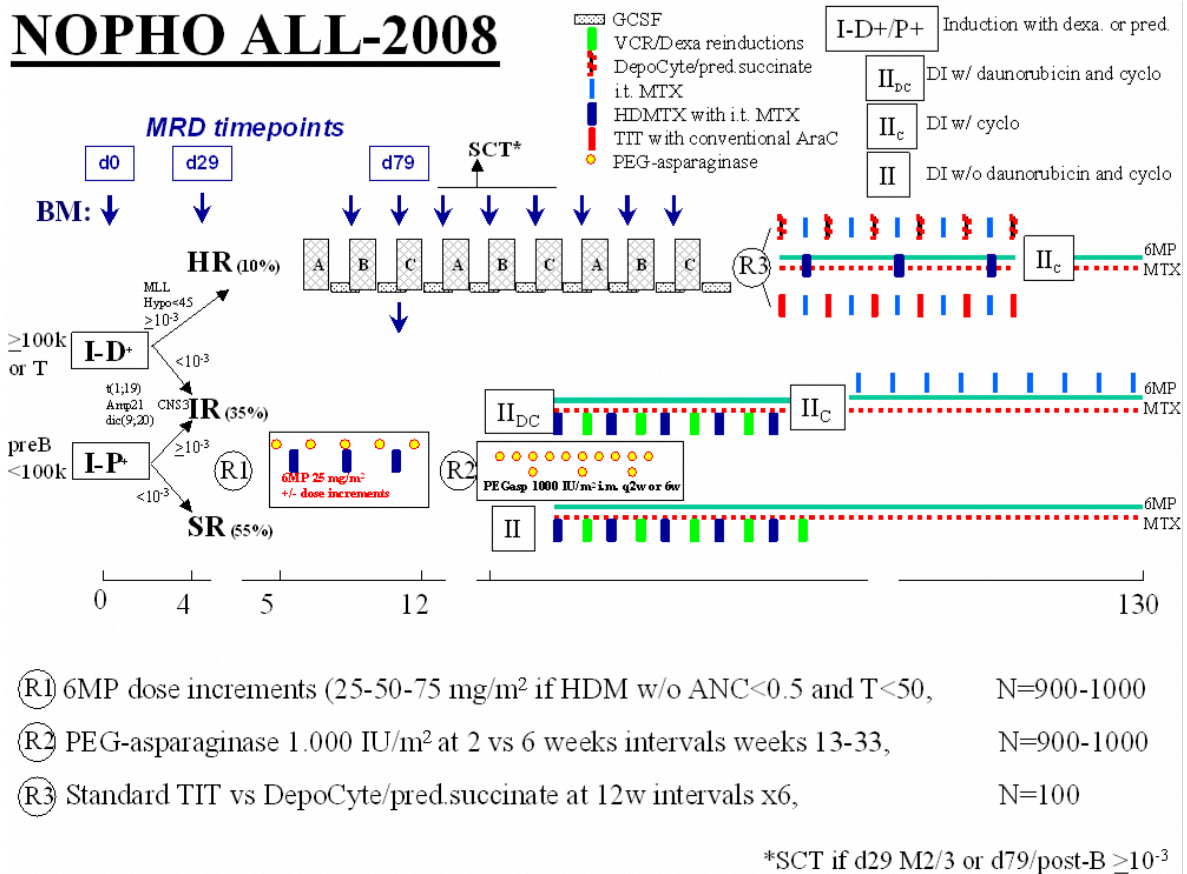


Figure 2: The NOPHO ALL-2008 protocol (reprinted with permission)

Abbreviations: ALL, acute lymphoblastic leukemia; ANC, absolute neutrophil count; BM, bone marrow; CNS, central nervous system; d, day; HDM, high-dose methotrexate; HR, high risk; I, induction; IR, intermediate risk; 6-MP, 6-mercaptopurine; MRD, minimal residual disease; MTX, methotrexate; NOPHO, Nordic Society for Paediatric Hematology and Oncology; PEG, Polyethylene glycol conjugated; R, Randomization; SCT, stem cell transplantation; SR, standard risk; T, Thrombocytes; TIT, triple intrathecal therapy; VCR, Vincristine.

virus (HIV), herpes virus, hepatitis B or C). Only a few drugs have been developed for the treatment of viral infections. The main difficulties in developing antiviral drugs arise because viral replication usually occurs before symptom onset, and therefore, the use of antivirals must be administered before or at least within a few days after symptom debut. Viral infections can range from both mild and transient infections to severe and persistent infection depending on both host factors (*e.g.*, susceptibility) and virus type (12). Respiratory viral infections are one of the most common causes of morbidity in the population, especially in children, and these viruses are a main focus in this thesis (26).

1.2.1.1 Respiratory viral infections

Respiratory viruses can cause both upper and lower respiratory tract infections with symptoms such as cough, nasal congestion, runny nose, sore throat, muscle ache, fatigue, and fever (Table 1). The infections can vary from mild with minor symptoms to severe and even fatal with excessive symptoms, especially in younger children (25). To date, by using polymerase chain reaction (PCR), 15 of the most common respiratory viruses are part of the standard analyses performed in many of the Swedish microbiological laboratories: adenovirus (HAdV), bocavirus (HBoV), coronaviruses (HCoV) NL63/OC43/229E/HKU1, enterovirus (EV), influenza virus A and B, metapneumovirus (HMPV), parainfluenza viruses (PIV) 1-3, respiratory syncytial virus (RSV), and rhinovirus (RV) (27) (Table 1). In addition, the newly emerging HCoV, Severe Acute Respiratory Syndrome (SARS) CoV-2 is also analyzed using PCR in the Swedish laboratories. RV is the most frequently detected virus in immunocompetent children with acute upper respiratory illness, followed by HBoV and PIV (28-30). The most common respiratory findings identified in children with lower respiratory tract illness is RSV, RV influenza virus, and HMPV (31, 32). However, the clinical interpretation of PCR positivity for certain respiratory viruses is still challenging, as some of the respiratory viruses have been reported to be found frequently in asymptomatic subjects and persist weeks after the acute infection. In asymptomatic children, RV is the most frequently detected virus, with a detection rate of ~30%, followed by HCoV, HBoV, and HAdV at ~6–13% (28, 29, 31-34), in contrast to some respiratory viruses that are rarely detected in asymptomatic children, such as influenza, RSV, and HMPV (29, 31, 32) (Table 1). Due to the vast number of different subtypes of respiratory viruses (*i.e.*, RV is abundant in over 100 subtypes) and frequently developed mutations in viral genomes, individuals can be repeatedly affected by respiratory viral infections.

1.2.2 Bacterial infections

Bacteria are prokaryotic microorganisms and are larger both in size and genome compared to viruses (12). Most bacteria consist of a circular chromosome with double-stranded DNA packed into a nucleoid. Bacteria can also carry extrachromosomal DNA, called plasmids, which contain genes important for virulence factors or antibiotic resistance. Almost all bacteria have a cell wall consisting of a peptidoglycan layer and one or two phospholipid membranes. Gram staining is a technique used to distinguish and broadly classify bacteria based on the structure of the cell wall as either Gram-positive (thick peptidoglycan layer, no outer membrane) or Gram-negative (thin peptidoglycan layer and an outer membrane). Specific structures are abundant in the membrane of the bacteria, such as lipopolysaccharide (LPS) (Gram-negative bacteria) and teichoic acid (Gram-positive bacteria).

Table 1: Summary of the most common respiratory viruses.

| Respiratory virus | Type of virus | Subtypes | Incubation | Seasonal peak | Respiratory disease | Documented shedding time* | Asymptomatic detection |
|----------------------------|-----------------------------|--|------------|---------------------------|--|---------------------------|------------------------|
| Adenovirus | Double-stranded DNA virus | Group A-G (>50 serotypes) | ~ 6 days | No seasonal differences | URTI Conjunctivitis Pneumonia | 2.5 weeks – months | Yes |
| Bocavirus | Single-stranded DNA virus | 1-4 | Unknown | Winter | URTI Bronchiolitis (pneumonia) | 10 weeks | Yes |
| Coronavirus | Positive-stranded RNA virus | NL63, OC43, 229E, HKU1, MERS, SARS-CoV-1 and 2 | 2-4 days | Winter | URTI Pneumonia | 3 weeks – months | Yes |
| Enterovirus | Positive-stranded RNA virus | Group A-D (>60 serotypes) | 7-14 days | Summer/Autumn | URTI Conjunctivitis | 2-3 weeks | Yes |
| Influenza virus | Negative-stranded RNA virus | A-C | 1-4 days | Winter | URTI Bronchiolitis Pneumonia | 1-2 weeks | Rarely |
| Metapneumovirus | Negative-stranded RNA virus | A-B | 3-6 days | Winter | URTI Bronchiolitis Pneumonia | 2 weeks | Rarely |
| Parainfluenza virus | Negative-stranded RNA virus | 1-4 | 1-7 days | Spring/autumn | URTI Pseudo croup Bronchiolitis Pneumonia | 3 weeks | Rarely |
| Rhinovirus | Positive-stranded RNA virus | A-C (>100 serotypes) | 1-3 days | Late spring /Early autumn | URTI Bronchiolitis (pneumonia) | 5 weeks – months | Yes |
| RSV | Negative-stranded RNA virus | A-B | 3-5 days | Winter | URTI Bronchiolitis Pneumonia | 3-6 weeks | Rarely |

*Few studies have reported the shedding times for different respiratory virus, therefore the specific shedding times may differ and are not exact numbers.

Abbreviations: CoV, Coronavirus; MERS, Middle East Respiratory Syndrome; RSV, Respiratory syncytial virus; SARS, Severe acute respiratory syndrome; URTI, Upper respiratory tract infection.

Bacteria can display many different shapes, of which the most common are cocci (round), bacilli (rod-shaped), and spirilla (spiral). Bacterial infections are usually rapidly cleared by the immune system, but can sometimes lead to host colonization (12). In contrast to antivirals, antibiotic treatment for bacteria is widely used, and many different drugs have been developed targeting specific bacteria. However, an increase in detected resistant bacteria (*e.g.*, methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* (VRE)) has become an issue due to extensive antibiotic use during the past decades; therefore antibiotic resistance is increasingly problematic (35, 36). Bacteria can cause mild and local infections, as well as severe bacterial bloodstream infections (BSIs) that in some cases can be lethal.

1.2.2.1 Bacterial bloodstream infections

Bacteria can enter the blood in several different ways (*e.g.*, through the respiratory or gastrointestinal tract, wounded skin, or through catheters). Presence of bacteria in the bloodstream, referred to as bacteremia, is a serious condition that may induce a massive inflammatory response. If the bacteremia leads to a systemic impact and organ dysfunction, a severe condition called sepsis has developed. Septic shock usually refers to a condition in which the patient with sepsis is not able to sustain an adequate blood pressure despite sufficient fluid supply, and this condition has a high mortality rate in pediatric patients (37). Many different types of bacteria, both Gram-positive and Gram-negative, can cause bacterial BSI. The most prevalent bacteria detected in blood cultures in immunocompetent children are *S. aureus* and *E. coli* (38).

1.2.3 Overview of fungal infections

Fungi exist all around us, from the ground to the air. They are microscopic spores and eukaryotic microbes with a cell wall. Most of them are harmless, although specific species can cause severe diseases in humans, particularly in immunosuppressed individuals. There are many types of different fungi, such as different *Aspergillus* species, dermatophytes, *Candida* species, *Histoplasma*, and *Pneumocystis* (12). Fungi spread through inhalation, orally, or through the skin. Fungi can colonize multiple sites such as the mouth, skin, and gastrointestinal tract, as well as cause invasive fungal disease (IFD) (39, 40).

1.2.4 Laboratory methods for pathogen detection

1.2.4.1 Methods for detecting viral infections

Just one decade ago, the standard methods for detection of viral infections were antigen detection by immunofluorescence staining and viral culture (27). However, reagents (antibodies) for immunofluorescence staining were not available for all viruses, manual handling of the viral cultures was time-consuming, and the results were usually obtained after the acute infection had resolved. Today, these methods have almost entirely been replaced by molecular-based methods such as PCR, a widely used method for detecting many viral infections (*e.g.*, respiratory, blood, and gastrointestinal). Thanks to its rapid response and high sensitivity, PCR is an important contribution in the microbiologic diagnostic work-up in clinical practice (12, 27, 41). As the method is based on amplification of nucleic acids, it can detect even small amounts of specific RNA or DNA fragments

(41). The most commonly used PCR technique today is the real-time PCR, a quantitative method with the ability to indirectly measure the amount of a specific microorganism in a given sample (12). In the real-time PCR analysis, a cycle threshold (Ct) value is calculated, and the lower the Ct value, the higher the viral load is in the original sample (41). One major limitation that remains with this method is the inability to differentiate viable pathogens from remnants of nucleic acid fragments from pathogens (27, 41). Therefore, due to viral remnants or asymptomatic infections, PCR findings can sometimes be difficult to interpret in a clinical setting. In addition, PCR can only identify pre-specified sequences of pathogens and thus it is sensitive to mutations in the primer or probe binding regions of the pathogens. Therefore, viral culture can still have an important role in detecting novel viruses that are not discovered with PCR. Another method for detection of viral infection is serology, a method based on detecting antibodies as a response to a pathogen, such as through enzyme-linked immunosorbent assays (ELISAs) (12). Levels of acute antibodies, such as immunoglobulin (Ig) M, rise and can be detected in serum a few days from infection onset, and long-term antibodies called IgG rise after a few weeks. The slow response hampers result interpretation in the acute phase of infection; therefore, serology is commonly used to determine immunity. Newer methods for detection, such as next-generation sequencing (NGS) (*e.g.*, RNA and DNA sequencing) have not yet been implemented in the clinical diagnostics of viral infection and are currently mainly used for research purposes.

1.2.4.2 Methods for detecting bacterial infections

Culturing (blood or local) is an older but still commonly used method in microbiology, mainly used for bacteria and fungi. By using cultures, antimicrobial resistance can be determined, leading to more individualized antibiotic treatment. Cultures can be collected either from a normally sterile compartment, such as blood or cerebrospinal fluid, or from compartments that might be colonized with bacteria. If a sample is taken from a possibly colonized compartment, it is important to separate those bacteria included in the normal flora from pathogenic bacteria. Culturing has several limitations. First, it usually takes 12–24 hours for the bacteria to grow (sometimes even longer). Second, some bacteria grow poorly or not at all in culture medium and therefore the ability to select therapy could be limited (42). Importantly, it is known that blood culture sensitivity is especially low in pediatric patients, often due to inadequate blood volume (43, 44). Other methods for bacteria detection are PCR (atypical bacteria, *e.g.*, mycoplasma and chlamydia) and serology (atypical bacteria). A few NGS methods, such as the 16 S sequencing method is used in the clinic. The method can detect bacteria at species levels and is of importance for bacteria that are not identified through culture (*e.g.* slow growing or uncultivable bacteria), bacterial infections at sterile compartments or to detect rare bacteria (45). However, many of the NGS methods are still used mainly for research purposes.

1.2.4.3 Methods for detecting fungal infections

Fungal infections can be diagnosed through culturing of blood or local compartments, microscopy, PCR, and in some cases, through radiology (*e.g.*, computed tomography scan) (46, 47). However, the diagnostic tools for fungal infections are still limited, as both the molecular diagnostic methods and cultures have limitations, such as low sensitivity (48-50).

1.3 INFECTIONS ASSOCIATED WITH FEBRILE NEUTROPENIA IN CHILDREN TREATED FOR CANCER

Due to the side effects of chemotherapy, mainly neutropenia and disrupted epithelial barriers, children treated for cancer are at increased risk of infections (51). Sometimes fever is the only symptom of a severe infection. Fever coinciding with neutropenia is a condition called **febrile neutropenia**. The most common reason of febrile neutropenia is thought to be infection. **Viral infections**, especially **respiratory viral** infections, are the most commonly detected infections during episodes of febrile neutropenia (52). **Bacterial BSIs** and **IFDs** are the most common causes of infection-related deaths in children with cancer (53-55). Infection-related deaths account for approximately 1–3% of the deaths during cancer treatment, and the relatively low number is probably due to more effective management of these patients (54-56). On the other hand, of all the deaths during cancer treatment, infections can account for as much as 70% (54). In addition, morbidity due to infections is still high with infection-related complications, delayed chemotherapy treatments, and hospitalization as consequences (57).

1.3.1 Febrile neutropenia

Febrile neutropenia is defined as a neutrophil count $\leq 0.5 \times 10^9/\text{L}$ on one occasion or $\leq 1.0 \times 10^9/\text{L}$ with a decrease to $\leq 0.5 \times 10^9/\text{L}$ over a subsequent 48-hour period together with fever, defined as a temperature $\geq 38.0^\circ\text{C}$, on two occasions 60 minutes apart or $\geq 38.5^\circ\text{C}$ on one occasion (58). Febrile neutropenia is a dreaded complication during chemotherapy treatment because an untreated infection, mainly bacterial or fungal, can worsen rapidly and progress to fatal sepsis. Therefore, when children treated for cancer contract a fever, they need immediate medical care. At admission, a thorough clinical examination should be performed, laboratory samples taken, and broad-spectrum antibiotics started within one hour from entering the emergency room (59). To distinguish between children with low or high risk of severe infection several predictive rules have been proposed. However, none of these rules has yet been fully validated (60-62). Further, not all episodes of febrile neutropenia are infection-related; the fever can also be caused by the cancer itself, transfusion of blood products, or treatment related (chemotherapy drugs). In many cases of febrile neutropenia, no underlying cause of the fever can be identified, and the fever is defined as fever of unknown origin (FOU) (63). Infection-related causes have been the main focus during this thesis and will therefore be discussed further. Febrile neutropenia episodes are more abundant at the beginning of the treatment (*e.g.*, induction and consolidation phases during ALL treatment) when the intensity of the chemotherapy treatment is highest and the children are not yet in remission of the disease (55, 56, 64). Children with febrile neutropenia are usually treated with broad-spectrum antibiotics for a median of 4–10 days and admitted to the hospital for a median of 4–8 days (52, 65-69). The long hospitalization has negative social effects for the child and their family, and the use of broad-spectrum antibiotics increases the risk of subsequent infection with antibiotic-resistant bacteria and fungi (70-72).

1.3.2 Viral infections during episodes of febrile neutropenia

Only during the last few years have viral infections been recognized when investigating febrile causes in children with cancer. In HSCT children, viral infections are associated with a high morbidity and mortality, whereas viruses are rarely reported as cause of infection-related deaths in children treated for cancer (54, 73-75). However, respiratory viral infections are still often detected during episodes of

febrile neutropenia in children treated for cancer and thus are suspected to cause high morbidity in this patient cohort. Other viruses, such as gastrointestinal viruses, different herpesviruses, and parvovirus B19 (B19V), are also detected.

1.3.2.1 Respiratory viral infections during episodes of febrile neutropenia

The role of respiratory viral infections in children with cancer has been subject to debate. One decade ago, viral infections were rarely reported during episodes of febrile neutropenia (63, 76). However, over the last few years, this has shifted, not only due to the introduction of PCR for detection of respiratory viruses, but also due to more frequent testing for respiratory viruses during episodes of febrile neutropenia. Currently, respiratory viruses are reported in 44–52% of all febrile neutropenia episodes (52, 65, 66), and the number is even higher (>75%) in patients with acute respiratory symptoms at the time of the febrile neutropenia episode (67). RV is the most frequently detected virus, followed by RSV and HCoV (52, 65–67, 77). Respiratory viruses are detected throughout the entire treatment period, notably even during the maintenance phase of ALL treatment (56). Respiratory viruses have in a few cases been reported as causes of death during pediatric cancer treatment, mainly RSV, influenza, HAdV, and one of the latest reports with HCoV OC43 (54, 78). In addition, secondary bacterial infection after viral infection have been reported with a few fatal cases (67). Nevertheless, in the majority of episodes with a positive respiratory viral finding, symptoms are mild and the outcomes often favorable (52, 65, 77). The interpretation of respiratory viruses, especially of RV, in terms of clinical significance remains challenging because these viruses have been detected in immunocompetent asymptomatic children and in children weeks after an infection (28, 29, 33, 34, 79) (Table 1, p. 7), and a simultaneous bacterial infection is difficult to rule out.

1.3.2.2 Other viral infections during episodes of febrile neutropenia

Gastrointestinal viruses (*e.g.*, norovirus, HAdV, and rotavirus) are also commonly detected viruses in children with cancer (56, 80). Some of these viruses have been detected even months after the acute infection, which hampers the interpretation of these viral findings in the clinic (81, 82). Herpesviruses, such as cytomegalovirus (CMV), Epstein-Barr virus (EBV), human herpes virus 6 (HHV6), and varicella, are relatively rare during episodes of febrile neutropenia in children treated for cancer, reported in ~1–10 % of the episodes, where HHV6 and CMV are more commonly and rarely detected, respectively (52, 76, 83). These viruses can either cause primary infection or be reactivated in immunosuppressed children, which in both cases can cause severe disease (84). B19V is another virus that can be detected during cancer treatment, detected in ~2% of febrile neutropenia episodes and ~15% during the entire course of ALL treatment (52, 85). B19V can be a cause of cytopenia during cancer treatment, leading to decreased dose intensity and complications during treatment (85).

1.3.3 Bacterial infections during episodes of febrile neutropenia

Bacterial infections in children treated for cancer have been well investigated, mainly due to the fact that bacterial BSI is a dreaded complication during cancer treatment, as an untreated infection can rapidly lead to sepsis development in this patient cohort. Local bacterial infections that are commonly reported during cancer treatment in children are *E. coli* urinary tract infection, *Clostridium difficile* gastrointestinal infection, and skin infections caused by *Streptococcus* species and *S. aureus* (57).

1.3.3.1 Bacterial bloodstream infections during episodes of febrile neutropenia

Bacterial BSIs have been a main focus area within the research field of febrile neutropenia (86, 87). However, although a bacterial agent is only identified in approximately 10–30% of febrile neutropenia episodes, almost 100% are treated with broad-spectrum antibiotics for several days (52, 63, 65, 76, 87, 88). The most common bacteria detected in blood during cancer treatment in children are Gram-positive bacteria (coagulase-negative staphylococci, viridans streptococci, and *S. aureus*) (56, 63, 76, 87, 88). Gram-negative bacteria are also detected, such as *E. coli* and *Pseudomonas aeruginosa*, but these are less common (56, 63, 76, 87, 88). However, because blood cultures have a limited sensitivity to detect bacterial BSIs in children, it is difficult to exclude a pathogen simply by a negative blood culture; other plausible methods for bacterial pathogen detection are thus needed (43, 87, 89).

1.3.4 Fungal infections during episodes of febrile neutropenia

Fungal infections mainly affect immunosuppressed patients. It is common with colonization of fungi during cancer treatment, not causing any active disease (90). However, if the fungi is spread into the bloodstream it can lead to IFD, causing severe disease in immunosuppressed patients (86).

1.3.4.1 Invasive fungal disease during episodes of febrile neutropenia

IFD can lead to disseminated disease, a severe condition in which multiple micro-abscesses are formed in otherwise sterile compartments in the body, such as the lungs, liver, spleen, brain, and kidneys (91). Diagnosis of IFD is complicated and hampered by the scattered clinical picture of fungal disease, low sensitivity of blood cultures, and difficulty in distinguishing fungal colonization from invasive disease (39, 92). Therefore, specific criteria for the diagnostics of invasive fungal infection have been developed by the European Organization for Research and Treatment of Cancer (EORTC) and National Institute of Allergy and Infectious Diseases Mycoses Study Group (MSG) (93). Through the EORTC/MSG criteria involving microbiological testing, risk factors, and radiology, fungal infections are categorized as “proven,” “probable,” and “possible” (93). The most commonly identified invasive fungal infections in children with cancer are *Candida*, *Aspergillus*, and *Pneumocystis jiroveci* (39, 94, 95). IFD is a prominent cause of infection-related deaths in pediatric patients treated for cancer (53–56, 86). Therefore, the start of antifungal treatment is crucial and should (if not already prophylactically given) be given to children treated for cancer on wide indications where continuation of fever despite antibiotic treatment and IFD is suspected (*i.e.*, should not be delayed by insufficient diagnostics) (59).

1.4 SUSCEPTIBILITY TO NEUTROPENIA AND FEBRILE NEUTROPENIA IN CHILDREN TREATED FOR ALL

There is considerable variety in the frequency and severity of side effects, such as neutropenia and febrile neutropenia, in children treated for cancer, implying that specific **genetic variants** might affect susceptibility to these events. Pharmacogenetics refers to the study of the individual variability in drug response due to genetic variations or heredity (96). Therefore, **pharmacogenes** have been suggested to impact the **susceptibility to neutropenia and febrile neutropenia** during pediatric cancer treatment. Moreover, proteins important in the **immune system** have also been suggested to influence the **susceptibility to episodes of febrile neutropenia and microbiological defined infections (MDI) during these episodes**. Considering that these events increase morbidity and delay further treatment, there is a need to identify risk factors for developing these conditions (63).

1.4.1 Genetic variants

In the next section, different genetic variants, also called sequence variants, in specific genes will be discussed. To facilitate further reading, adequate basic genetic principles and terminology are introduced in this section. In general, the term “polymorphism” refers to a sequence variant in the DNA chain that occurs in >1 % of the population, and the term “mutation” if it occurs in <1%. The terms “genetic variant” or “sequence variant” is used in this thesis to describe both polymorphisms and mutations (97).

The **genome** refers to an individual’s complete genetic material, and the human genome is stored in 23 pairs of **chromosomes**. A chromosome is a long chain of **DNA**, which consists of two strands of nucleotides bases (A, T, C, and G) (98, 99). The coding regions of a DNA chain are called **exons** and non-coding regions **introns**. Through a process called **transcription**, the DNA functions as a template to yield complementary **RNA**. The introns of the RNA chain are then spliced, forming messenger RNA (**mRNA**). The mRNA codes for amino acids, and these are formed together in a specific order to produce a **protein** in a process called **translation**. The **transcriptome** is the term for RNA expressed by an organism or refers to the RNA transcripts produced by a particular cell. The transcriptome is actively changing, depending on which genes that are activated at a given point in time; hence, it can be considered a more precise measure of the cell’s current activity compared to the measurement of proteins, called **proteomics**. However, not all RNA transcripts are translated to proteins, and therefore, both the transcriptomics and proteomics are needed to better understand the functionality.

A **gene** is a sequence of DNA that can encode a protein and thereby determines a specific trait. The human genome is estimated to encode ~20,000–25,000 genes (100). Each gene has a specific location on a chromosome called a **locus** and exists in two copies, one inherited from each parent. These two copies are not necessarily the same, and there may be small differences in the genes. The different copies are called **alleles**. An allele is a specific form of the gene, located at the same site on the two paired chromosomes, one inherited from each parent. Together, the two alleles are called a **genotype**. A genotype can either be **heterozygous** or **homozygous**, meaning it either consists of two different alleles or two of the same alleles, respectively. Further, the homozygous alleles can be either “**wild type**”, meaning that it consists of the two most commonly occurring alleles in a population, or “**mutant/polymorphic**”, meaning it consists of the two less occurring alleles. An allele is responsible for variations in which a given trait, *i.e.*, **phenotype**, can be expressed. In addition, alleles can be dominant, act in a co-dominant fashion, or be recessive (97).

Several different genetic variants exist, with the most common being the single nucleotide polymorphisms (**SNPs**), meaning a substitution of a single nucleotide at a specific position in the genome that constitutes a **sequence variant** in the DNA chain (97, 101). Therefore, the different sequence variants give rise to differences in the alleles. SNPs are common in the human genome, and depending on the location of the SNP, it can either have dramatic consequences for the protein or not affect the protein at all. Within a cohort, some sequence variants are more common than others and therefore are called the “**major allele**,” whereas the less common variant is called the “**minor allele**” (97). The **allele frequency** is the relative frequency of an allele at a specific position in a defined population, expressed as either a fraction or percentage (97). The allele frequency is not the same as genotype frequency. However, it can be calculated if the genotype frequency is known. SNPs are identified in different frequencies in populations, and therefore, the “major allele” in one population may be the “minor allele” in another population. Other genetic variants can be **tandem repeat polymorphisms**. Tandem repeats occur in DNA when nucleotides are repeated directly adjacent to each other in a specific number of base pairs. Tandem repeats can then be abundant in variable copies of the repeats (*e.g.*, two or three copies) and therefore affect the phenotype.

Genotyping is a method for SNP detection. Several different genotyping methods can detect novel/unknown SNPs, as well as methods whereby already known SNPs can be detected. Genome or exome sequencing can detect novel/unknown SNPs throughout the entire genome, whereas methods such as pyrosequencing or PCR-based methods can detect specific and already known SNPs. Gel electrophoresis can be used for detecting tandem repeats (101, 102). Some of these specific methods will be described in more detail in the method section.

1.4.2 Sequence variants in important pharmacogenes

Common side effects of chemotherapy (as described above) are neutropenia and febrile neutropenia. Despite identical treatment protocols for ALL, some patients suffer from more frequent episodes of neutropenia and febrile neutropenia, suggesting a genetic predisposition in genes crucial for drug metabolism. Thiopurines, mainly **6-MP**, are drugs commonly used for the treatment of ALL, and a common side effect is cytotoxicity of the host’s blood cells. Thiopurine methyltransferase (**TPMT**) is an enzyme with a documented ability to affect thiopurine metabolism, and sequence variants in this gene have been identified to increase the risk of side effects during ALL treatment (103-105). Dose reduction for TPMT-deficient children have already been implemented in the NOPHO ALL 2000 and 2008 treatment protocols. However, despite these implementations, some children still experience various numbers and degrees of side effects, and other enzymes in thiopurine metabolism, such as inosine triphosphate pyrophosphatase (**ITPA**) and Nudix hydrolase 15 (**NUDT15**), have also been suggested to affect the cytotoxic effect of thiopurine through altered drug metabolism.

1.4.2.1 6-MP

The thiopurine drugs, such as 6-thioguanine, azathioprine, and 6-MP, are purine analogs that can be incorporated in RNA and DNA as “fake” monomers. The main thiopurine used during ALL treatment is 6-MP, administered almost every day of the 2.5 years of treatment and is therefore discussed in more detail in this thesis. 6-MP was discovered in the 1950s and was almost immediately introduced as a treatment for ALL (106). However, thiopurines are also incorporated in normal cells (mainly rapidly dividing cells such as blood cells), leading to side effects. Furthermore, 6-MP is a pro-drug

that needs to be metabolized to exert cytotoxicity. The metabolism of 6-MP is a complex procedure that depends on several different enzymatic reactions (Figure 3) (107-112). The final active products produced are: (i) deoxy thioguanosine triphosphate (TdGTP) and thioguanosine triphosphate (TGTP), which can be incorporated in DNA or RNA, respectively, as purine antagonists to interfere with replication and induce apoptosis of the cell, (ii) TGTP that binds to RAC1, which induces apoptosis of activated T cells, and (iii) formation of methyl-thioinosine monophosphate (meTIMP), which inhibits the *de novo* synthesis of purine nucleotides (110, 112-115). Tioguanine nucleotides (TGNs) generically describes all the thiopurine drug metabolites (TGMPs, TGDPs, TGTP, and the deoxy variants). Enzymes convert 6-MP to its active product or can degrade 6-MP to a non-active metabolite, such as TPMT, NUDT15, and ITPA (106, 107, 110, 112, 116, 117). Hence, these enzymes may therefore regulate the cytotoxic effect of 6-MP (105, 107, 118-129).

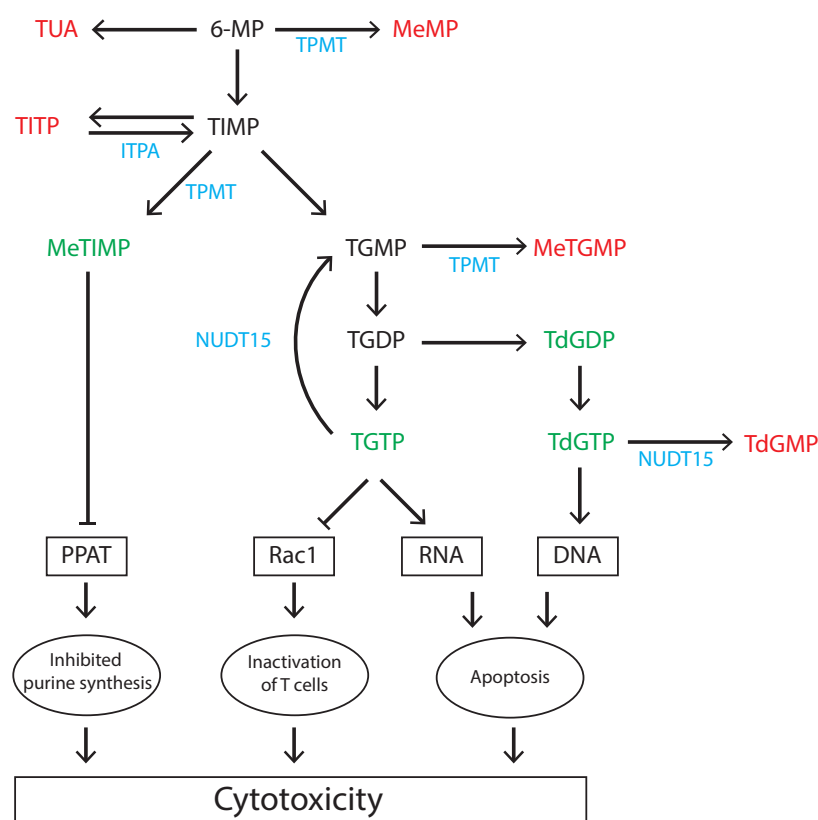


Figure 3: 6-MP metabolism. 6-MP can exert its cytotoxic affect through three different pathways: (i) by affecting RNA and DNA, leading to apoptosis, (ii) RAC1 inhibition leading to inactivation of T cells, and (iii) formation of meTIMP, which inhibits *de novo* purine synthesis.

Abbreviations: ITPA, inosine triphosphate pyrophosphatase; MeMP, methylated mercaptopurine; MeTIMP, methylated thioinosine monophosphate; 6-MP, 6-mercaptopurine; NUDT15, Nudix hydrolase 15; PPAT, phosphoribosyl pyrophosphate amidotransferase; RAC1, Rac family small GTPase 1; TdGDP, deoxy thioguanosine diphosphate; TdGMP, deoxy thioguanosine monophosphate; TdGTP, deoxy thioguanosine triphosphate; TGDP, thioguanosine diphosphate; TGMP, thioguanosine monophosphate; TGTP, thioguanosine triphosphate; TIMP, thioinosine monophosphate; TITP, thioinosine triphosphate; TPMT, thiopurine S-methyltransferase; TUA, thiouric acid.

1.4.2.2 TPMT

The enzyme TPMT has the ability to inactivate 6-MP by metabolizing it to non-toxic compounds through methylation of the drug to the inactive product: methylated mercaptopurine (meMP) (Figure 3) (130). However, TPMT can also produce an active product (meTIMP) that is toxic for the cell because it inhibits *de novo* purine synthesis in the cell (Figure 3) (131, 132). There are many known sequence variants in the TPMT gene, and the most frequently studied alleles are TPMT*2 (238G>C), TPMT*3A (460G>A and 719A>G), TPMT*3C (719A>G), and the more rare allele TPMT*3B (460G>A) (105, 133-135). TPMT*2, *3A, and *3C are together responsible for ~95% of variant alleles (105, 135-137). Sequence variants in the TPMT gene are identified in ~2–10%, with lower and higher frequencies in Asian and Caucasian populations, respectively (105, 138). A sequence variant in the TPMT gene results in reduced levels of TPMT enzyme activity, which in turn accumulates the end products of 6-MP (TGPT and TdGTP) and the risk of cytotoxic events increases during thiopurine treatment (103, 135, 139-144).

TPMT has been well-investigated in children treated for ALL. TPMT-deficient children have an increased susceptibility to thiopurine-induced toxicity, such as myelosuppression (Table 3, p. 40) (103, 105). Therefore, implementations were made in the NOPHO ALL 2000 and 2008 protocols with dose reduction for children deficient in the TPMT gene (23, 24). Interestingly, studies have shown a correlation with a higher probability for relapse when TGN levels are low (hence in individuals with high TPMT activity) and a decreased risk of relapse in TPMT-deficient children (103, 145-147). Follow-up studies after the implementation of dose adjustments have also indicated an increased risk of relapse in TPMT-deficient patients (148).

1.4.2.3 ITPA

The ITPA enzyme has the ability to hydrolyze thioinosine triphosphate (TITP) to thioinosine monophosphate (TIMP) nucleotides where TIMP is an important intermediate in the purine synthesis (Figure 3) (149). Accumulation of TITP is thought to be toxic for the cell, and ITPA protects the cell from accumulating this product (150). Two different sequence variants have been more frequently investigated and associated with enzyme deficiency: ITPA rs7270101 (IVS2+21A>C) and ITPA rs1127354 (94 C>A) (150). Sequence variants in ITPA are identified in 5–19% of the population and are more common in Asian compared to Caucasian populations (151). Sequence variants in ITPA rs1127354 and rs7270101 have shown decreased ITPA activity and therefore ITPA deficiency may result in the accumulation of toxic TITP (107, 118, 150). No effect on TGNs levels have been identified in ITPA deficiency (107, 118, 152).

In children treated for ALL, ITPA deficiency has in some studies been associated with side effects during 6-MP treatment, whereas other studies have failed to show any significant association between *ITPA* sequence variants and side effects (Table 3, p. 40) (107, 118, 119, 123, 125, 152-155). Therefore, the clinical significance of ITPA deficiency is still debated, and the contradictory results reflect the complex nature of thiopurine metabolism. No dose adjustment for ITPA-deficient patients has been implemented in the NOPHO ALL protocols.

1.4.2.4 *NUDT15*

The NUDT15 enzyme is thought to convert the active thiopurine metabolites TGTP and TdGTP through dephosphorylation to thioguanosine monophosphate (TGMT) and deoxy thioguanosine monophosphate (TdGMP) (Figure 3) (156, 157). Therefore, NUDT15 prevents cytotoxic TGTP and TdGTP from being incorporated into DNA or RNA (128, 157, 158). One sequence variant in the NUDT15 gene, NUDT15 rs116855232 (c.415C>T), is the most commonly detected and investigated variant (128, 157). NUDT15 sequence variants are identified in ~0.2–20 % of the population, with the highest and lowest numbers identified in Asian and European populations, respectively (128, 157). Sequence variants in NUDT15 directly result in excessive levels of thiopurine active metabolites, such as TGTP and TdGTP (but not the total TGN) (120, 157, 159). Therefore, NUDT15 deficiency may lead to an increased risk of thiopurine toxicity (157, 159).

Investigating sequence variants in the gene coding for NUDT15 in children with ALL has had an emerging role in recent years. The studies conducted have consequently identified NUDT15 as a risk factor for side effects such as myelosuppression and showed the need of 6-MP dose reduction in deficient patients (Table 3, p. 40) (119, 125, 128, 160, 161). These studies indicate an important role of NUDT15 deficiency. NUDT15 was recently implemented in the treatment protocols for ALL (ALLTOGETHER) currently used in Sweden (162).

1.4.3 The immune system

Because genetic variants in important proteins of the immune system are investigated in this thesis, some basic immunology is briefly described in this section to facilitate further reading. Pathogens are present all around us, trying to invade our barriers. The immune system is a defense barrier against these pathogens, preventing us from constantly being infected by these microbes. In most cases, encountered microbes are eliminated before symptom onset, while sometimes the immune system fails to eliminate microbes, thus allowing an infection to be established. The immune system is usually divided into the innate and adaptive immune systems, with several overlapping cell types, as well as the complement system. In children treated for cancer, the regular immune response is weakened or missing due to the chemotherapy treatment and sometimes by the cancer itself.

1.4.3.1 *The innate immune system*

The innate immune system is the first line of defense against microbes and can recognize pathogens directly, react to host cells that have been damaged, and activate the adaptive immune system (163). The innate immune system consists of epithelia, functioning both as a barrier and a reservoir of immune proteins against infection, cells in the blood and peripheral tissues, and plasma proteins. Important cells in the innate immune system are phagocytes, which includes both neutrophils and monocytes that differentiate to macrophages or dendritic cells in tissue (Figure 1, p. 3). Another important cell type is the natural killer (NK) cell, which can recognize damaged or infected host cells and induce apoptosis. The cells of the innate immune system use different receptors, such as Toll-like receptors (TLRs), cytokine receptors, formyl methionine peptides, mannose receptors, and scavenger receptors to recognize microbes. When a pathogen attaches to these receptors, a cascade of events follow that ultimately lead to transcription of genes involved in the inflammatory response, during which inflammatory cytokines are produced, such as ILs (especially 1, 6, and 12), tumor necrosis

factor (TNF), chemokines, and interferon (IFN)- γ . The cytokines then activate and mediate many of the reactions involved in innate immunity, such as inflammation, communication between leukocytes, and activation of the adaptive immune response (163).

1.4.3.2 The complement system

The complement system is an important part of the immune system that can enhance the role of phagocytes and antibodies (163). The complement system is activated in three different ways: alternative pathway (innate immune response), lectin pathway (innate immune response), and the classical pathway (adaptive immune response). The complement system can initiate a cascade of events in which the end product enables opsonization to facilitate phagocytosis, cytolysis of microbes by forming pores in the membrane, and stimulation of the inflammatory response from various leukocytes (163).

1.4.3.3 The adaptive immune system

The adaptive immune system is subsequently activated and constitutes a more specific defense against pathogens (163). The adaptive immune system consists of lymphocytes: B cells (humoral immunity) and T cells (cell-mediated immunity) (Figure 1, p. 3), which are activated after microbes have passed through the epithelial barrier and reached the lymph nodes. Although, during recent years the distinctions between the innate and adaptive system are not mutually exclusive, since some of the cells have been identified to affect both systems (*e.g.* NK memory cells) (164, 165). Lymphocytes express receptors that recognize molecules called antigens that are produced and expressed on microbes (163). When an antigen binds to the naive B cell, the cell differentiates into an effector cell called a plasma cell, which can produce antibodies that can neutralize and eliminate microbes. Plasma cells can be long-lived and produce antibodies in the absence of antigen, therefore providing a level of protection if the antigen is re-encountered. Some B cells also differentiate into memory B cells, which do not secrete antibodies but respond rapidly if the antigen is re-introduced. T cells can be grouped as CD4⁺ T cells (T helper cells and regulatory T cells) or CD8⁺ T cells (cytotoxic T cells). T helper cells and cytotoxic T cells are effector cells. T helper cells produce cytokines that activate B cells and macrophages. Cytotoxic T cells eliminate host cells infected with intracellular microbes. A fraction of the T cells become memory T cells, which can be more long-lived and be rapidly activated if the antigen is re-introduced (163).

1.4.4 Sequence variants in important proteins in the innate immune system

Sequence variants in genes important in the immune system are common, and some of these variants may affect infection susceptibility and side effects to different extents. Mannose-binding lectin (MBL), TLR4, and IL-1 receptor antagonist (IL-1Ra) are three different proteins important in the immune system that have been identified as potential risk factors for episodes of febrile neutropenia and associated MDI.

1.4.4.1 *MBL*

MBL is a plasma protein produced in the liver that is an important protein for the innate immune system, as it activates the lectin pathway of the complement system (163). The *MBL2* gene codes for the MBL protein (166). Three sequence variants in the *MBL2* gene located in an exon are commonly detected and investigated: codon 52 (allele D), codon 54 (allele B), and codon 57 (allele C) (167-169). In addition, three sequence variants in the promotor region of the *MBL2* gene have also been shown to affect the protein levels to different extents: H/L, Y/X, and P/Q (170, 171). However, a strong linkage disequilibrium between the sequence variants in the promotor and the exon regions has been observed (170). MBL sequence variants are fairly common with a detection rate of 20–50%, whereas variant and frequencies vary within different populations (168, 169, 172). Sequence variants in the MBL gene, mainly the exon regions and one of the promotor regions, have been correlated with lower serum levels of MBL protein (170, 171, 173, 174).

In some studies of immunocompetent pediatric and adult cohorts, sequence variants in the MBL gene have been correlated with an increased risk of infections, such as higher risk for systemic inflammatory response, sepsis, and respiratory infections (173-178). However, studies have also shown discrepant results in the role of MBL during infectious episodes (179, 180). Several studies have investigated sequence variants in the MBL gene in children undergoing treatment for cancer (Table 4, p. 44). Similar to the immunocompetent patient group, results have been contradictory when investigating the association between sequence variants in the MBL gene and episodes of febrile neutropenia and MDI (181-186).

1.4.4.2 *TLR4*

TLRs are receptors used by the innate immune system to recognize molecules presented on different pathogens (163). These receptors are present both extra- and intracellularly and in many different cell types, such as phagocytes, dendritic cells, lymphocytes, and endothelial cells. TLRs are specific to different molecules shared by classes of microbes, such as LPS presented on Gram-negative bacteria and single- or double-stranded RNA of viruses. Immune cells are activated when the specific ligand on the pathogen attach to the corresponding TLR. When a pathogen is discovered by a TLR, immune cells are activated, leading to transcription of genes involved in the inflammatory response (Figure 4) (163). TLR4 is present on the cell surface and is one of the most important TLRs for the recognition of LPS presented on Gram-negative bacteria. Several sequence variants in the TLR4 gene are known, with rs4986790 (Asp299Gly) and rs4986791 (Thr399Ile) more commonly studied (187). The frequency of sequence variants in the TLR4 gene (for rs4986790 and rs4986791) varies from 0–18% and differs within populations (188). It has been reported that sequence variants in the TLR4 gene may alter the extracellular structure of the receptor, resulting in conformational changes of the binding site that may affect the binding of pathogen ligands such as LPS (189-191). Another possibility is that TLR4 inhibits neutrophil apoptosis, wherefore dysfunctional TLR4 may lead to a shorter neutrophil lifespan (192-194). However, due to the complex nature of the TLR4 receptor and difficulties investigating the exact function, the phenotypes of different sequence variants in TLR4 are not fully understood.

Sequence variants in the TLR4 gene have been correlated with an increased risk of infection, such as an increased risk of Gram-negative BSI, RSV infection, and pulmonary aspergillosis (195-199). However, contradicting results of the role of sequence variants in rs4986790 and the risk for Gram-

negative BSI have also been reported (200, 201). During the past decade, several other sequence variants in the gene coding TLR4 have been discovered, but because these are relatively recently discovered, they are not as well-investigated as rs4986790 and rs4986791. Some studies have investigated sequence variants in the TLR4 gene and the risk of febrile neutropenia and associated MDI in patients treated for cancer (181, 202) (Table 4, p. 44). The studies conducted in adults indicate increased risks of Gram-negative BSIs, sepsis, and pneumonia (181, 202). One study of a pediatric cohort treated for cancer showed an increased risk of developing chemotherapy-induced neutropenia in children deficient in the TLR4 gene (203).

1.4.4.3 *IL-1Ra*

ILs are secreted as a response to pathogens and play essential roles in both the activation and maturation of immune cells. ILs can have both pro- and anti-inflammatory properties. The IL-1 family is an important group of ILs, with IL-1 α and IL-1 β as the two most studied proteins. Both proteins have a strong pro-inflammatory effect and are secreted in response to pathogens. IL-1 α and IL-1 β have a natural antagonist called IL-1Ra that is produced in the same cells as IL-1 α and IL-1 β (204). IL-1Ra can also bind to the IL-1 receptor, but the binding does not alter downstream signaling (205, 206). IL-1Ra therefore regulates the inflammatory response by competing with IL-1 α and IL-1 β for the binding site of the IL-1 receptor; consequently, it has an anti-inflammatory action. IL-1Ra is encoded by the *IL1RN* gene (207). There are four commonly studied sequence variants within intron 2 of the *IL1RN* gene (*IL1RN**2-5), which consists of tandem repeats of an 86-base pair (bp) sequence. The *IL1RN**1-5 alleles correspond to 4, 2, 5, 3, and 6 copies of the 86-bp sequence, respectively, and four copies is the most common allele (102). The frequency of the *IL1RN**2 allele is reported in ~20% of the population, while the *IL1RN**3 allele is more uncommon (~4%) (102). Presence of the *IL1RN**2 allele has been associated with an increased production of serum IL-1Ra (208, 209). However, contradictory results have also been presented, showing that homozygosity for the *IL1RN**2 allele led to a decrease in IL-1Ra production from cultured peripheral blood mononuclear cells, while no difference was seen in its plasma levels of healthy donors or sepsis patients (210). Sequence variants in the IL-1Ra gene have been associated with an increased susceptibility to sepsis and also more severe sepsis with a higher mortality in cohorts of otherwise healthy adults (210, 211).

Studies investigating IL-1Ra in children with cancer are limited. However, one described an increased risk of septic shock in children with the presence of an *IL1RN**2 allele (212) (Table 4, p. 44). Another study focusing on adults with acute leukemia found no correlation between sequence variants in the IL-1Ra gene and infection risk (181).

1.5 MICROBIOLOGICAL ACTIVATION OF THE IMMUNE SYSTEM

When a pathogen penetrates the mucosal barriers and enters the host, the immune system is activated in **different pathways, depending on the type of pathogen**. The patterns can be specific for viruses and bacteria (but also overlapping) to eliminate the pathogen as effectively as possible. These **pathogen specific innate immune signatures** have recently been highlighted as potential new methods for pathogen detection.

1.5.1 General innate pathway as a response to pathogens

The first step in innate immune activation is when a pathogen is recognized by a specific receptor, called a pattern recognition receptor (PRR). PRRs recognize molecules on either bacteria or viruses and can be localized on the cell surface, such as TLRs, or intracellularly, such as retinoic acid-inducible gene I like receptors (RLRs) and Nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (213-219) (Figure 4). Binding to TLRs and RLRs is important for the production of IFN (type I) and other cytokines, whereas NLRs are involved in regulating IL-1 β (213, 220, 221) (Figure 4). The most common and well-described pathway is through TLR signaling, which the following described pathway focuses on. The binding of a pathogen to the receptors causes a cascade of intracellular signaling through different kinases (MYD88 or Toll/IL-1 receptor (TIR)-domain) resulting in the production of interferon-regulating factor (IRF) (mainly 3 and 7) and nuclear factor kappa-light-chain-enhancer of activated B-cells (NF- κ B) (12, 213-219) (Figure 4). These factors can

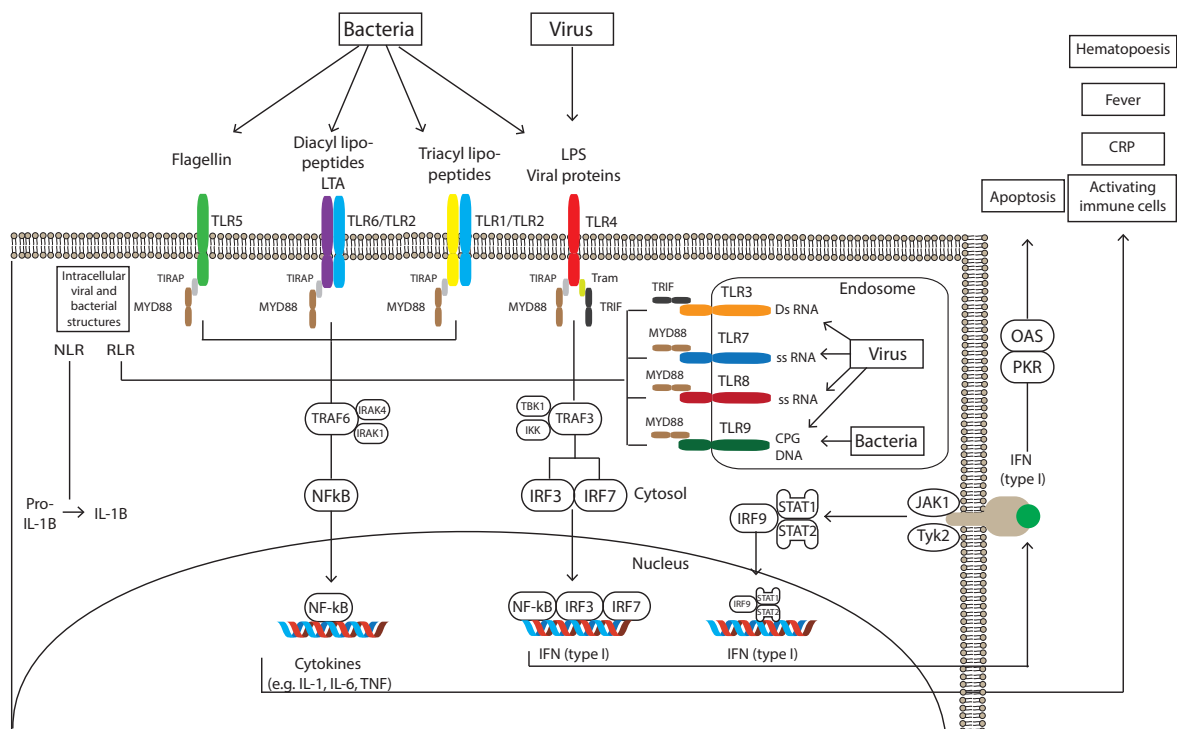


Figure 4: Viral and bacterial innate immune activation.

Abbreviations: CRP, C-reactive protein; Ds, Double stranded; IFN, Interferon; IL, Interleukin; IRAK, Interleukin-1 receptor-associated kinase 4; IRF, Interferon regulatory factors; JAK1, Janus Kinase 1; LPS, Lipopolysaccharide; Myd88, myeloid differentiation primary response 88; NF- κ B, Nuclear factor kappa-light-chain-enhancer of activated B cells; NLR, Nod-like receptor; OAS, Oligoadenylate synthetase; PKR, Protein kinase R; RLR, Retinoic acid-inducible gene-I-like receptor; Ss, Single stranded; STAT, Signal transducer and activator of transcription; TLR, Toll-like receptor; TNF, Tumor necrosis factor; TRAF, TNF receptor associated factor; TRIF, TIR-domain-containing adapter-inducing interferon- β ; Tyk2, Tyrosine kinase 2

then enter the cell nucleus and induce the transcription of immune genes, thus stimulating the production of cytokines (*e.g.*, IL-1, TNF, IL-6, and IFN type I) (Figure 4). IFN is an important cytokine, and high levels are produced during viral infection and also during bacterial infection, but to a lesser extent (222). IFN can induce intracellular signaling through Janus Kinase 1 (JAK1) and Signal transducer and activator of transcription (STAT) and inhibit protein synthesis (both viral and cellular), leading to cell death. Two important pathways of inhibiting protein synthesis are through activating protein kinase R (PKR) and oligoadenylate synthase (OAS) (Figure 4) (12, 223-225). The main focus of this chapter is the innate immune system; however, the adaptive immune system is essential to clear an infection and generate immunity. The pathways are very simplified, with the intention to briefly describe the complex system so both the direct immune genes and clusters of associated genes can be identified and associated with an active immune response. Different signaling pathways are activated through PRRs, and all can tune the intensity, duration, and quality of the signaling cascades, thus generating an innate immune response very specific for the pathogen they are sensing.

1.5.2 Diagnosing pathogens through host innate immune signatures

New methods have recently been developed focusing on the specific gene expression patterns of the innate immune system elicited by certain pathogens. RNA sequencing and microarrays can measure the amount of RNA (*i.e.*, the transcriptome) expressed by an organism or a cell type (*e.g.*, leukocytes) at a given point in time. As RNA is produced upon activation of specific genes, measurements of RNA serve as an indirect estimate of which genes are currently activated, giving specific gene-expression profiles. Therefore, using gene-expression profiling during infectious episodes could in the future be a promising alternative approach to traditional diagnostic methods of infectious diseases.

1.5.2.1 Gene-expression profiling

Specific signatures or gene expression patterns have been studied and identified in both adult and pediatric immunocompetent cohorts during viral and bacterial infections (226-230). Zaas *et al.* (2009) used a cohort of adults inoculated with RV, RSV, and influenza virus and compared those developing symptoms and those who did not. The study was able to identify a blood mRNA expression profile that could classify symptomatic respiratory viral infections (226). The specific viral signature identified was compared to gene expression specific for bacterial infections, and the authors thereby confirmed that their signature was viral-infection-specific and could distinguish between patients with viral and bacterial infections, as well as healthy controls. In a follow-up study, Zaas *et al.* (2013) selected gene sets to classify respiratory viral infections by using reverse transcription-PCR (RT-PCR) and were able to identify influenza with a sensitivity of 89% and specificity of 94% (231). However, the standard dogma in pediatrics that “children are not little adults” is likely true also for the immune response, so it is important to also perform such studies in pediatric cohorts. Ramilo *et al.*, Hu *et al.*, and Herberg *et al.* were able to identify specific pathogen-response signatures for viral and bacterial infections in children (227, 229, 230). To our knowledge, no study has yet investigated these specific signatures in a cohort of immunosuppressed children. If similar signatures could be identified in immunosuppressed children, this information could be used to distinguish different microbiological findings and specifically assess children with FOU to investigate potentially undetected pathogens during febrile neutropenia episodes.

2 AIMS

This thesis had two overall aims:

- (i) To increase the knowledge of the causative agents in episodes of febrile neutropenia in children treated for cancer, with the long-term goal of improving the interpretation of microbiological findings in a clinical setting during these episodes (**papers I and IV**).
- (ii) To assess risk factors of neutropenia and febrile neutropenia during pediatric ALL treatment, with the long-term goal of developing a more individualized treatment strategy and risk stratification for these events (**papers II and III**).

The more study specific aims were:

Paper I

- To investigate the role of respiratory viruses as causative agents during episodes of febrile neutropenia in children treated for cancer using a longitudinal study design with repeated sampling in virus positive children.

Paper II

- To evaluate if sequence variants in genes encoding enzymes important in the 6-MP metabolism, such as TPMT, ITPA and NUDT15, increase the risk of neutropenia and febrile neutropenia during pediatric ALL treatment.
- To determine if the end dose of 6-MP during pediatric ALL treatment is altered depending on the child's specific TPMT, ITPA, and NUDT15 genotypes.

Paper III

- To retrospectively define and characterize the etiology of febrile neutropenia episodes during the 2.5 years of treatment for pediatric ALL.
- To investigate if sequence variants in genes coding for proteins important in the innate immune system, namely TLR4, MBL, and IL-1Ra, increase the risk of suffering febrile neutropenia episodes and associated MDI.

Paper IV:

- To investigate the feasibility of gene-expression profiling as a diagnostic tool during febrile neutropenia episodes in a cohort of children treated for cancer.
- To improve the knowledge of respiratory viruses role as infectious pathogens' during febrile neutropenia episodes by the assessment of their specific gene expression profiles.

3 STUDY DESIGNS

3.1 STUDY POPULATION AND SAMPLING

Two different cohorts were included in this thesis. The first was a prospective cohort of children presenting with febrile neutropenia from which samples for **papers I and IV** were collected. The second was a retrospective cohort in which children were identified using the NOPHO ALL registry and from whom samples had already been collected for **papers II and III**.

3.1.1 Cohort I

Papers I and IV: Children aged 0–18 years treated for cancer at Astrid Lindgren Children’s Hospital, Stockholm, Sweden between January 2013 and June 2014 were eligible for enrollment. All children who met the criteria for febrile neutropenia ($\geq 38.5^{\circ}\text{C}$ on one occasion or $\geq 38.0^{\circ}\text{C}$ on two occasions at least 60 minutes apart, combined with an ANC of either $\leq 0.5 \times 10^9/\text{L}$ on one occasion or $\leq 1.0 \times 10^9/\text{L}$ with a decline to less than $\leq 0.5 \times 10^9/\text{L}$ over a subsequent 48-hour period) were prospectively enrolled in the study. Children could be included multiple times if they had recurrent episodes of febrile neutropenia, and a total of 87 episodes in 54 patients were included. All included samples were collected within 72 hours from fever onset. Nasopharyngeal aspirates (NPAs) (**papers I and IV**) and blood samples (**paper IV**) were taken at time of enrollment, and follow-up samples were collected after 1–8 weeks (blood samples were collected from all children and NPAs from all virus positive children) (Figure 5). Blood cultures were collected as part of the clinical routine, and information about microbiological and biochemical results was collected from the medical records. For **paper IV**, all available samples from virus- and bacteria-positive and FOU children were included for further analysis ($n = 67$), of which four of these were excluded due to sample collected >72 hours from fever onset (Figure 5). Of the 51 follow-up blood samples, 31 represented new episodes of febrile neutropenia or had clinical signs of symptoms and were therefore excluded as controls. Of the remaining 20 samples, 12 children were randomly selected as controls (Figure 5).

3.1.2 Cohort II

Papers II and III: Children were retrospectively identified and included using the NOPHO-ALL registry. Children diagnosed with ALL, treated either within the NOHPO ALL 2000 or the 2008 protocol at Astrid Lindgren Children’s Hospital between May 2004 and April 2014 were included in the study (Figure 5). In **paper II**, the HR patients were excluded due to the differences in the treatment protocols, with a focus on differences in the 6-MP treatment. The HR group was included but adjusted for in **paper III** (Figure 5). Patient characteristics and numbers of episodes of febrile neutropenia were retrospectively collected from medical records for both **paper II** and **paper III**. End doses of 6-MP and numbers of episodes of neutropenia were collected only for **paper II**, and microbiological findings and other infectious parameters during the episodes of febrile neutropenia only for **paper III**. Neutropenia was defined as a neutrophil count $\leq 0.5 \times 10^9$ cells/L. A neutropenia episode had to be resolved ($\geq 1.0 \times 10^9$ cells/L) before a new episode could be registered. Febrile neutropenia was defined as two or more spikes at least 1 hour apart of temperature of $\geq 38.0^{\circ}\text{C}$ or a single spike of $\geq 38.5^{\circ}\text{C}$, with a neutrophil count of $\leq 0.5 \times 10^9$ cells/L at the time of the fever or decreasing to $\leq 0.5 \times 10^9$ cells/L within 48 hours of fever onset (58). The patients were followed from diagnosis until end of treatment, to relapse, until HSCT, or death.

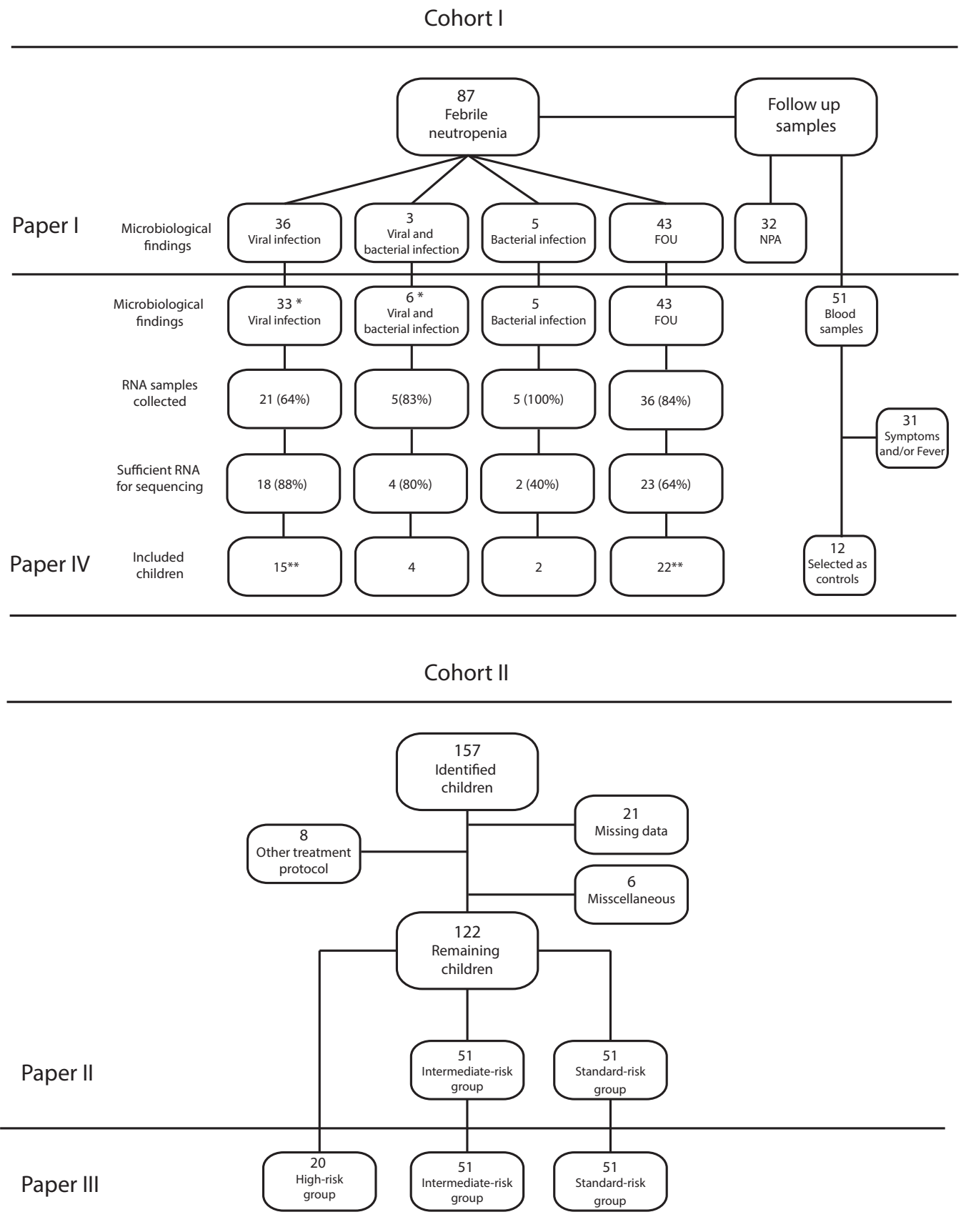


Figure 5: Outline of cohort I, used for papers I and IV and cohort II used for papers II and III.

*Three episodes were redefined from only viral infection to co-infection with virus and bacteria.

**Four children were excluded due to sampling >72 hours from fever onset.

The selection of sequence variants for **papers II and III** was made after a thorough literature review in 2014 that was updated in 2018 using the following search strategy. A search in the PubMed database between 1950-01-01 and 2018-04-30 was performed with the filter “child,” “young adults,” “humans,” and “English” for keywords “((acute lymphoblastic leukemia) OR (acute lymphocytic leukemia)) AND (polymorphism OR mutation) AND (febrile neutropenia OR infection),” and 271 papers were identified. Out of the 271 hits, 63 titles were of interest, and 18 abstracts were chosen for further reading. An additional search was also made including the keywords “neutropenia” and “sepsis,” increasing the number of hits to 313. In addition, earlier known and investigated sequence variants within the research group were included. Based on the literature review and prior knowledge TPMT, ITPA, and NUDT15 were identified as pharmacogenetic enzymes to further investigate in **paper II** and MBL, TLR4, and IL-1Ra as immunological proteins of interest in **paper III**. The literature review was also updated 2019-12-20 with 325 papers identified (search path “((acute lymphoblastic leukemia) OR (acute lymphocytic leukemia)) AND (polymorphism OR mutation) AND ((febrile neutropenia) OR (infection) OR (neutropenia) OR (sepsis))”). No new enzymes or other proteins of interest for this thesis were identified during this time period.

3.2 MOLECULAR ANALYSES

3.2.1 Real-time PCR

In **paper I**, the NPAs were sent to the accredited Karolinska University Laboratory (ISO: 15189:2012) for analysis according to their standard PCR protocol for detecting respiratory viruses (27). In brief, viral nucleic acids were extracted from the NPAs and then analyzed with real-time PCR for 15 viruses: HAdV, HBoV, HCoV (NL63/OC43/229E/HKU1), EV, influenza virus (including types A(H1N1)pdm09 and B), HMPV, PIV 1-3, RSV, and RV (19). This method can be described as semi-quantitative and has not been validated for quantification of viral load. Although viral load cannot directly be inferred from the Ct values and should be carefully interpreted, low Ct values still indicate higher viral loads. These Ct values were not provided in the medical record but were collected retrospectively for **paper I** in collaboration with the laboratory.

3.2.2 Genotyping

3.2.2.1 RV/EV genotyping

For **paper I**, Sanger sequencing was performed for samples positive for RV/EV by sequencing the *VP4/VP2* region as previously describes by Dyrdak *et al.* and Wisdom *et al.*, and the RV/EV species and type were determined by maximum likelihood phylogenetic trees constructed using PhyML (232-234). Through this method, it was possible to distinguish RV and EV in **paper I** and also determine the specific RV genotype. Since some RV still remained present in follow-up samples, the *VP4/VP2* sequencing could determine if the infection was caused by the same RV genotype or a new genotype.

3.2.2.2 Pyrosequencing, TaqMan SNP genotyping assay, and agarose gel electrophoresis

Different methods were used to determine the genotypes for the investigated proteins and enzymes in **papers II** and **III**. *Pyrosequencing* was used for *ITPA* rs1127354, *ITPA* rs7270101, *NUDT15* rs116855232, *MBL* rs1800450, *MBL* rs5030737, *MBL* rs1800451, *TLR4* rs2737190, *TLR4* rs1927911, *TLR4* rs10759931, and *TLR4* rs11536889. Information regarding the presence of *TPMT* rs1800462, *TPMT* rs1800460, and *TPMT* rs1142345 variants analyzed by *pyrosequencing* as part of the clinical routine at Linköping University was collected retrospectively (137). *Pyrosequencing* is a rapid and accurate quantification method that can accommodate large samples sizes. Also, *pyrosequencing* gives qualitative sequence data, which confirms that the correct sequence is genotyped. A limitation of this method is that only known sequence variants can be detected; however, there are a few examples in which the genotype and phenotype can be combined and other less common alleles can be detected (through a mismatch with the genotype and phenotype). In **paper II**, genotyping was performed with the TaqMan™ SNP Genotyping Assay for *TLR4* rs4986790 and *TLR4* rs4986791. To determine the sizes of amplicons (how many tandem repeats of the 86-bp sequence) for the *IL1RN* in **paper III**, *agarose gel electrophoresis* was used (102). In **papers II** and **III**, the sequence variants for the genes were pre-determined, and because *pyrosequencing* was not possible for detection of tandem repeats, *agarose gel electrophoresis* was chosen for *IL1RN*. When using *agarose gel electrophoresis*, the specific allele in which the sequence variant is located can be detected, giving a specific genotype. With *pyrosequencing*, the result only reveals one specific sequence variant at a specific locus, so when investigating several different sequence variants (as with *TLR4*) within the same gene, the method cannot always determine if the sequence variant is located on the same or opposite allele.

3.2.3 RNA sequencing

RNA sequencing is a NGS method that was relatively recently developed (235). RNA sequencing was used in **paper IV** as an expansion to **paper I** to investigate if the immune response correlated to the MDI identified using conventional methods. RNA sequencing was performed using the Illumina platform according to the manufacturer's instructions, and the data were thereafter analyzed in collaboration with a bioinformatician (described in the statistical method section below). One initial challenge during RNA sequencing was that, even though 5 ml of blood was collected from all children, no RNA could be extracted in 20 samples. This was a concern in the study, as RNA from blood samples mainly originates from WBCs, and the children included could be severely leukopenic. However, in the cases with sufficient RNA the RNA had good quality (RNA integrity number, RIN >7). Because many of the methods currently used for pathogen detection still have limitations, the use of information from RNA sequencing would be of great interest with regards to clinically diagnosis of pathogens. RNA sequencing has the ability to detect novel transcripts and does not require any specific transcript probes, it can detect genes with low expression, and it can reveal SNPs in the transcribed regions (235). Even though RNA sequencing is a promising method, it still has its limitations. One challenge is that the transcriptomes generated require large amounts of data capacity to store the information. In addition, the data analysis is a very complex procedure, and no gold standard method has been implemented (235, 236).

3.3 STATISTICAL ANALYSES

Data was analyzed using GraphPad Prism 6.0 software (**papers I and IV**) and R version 3.4.4. (**papers II and III**). A p-value of < 0.05 was considered statistically significant.

In **paper I**, Fisher's exact test and the Mann-Whitney U test were used for group comparisons of categorical and continuous data, respectively. For **paper II**, the Andersen-Gill model, a proportional hazards model allowing for recurrent events, was applied to evaluate the associations between age, protocol, treatment intensity (risk group), gender, and sequence variants and the risk of neutropenia or febrile neutropenia (237). The function *coxph* in the R package *survival* was used to fit the models (238, 239). Doses of 6-MP were compared between protocols, risk groups, and genders using independent sample *t*-tests. Pearson correlation coefficients were calculated to assess the correlation between the age at diagnosis and 6-MP dose. In **paper III**, the Andersen-Gill model was also used to evaluate the associations between age, protocol, risk group, gender, ALL immunophenotype, and sequence variants and the risk of febrile neutropenia and MDI (237). The function *coxph* in the R package *survival* was used to fit the models (238, 239). Statistical analyses for **papers II and III** were performed in collaboration with a statistician. In **paper IV**, the analyses were performed in a collaboration with a bioinformatician. Samples were mapped using a human reference genome, and the data was normalized using DESeq2 (false discovery rate (FDR) < 0.05 and fold-change of 1.5 were used to identify significant genes). Ingenuity Pathway Analysis (IPA) software was used for pathway analysis (Content version: 51963813; Release Date: 2020-03-11; Ingenuity Systems).

3.4 ETHICAL CONSIDERATIONS

All studies were approved by the Regional Ethical Review boards in Stockholm and Linköping (2008/648-31/4, 2009/286-32, 2010/1652-32, 2014/1012-31/4, 2016/442-31/2 and 01-297 Linköping).

Children treated for cancer experience intensive treatment and regular repeated sampling. It was of importance that our studies would not require additional hospital visits and that sampling would cause as little distress as possible. Therefore, samples were taken together with the routine samples as often as possible and in some cases previously collected samples were used. In **papers I and IV**, NPA collection could cause minor distress. However, these samples were collected as quickly as possible and sometimes as a part of the clinical routine. The results were also provided in the medical records for the clinician. The blood samples were always synchronized with other clinical samples. We believe that the short discomfort is outweighed by the positive long-term effects (on a group level) of increasing knowledge of optimal management of febrile neutropenia, with potentially fewer days of in-hospital care and broad-spectrum antibiotics, as well as improved diagnosis. In **papers II and III**, data were retrospectively collected from medical records and results presented on a group level. The samples were already collected as part of the clinical routine. This study tested genetic sequence variants, which could be seen as a violation of the personal integrity. However, the children had ended their treatment when results were published, and patients did not receive any information about the results. We believe that increased knowledge about children at risk of infection and children more sensitive to specific chemotherapy drugs could lead to more individualized treatment strategies, and that such positive outcomes outweigh the possible negative experiences during this study.

4 RESULTS AND DISCUSSION

4.1 ETIOLOGY OF FEBRILE NEUTROPENIA

Just one decade ago, respiratory viral infections were rarely assessed in children with cancer, presumably due to lack of sensitive and rapid detection methods. In addition, respiratory virus was not acknowledged as a potential and important cause of febrile neutropenia and diagnostics were mostly focused on severe bacterial infection. However, the role of respiratory viral infections during this condition has gained interest in the past 10 years. In **papers I and IV**, the etiology of febrile neutropenia was investigated with respiratory viral infection as the main focus. For **paper I**, PCR and blood cultures were used to determine the presence of respiratory viruses and bacterial BSIs, respectively. Due to the inherent limitations with both PCR and blood cultures discussed in this thesis (*e.g.*, asymptomatic patients and viral remnant findings in PCR and low sensitivity for blood cultures in pediatric patients), challenges still remained in interpreting the results generated by these methods. Therefore, in **paper IV**, the MDI and FOU were further investigated to identify a possible transcriptional pathway in the innate immune response using RNA sequencing. Unfortunately, the results from **paper IV** revealed that gene-expression profiling is difficult to use in this patient cohort and a pathogen-specific innate response was not identified. Despite the current lack of antiviral treatment options, a correct diagnosis and increased knowledge of viral infections in children treated for cancer is of importance and could lead to a more accurate prediction of the clinical course, possibly with fewer days of in-hospital care and decreased use of broad-spectrum antibiotics.

4.1.1 Paper I

In **paper I**, all children admitted to the hospital for febrile neutropenia during a period of 18 months (January 2013–June 2014) were asked to participate in the study. Therefore, at least two infectious disease seasons were covered during the sampling period. This was of importance with regards to respiratory viral infections, since the circulation of most virus follow distinct epidemiological patterns and largely vary in incidence over the year (Table 1, p. 7). In total, 54 children with 87 episodes of febrile neutropenia were included. One or several respiratory viruses was detected in 45% of all episodes. RV was the most commonly detected virus ($n = 21$, 24%), followed by HCoV ($n = 7$, 8%) and influenza virus ($n = 4$, 5%). Ct values were collected for the different viruses to estimate viral load. The low Ct values in most of the cases (<30 in 27/39, presented in **paper I**) indicated a high viral load. In 86% of the respiratory virus-positive episodes the child also presented with respiratory symptoms, providing further evidence that the identified virus was indeed related to the febrile episode. In contrast, respiratory symptoms were only presented in 40% and 58% of children with only bacterial BSI and FOU, respectively, which was significantly different compared to the virus-positive group. However, the bacterial BSI group ($n = 5$) was relatively small, which must be considered when interpreting the results. Follow-up samples were collected in 32 of the 39 virus-positive episodes (median time to follow up, 28 days; range, 9-74 days), and as many as 84% had cleared the respiratory virus; only HCoV and RV persisted in one and four episodes, respectively. Initially, six RV samples remained positive after the regular PCR was performed at the microbiology unit (Karolinska University Laboratory), but sequencing of these samples revealed that two of these episodes were a new RV genotype.

Bacterial BSI was confirmed in eight (9%) episodes. Of these, a respiratory virus was also detected in three. The bacteria identified in episodes without viral co-infection were *S. epidermidis*, *coagulase-negative staphylococci*, *E. coli*, and two *S. aureus*. The three bacteria co-detected with a virus were *alpha-hemolytic Streptococcus* and two *S. epidermidis* cases. It should however be noted that blood cultures have low sensitivity in pediatric patients (43, 44). Interestingly, all of the children with a bacterial BSI were treated for a hematological malignancy.

4.1.1.1 Microbiological findings during febrile neutropenia episodes

The results from **paper I** were consistent with previous studies, both for frequency and subtype of respiratory virus (52, 65-68, 77, 240, 241) (Figure 6 and Table 2). Koskenvuo *et al.* performed the first study, which included respiratory viral testing in children with febrile neutropenia using PCR, and identified a respiratory virus in 44% of the cases (77). Subsequent studies also concluded that respiratory viruses could be detected in 45–52% of the febrile neutropenia episodes (52, 65, 66) (Figure 6). Similar to our study, respiratory viral findings were more often associated with respiratory symptoms (65, 66). In a recently published study by Meena *et al.*, children with episodes of febrile neutropenia with respiratory symptoms were considered as cases and children without symptoms as

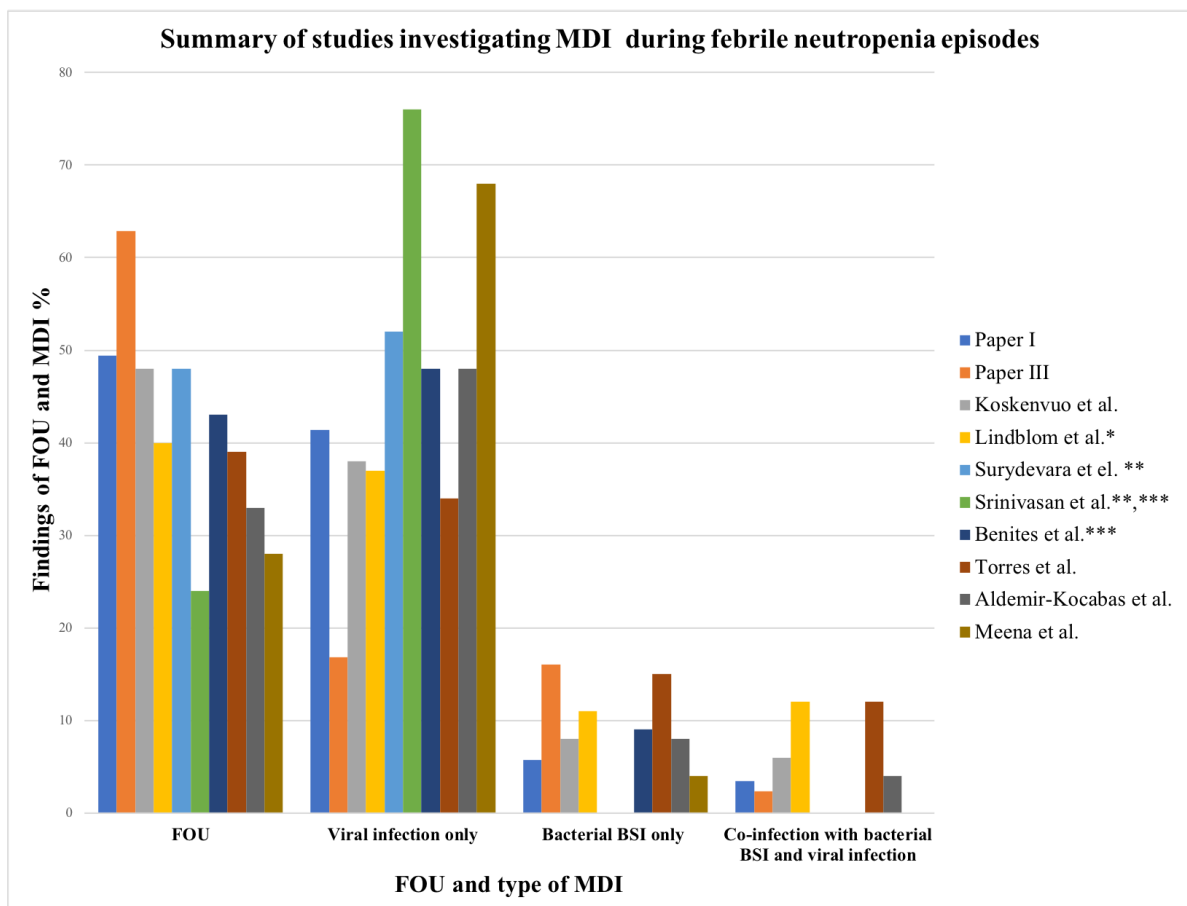


Figure 6: FOU and MDI during febrile neutropenia episodes in pediatric patients treated for cancer. Gaps represent either not reported or no cases. *Also reported four blood-borne viruses which are not included in the numbers, **Did not report bacterial BSI, ***Respiratory symptoms/infection were inclusion criteria in these studies.

Abbreviations: BSI, bloodstream infection; FOU, Fever of unknown origin; MDI, Microbiological defined infection

controls. In the group with respiratory symptoms, 76.5% of the patients had a detected respiratory virus (compared to 48.6% in the control group). The correlation between respiratory symptoms and viral findings supports the hypothesis that respiratory viral sample collection should be part of the routine clinical management in children presenting with respiratory symptoms during febrile neutropenia. In **paper I**, fewer bacterial BSIs were detected compared to previous studies, in which such infections have been reported in 13–27% (Figure 6) (52, 65, 77). One reason may be that we excluded children with positive blood cultures (n = 5) that were considered as contamination or of no clinical relevance by the laboratory staff and the treating physician. Other potential sources of bias may be that the children suffering from a bacterial BSI had a more severe condition and therefore more often declined participation in the study (no such data available).

4.1.1.2 Asymptomatic infection and prolonged viral shedding

Certain respiratory viruses have been reported as commonly detected by PCR in asymptomatic children and persist weeks after an acute infection, which hampers clinical interpretation (29, 33, 79, 242). Respiratory viruses and their presence in asymptomatic, immunocompetent children have been investigated in a series of studies. Rhedin *et al.* conducted a case-control study in children presenting to the emergency room with respiratory symptoms (29). The control group included asymptomatic children at their routine visits at child healthcare centers. The authors identified a respiratory virus in 35.4% of asymptomatic individuals in the control group, with RV and HCoV the most commonly detected viruses in this group. RSV, HMPV, and PIV were only rarely identified in the asymptomatic individuals. These results were consistent with those of previous investigations conducted in similar patient cohorts (33, 34, 243-245). The authors of these studies concluded that HMPV, PIV, influenza, and RSV are likely to play an etiologic role and suggested that findings of RV and HCoV should be more carefully interpreted (29, 33, 34, 243-245). To our knowledge, no similar studies investigating asymptomatic infections in children treated for cancer have been published. In **paper I**, the results were in line with those of the aforementioned studies in immunocompetent cohorts; RV and HCoV were the only viruses identified in children without reported symptoms during febrile neutropenia episodes (29, 33, 34, 243-245).

Table 2: Most commonly identified respiratory viruses and follow-up samples during episodes of febrile neutropenia in children treated for cancer.

| | Most commonly detected respiratory virus | Follow-up samples | Persistent virus |
|-------------------------------|--|-------------------|---------------------|
| Koskenvuo et al. | RV (22%), RSV (11%), HBoV 5%) | 27/61 | 3RV, 1 RSV |
| Lindblom et al. | RV (17%), HCoV (9%), HAdV (4%) | 8/41 | 2 RV, 1 HCoV |
| Suryadevara et al. | RV/EV (32%), Influenza (10%), PIV (6%) | 15/23 | 0 |
| Srinivasan et al. | RV (49%), PIV (12%), RSV (11%) | 119/193 | 6 RV, 2 influenza* |
| Benites et al. | RV (23%), RSV (9%), HCoV (7%) | N/A | N/A |
| Söderman et al. | RV (24%), HCoV 8%, influenza (5%) | 32/39 | 4 RV, 1 HCoV |
| Torres et al. | RV (16%), RSV (9%), PIV (6%) | N/A | N/A |
| Aldemir-kocabas et al. | RV (16%), RSV (10%), HCoV (10%) | 28/43 | 2 RV, 1 HCoV, 1 PIV |
| Meena et al. | RV (25%), HCoV (12%), PIV (12%)** | N/A | N/A |

*persistent >100 days

**combined cases and controls

Abbreviations: EV, Enterovirus, HBoV, Human Bocavirus; HCoV, Human Coronavirus; N/A, Not investigated/reported; PIV, Parainfluenza virus; RSV, Respiratory syncytial virus; RV, Rhinovirus

In **paper I**, there was no control group. However, the study participants served as their own control, as follow-up sampling was performed. This study design identified children still positive for a respiratory virus weeks after the first sample was collected. All children who were repeatedly positive for RV (n = 4) still reported respiratory symptoms at follow-up, which occurred after ~1.5-7 weeks, which raises the question of whether these children had prolonged viral shedding due to their immunosuppression. Studies have been conducted following virus-positive children in immunocompetent cohorts (79, 242, 246-248). Jartti *et al.* studied both EV and RV and reported shedding times of 2–3 and 5–6 weeks, respectively (79). However, they did not genotype the RV samples, so the RV-positive follow-up samples could have represented a new infection because >100 different RV subtypes are known. Another study by Martin *et al.* followed a cohort of children at daycares with repeated sampling (after 7–10 days) and registered respiratory symptoms (242). They identified RV 41 days after symptom debut, HBoV and HAdV after 44 days, and RSV virus after 47 days. The other viruses detected had a shorter shedding time of 7–26 days (Table 1, p. 7). Hence, shedding time appears to differ between viruses even in immunocompetent cohorts, not only in immunosuppressed cohorts. Also in this respect, the results are in concordance with those of **paper I** and similar studies that investigated viral infections during febrile neutropenia, during which viruses such as influenza, PIV, and HMPV seem to be rare weeks after the acute infection, while RV and HCoV are more frequently detected at follow-up (Table 2) (52, 68, 77, 240). In **paper I**, the shedding times for different viruses did not seem to differ from what had been reported in immunocompetent children. However, the cohort in **paper I** was rather small, children included had a higher median age than many of those studies involving immunocompetent children, and only a few cases of each virus were detected, making it difficult to draw definitive conclusions regarding the shedding times.

4.1.1.3 Limitations

One limitation in **paper I** was the lack of a control group. A matched control group of children who were neutropenic but without fever would have been desirable to investigate the presence of asymptomatic detection of viruses in an immunosuppressed cohort. A control group was included early in the study. However, all of these patients developed a fever within a few days from sampling and therefore had to be excluded. Due to this issue, the study had to proceed without a control group. However, the study participants served as their own control group. Furthermore, the design of **paper I** was not ideal for determining the exact shedding times of different respiratory viruses, as such a study would have demanded at least weekly (preferably daily) sampling, which was not possible in this study. Lastly, the cohort was rather heterogeneous, with children treated for diverse malignancies and different age groups, and children included at different time points of their treatment.

4.1.1.4 Summary

Paper I added new perspectives to the field; its longitudinal study design followed all virus-positive children, presented the Ct values, which helped to further address the respiratory viral findings, and sequenced the RV/EV-positive episodes to investigate persistent or reinfection with a new genotype. In addition, with the knowledge that: (i) respiratory viral infections are commonly detected, (ii) respiratory viruses are mainly cleared within a shorter period (with the exception of RV and HCoV), (iii) low Ct values indicate a high viral load, and (iv) the detection rate of bacterial BSI was low, the results in **paper I** strengthen the evidence that the respiratory virus are a common cause of febrile

neutropenia episodes. However, some issues still remain: (i) asymptomatic virus detection is common, (ii) there is a prolonged shedding time (mainly for RV and HCoV) and (iii) simultaneous infection with bacteria occurs wherefore causality could not be proven. Against this background, clinical management changes should not be implemented solely based on the results in **paper I**. One remaining challenge is to develop microbiological methods that can correlate a positive viral sample with an actual infection. **Paper IV** was an attempt to further address the etiological role of the viral and bacterial findings during episodes of febrile neutropenia.

4.1.2 Paper IV

Paper IV took advantage of the same cohort used in **paper I** for all children where a blood sample was collected within 72 hours from fever onset ($n = 63$). In 20 of these, there were insufficient amounts of RNA, leaving 43 case samples collected from 35 unique individuals (Figure 5, p. 25). A majority of the children diagnosed with only a bacterial infection did not express sufficient amount of RNA (60%) (Figure 5, p. 25). The control samples were collected in the same cohort, but sampled at a time when the children were asymptomatic and 12 episodes, representing 12 unique individuals were included in the final analysis (Figure 5, p. 25). First, the RNA insufficient group ($n = 20$) was compared to the case group ($n = 43$) and lower total WBC count and ANC (p -value <0.001 for both), a higher C-reactive protein (CRP) and higher number of days of antibiotics (p -value <0.01 and <0.05 , respectively) were identified for the RNA insufficient group. Second, the different case groups (viral, co-infection and FOU, respectively) were compared to the control group. Results showed a higher WBC count and ANC in the control group compared to viral and FOU (presented in **paper IV**). When comparing the control group to the co-infection group, only a higher ANC in the control group was identified (presented in **paper IV**). These findings were expected, as the samples from the control group were collected at a point in time when the children are usually not neutropenic.

The case groups with viral, co-infection, bacterial and FOU were then pairwise compared to the control group to investigate whether a specific transcriptional profile in the immune response during the febrile neutropenia episodes could be identified as compared to when the children were asymptomatic. For the viral and FOU group, the majority of differentially expressed genes were up-regulated (178/231 and 261/443, respectively), while for the co-infection group the majority was down-regulated (124/219). For bacterial infection compared to the control group, there were only two genes that were differently expressed and therefore no further analysis was performed in this group. When performing pathway analysis for the viral, co-infection and FOU groups, none of the up-regulated genes could be enriched to the innate immune response for any of the groups. Interestingly, for the co-infection group, genes involved in the IFN signaling pathway was identified as the top ranked pathway of down-regulated genes (Figure 7). This results was surprising and contradictory to both our hypothesis as well as earlier published findings in immunocompetent children (229).

4.1.2.1 Innate immune signatures in children

A few studies have investigated blood innate immune signatures in children with fever with a diagnostic perspective, mainly by using microarrays. Microarrays constitute a slightly different but comparable method to RNA sequencing because both methods measure the transcriptome (249, 250). One of the first studies performed in a pediatric cohort was by Ramilo *et al.*, in which the

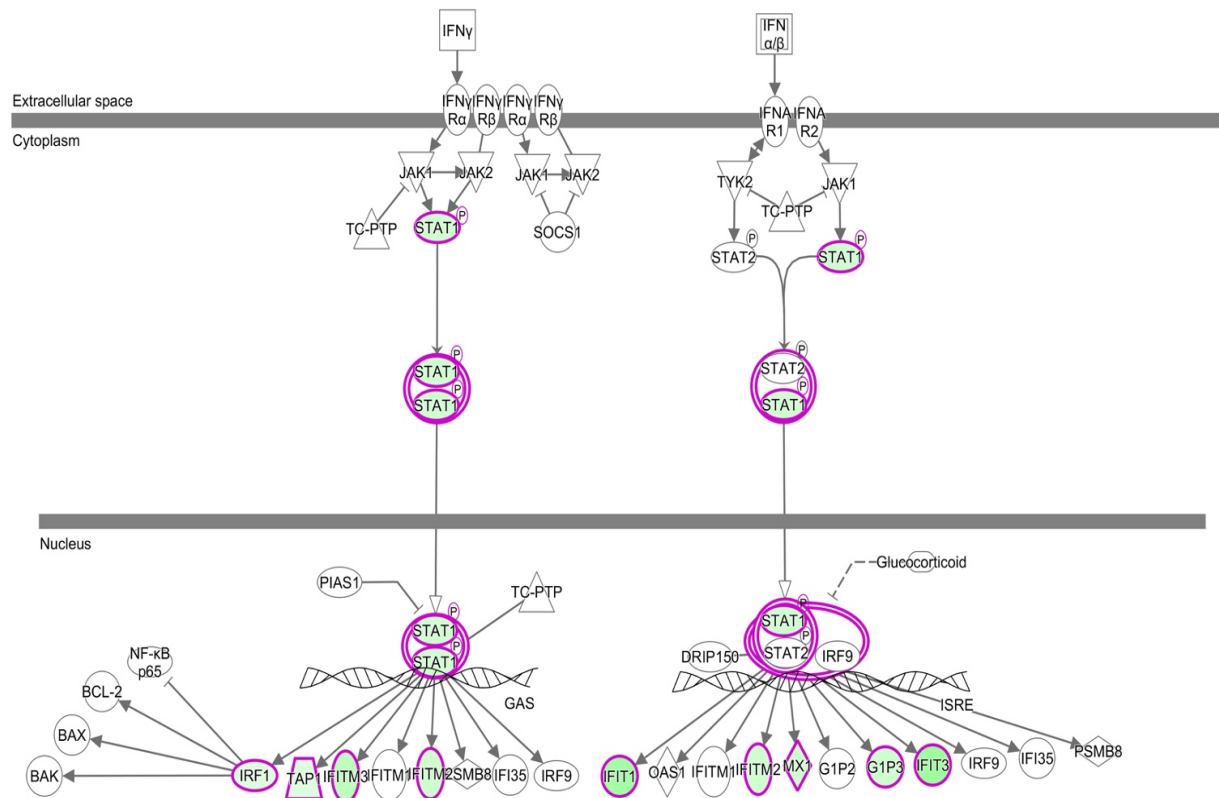


Figure 7: IFN-signaling pathway for co-infection versus control group. Green indicates the significant down-regulated genes (figure generated from IPA)

Abbreviations: GAS, gamma-activated sequence; IFN, Interferon; IFN γ , Interferon gamma, IRF, Interferon regulatory factors; JAK, Janus Kinase; NF- κ B, Nuclear factor kappa-light-chain-enhancer of activated B cells; OAS, Oligoadenylate synthetase; PIAS1, Protein Inhibitor Of Activated STAT 1; SOCS1, Suppressor of cytokine signaling 1; STAT, Signal transducer and activator of transcription. TC-PTP, T-Cell Protein Tyrosine Phosphatase, Tyk, Tyrosine Kinase 2.

authors demonstrated that they could distinguish children with acute infection by influenza, *E. coli*, *S. aureus*, and *S. pneumoniae* using gene expression patterns. Another study by Hu *et al.* included both febrile children (with different viruses and bacterial infections) and afebrile children (both positive for viral infections and negative controls) (229). The latter study could identify specific signatures that could distinguish between viral and bacterial infections. Interestingly, the transcriptional profiles were different between febrile and afebrile children positive for the same viruses, and the afebrile group was indistinguishable from the profile of virus-negative children. This was a relevant finding, as one of the issues discussed in this thesis is how to interpret the viral findings, especially those of respiratory viruses. Such results could therefore be of practical importance to distinguish asymptomatic and symptomatic infections (229). In a later study by Herberg *et al.*, a larger cohort was included, and both test and validation sets were assessed. The authors were able to identify a 2-transcript signature to distinguish viral from bacterial infection. Expression of IFI44L (viral) and FAM89A (bacterial), were increased as compared to healthy children. These specific 2-transcript signatures were also identified in subsequent studies in febrile children (251, 252). Another important issue to address is how the WBC count affects the results from transcriptomics. Hu *et al.* was not able to identify a correlation between the total WBC count and the gene expression levels. However, the

authors did identify that the expression of specific clusters of genes were associated with ANC, lymphocyte, and monocyte counts and therefore suggested that the clusters might be activated by specific cells types (229).

In **paper IV**, we wanted to investigate if gene-expression profiles could be identified in a cohort of children treated for cancer, as we were unsure of whether these children would have a detectable immune response with these methods due to immunosuppression. First, almost one third of the samples collected did not contain sufficient amounts of RNA to be able to perform the analysis, due to low amount of WBC count and ANC. Second, even in those samples with sufficient RNA, we could not identify any specific innate signatures for the different microbiological pathogens. To be able to correctly diagnose bacterial infections during febrile neutropenia is of utter most importance, as many of the treatment related deaths are due to these infections. However, in our study, the cases with bacterial infection were overrepresented in the group with insufficient RNA for RNA sequencing and when comparing the bacterial group with sufficient RNA to the control group there were only two genes that differentiated (Figure 5), which hampers the interpretation of the results. As for respiratory virus roles during these episodes, the results from **paper IV** could not answer whether the detected virus in nasopharynx was indeed causing the febrile neutropenia episode as no viral specific gene-expression signatures in blood were identified. The results that the co-infection group had a down-regulated innate immune response was, as mentioned, a surprise. We cannot fully explain the results identified. Plausible theories that could explain the findings are that the innate immune cells were redistributed to the affected tissues and therefore not abundant in the circulation or that the immune response was downregulated due to the immunosuppressive treatment. Lastly, it is still not fully known how chemotherapy affects the immune response. Is it possible that the chemotherapy affects the few remaining cells still present, and hamper the generation of a proper immune response? Is that the reason why we did not see the same pattern as in studies with immunocompetent children? In an earlier study performed by us, prolonged viral shedding did not seem to be a major issue in this patients cohort; hence, these children do have an immune response that is able to clear respiratory viruses (69). Therefore, is the immune response at the local site sufficient and could specific innate immune signatures be detected in mucosal tissue? Yu et al. identified an IFN response at the local site during viral infection, much like the signature in blood. Therefore, in future studies both blood samples and samples from relevant mucosal sites should be collected to fully evaluate the response in this patient cohort.

4.1.2.2 Limitations

In general, there is still limited data from the use of blood innate immune signatures as a diagnostic tool and the present study was also subject to a few limitations that should be considered. First, the samples size was small and the cohort was heterogeneous as it consisted of children treated for different cancer diagnoses. Another issue was that one-third of the blood samples did not contain sufficient RNA and therefore had to be excluded. Therefore, based on the limited size of our study, it would be difficult to assess this method as a diagnostic tool, since there would be a significant risk (over 30%) that the samples would not contain sufficient amounts RNA to perform the analysis.

4.1.2.3 *Summary*

Results from **paper IV** did not reveal similar transcriptional profiles as in cohorts of febrile immunocompetent children. In fact, results indicate that gene-expression profiling is difficult to use in a cohort of children treated for cancer, since the amount of RNA from blood was insufficient and blood immune response seems to differ from that of otherwise healthy children. Due to these issues, the feasibility of using transcriptomics in children presented with febrile neutropenia during cancer treated remains challenging. Additional studies are needed to further investigate this response and the feasibility of using gene-expression profiling as a diagnostic method in this patient cohort, preferably using both local and blood samples.

4.2 RISK FACTORS OF FEBRILE NEUTROPENIA

In **papers II** and **III**, risk factors of side effects during treatment for ALL were assessed by investigating genes coding for important enzymes and proteins in drug metabolism and the immune system. Both papers included a homogeneous cohort of children treated for ALL within the NOPHO ALL 2000 and 2008 protocols. In **paper II**, the main focus was enzymes important in drug metabolism (*i.e.*, *TPMT*, *ITPA*, and *NUDT15*). These enzymes were of interest because the hypothesis stated that sequence variants in the genes increased the risk of neutropenia and febrile neutropenia and also led to lower end doses of 6-MP. **Paper III** assessed the characterization of microbiological findings during episodes of febrile neutropenia in children during the entire 2.5 years of treatment for ALL. In addition, in **paper III**, investigated proteins important in the immune system (*i.e.*, TLR4, MBL, and IL-1Ra) with the hypothesis that sequence variants in these genes may lead to an increased risk of episodes of febrile neutropenia and associated MDI. The knowledge of a child's individual risk of febrile neutropenia and specific infections during these episodes could improve risk stratification during cancer treatment and thereby prevent these events with regard to dose reduction in children deficient in drug metabolism genes as well as to tailor prophylaxis.

4.2.1 Paper II

In **paper II**, 102 children were included in the final analysis. Younger age and the NOPHO ALL 2008 protocol were associated with neutropenia and febrile neutropenia during the entire treatment period and the maintenance II phase (only associated with age). Therefore, these factors were adjusted for in the final analysis. Both factors were expected because infections are more common at young age (with fever as a consequence), and the NOPHO ALL 2008 protocol contained slightly higher doses of chemotherapy during the entire treatment period but not during the maintenance II phase. Because 6-MP and MTX are the only drugs administered during the maintenance II phase, investigating this phase separately was of great interest. The results from **paper II** revealed that children deficient in any of the *TPMT* sequence variants had a decreased risk of both neutropenia and febrile neutropenia during the maintenance II phase. In fact, none of the 10 patients with at least one *TPMT* sequence variant had any episodes of febrile neutropenia during the maintenance II phase. These results were unexpected because the hypothesis stated that, due to dose reduction, *TPMT*-deficient children would have an increased or the same risk of neutropenia and febrile neutropenia compared to *TPMT*-wild-type children. No significant difference in 6-MP end dose was identified for *TPMT*-deficient children as compared to *TPMT* wild-type children (mean end doses 53.0 mg/m² and 61.4mg/m², respectively; $p = 0.098$). Another notable result in **paper II** was that children deficient in *NUDT15* received lower end doses of 6-MP (mean end dose 40.4 mg/m²; $p = 0.0097$), which was in line with our hypothesis. A *NUDT15* variant is rare in European cohorts, reported only in 0.2% of the population (128). The frequency of 4% observed in the study was therefore higher than expected. It should be emphasized that these results must be carefully interpreted because of the small sample size. For *ITPA*, the results revealed that children deficient in *ITPA* rs1127354 had a decreased risk of febrile neutropenia during the entire treatment period (unadjusted). These results were also unexpected and contrary to our hypothesis. No significant associations with the end doses of 6-MP and *ITPA* deficiency were seen.

4.2.1.1 *The role of TPMT in children treated for ALL*

Early studies investigating *TPMT* deficiency revealed that patients had an increased risk of myelosuppression and were more intolerant to 6-MP treatment (Table 3) (139). This resulted in implementations of recommended 6-MP dose reduction for deficient patients in many of the ALL treatment protocols. Subsequent studies also found *TPMT*-deficient patients to be less tolerant to 6-MP, and they receive lower doses when adjusted according to WBCs (107, 119, 128) (Table 3). In contrast, the analysis in **paper II** showed *TPMT*-deficient children to have decreased risks of neutropenia and febrile neutropenia during the maintenance II phase. One reason for this observation could be that the clinicians were aware of the child's *TPMT* genotype and more careful when increasing the 6-MP dose (adjusted according to WBC during maintenance II). There was a trend towards lower end-doses of 6-MP in *TPMT*-deficient children as compared to wild-type patients, but this was not statistically significant. However, solely based on the results from **paper II**, we cannot fully understand the decreased risk of these conditions, and this need to be addressed in larger cohorts.

4.2.1.2 *The role of NUDT15 in children treated for ALL*

In study populations of Asian origin, the frequency of *TPMT* deficiency is low and the *NUDT15* deficiency is higher. Consequently, *NUDT15* has become a topic interest during 6-MP treatment in this population (105, 128, 138). *NUDT15* was identified as a potent predictor for myelosuppression and 6-MP intolerance, and dose reduction is often needed in deficient patients (Table 3) (119, 125, 128, 160, 161). Therefore, recommendations to detect sequence variants in *NUDT15* and make dose adjustments in *NUDT15*-deficient patients have been implemented in some of the newer ALL treatment protocols (162). Even though *NUDT15* was rarely identified in **paper II** ($n = 4$), the results indicated the same conclusion: patients deficient in *NUDT15* were more intolerant to 6-MP and therefore received lower end doses. In **paper II**, no correlation with neutropenia or febrile neutropenia was identified. This finding could possibly be explained by the fact that deficient children received lower doses, hence neutropenia did not occur. Another factor that could explain the findings is that the sample sizes were too small ($n = 4$), and therefore, differences were not discovered.

4.2.1.3 *The role of ITPA in children treated for ALL*

Studies investigating *ITPA* have presented contradictory results. Some identified a correlation between reduced doses of 6-MP and an increased risk of febrile neutropenia or more severe febrile neutropenia episodes, while other have not identified any such association (Table 3) (107, 118, 119, 123, 125, 152, 154, 155). In **paper II**, no increased risk of neutropenia or febrile neutropenia or dose reduction of 6-MP was identified. On the contrary, *ITPA* rs1127345 was associated with decreased risk of febrile neutropenia (unadjusted for age at diagnosis and treatment protocol). Interestingly, Hareedy *et al.* reported that children with wild-type *ITPA* rs1127345 had an increased risk of leukopenia compared to deficient patients (Table 3). Similar results were described in a study by Ban *et al.* where *ITPA* rs1127345 was investigated in patients with inflammatory bowel disease treated with 6-MP (reported as not significant) (253). However, the different degrees of immunosuppression between inflammatory bowel disease patients and children treated for ALL makes the results difficult to compare. Due to the contradicting results in earlier studies as well as our own study, and as thiopurine metabolism is complex, the role of *ITPA* still needs further evaluation.

Table 3: Sequence variants in TPMT, NUDT15 and ITPA and risk of neutropenia, febrile neutropenia and affected doses of 6-MP during pediatric ALL treatment

| STUDY | SEQUENCE VARIANT | COUNTRY | LOW WBC | FN | DOSES |
|-------------------|------------------|--------------------------------|--|--------|-------|
| Relling et al. | TPMT | USA | ↑ ¹ | → | ↓ |
| Stocco et al. | TPMT | USA | N/A | → | ↓ |
| Yang et al. | TPMT | USA | N/A | N/A | ↓ |
| Liang et al. | TPMT | China | N/A | N/A | ↓ |
| Zgheib et al. | TPMT | Lebanon | N/A | N/A | ↓* |
| Gerbek et al. | TPMT | Denmark | N/A | N/A | N/A |
| Zhou et al. | TPMT | China | N/A | N/A | ↓ |
| Moradveisi et al. | TPMT | Lebanon and Iran | N/A | ↑** | ↓* |
| Stocco et al. | ITPA | USA | N/A | ↑ | → |
| Tanaka et al. | ITPA | Japan | → | N/A | ↓ |
| Azimi et al. | ITPA | Iran | ↑ ² | N/A | N/A |
| Hareedy et al. | ITPA | Egypt | ↑ ^{a, 1, 3} ↓ ^{b, 3***} | N/A | N/A |
| Chiethong et al. | ITPA | Thailand | → | N/A | → |
| Gerbek et al. | ITPA | Denmark | N/A | N/A | N/A |
| Zhou et al. | ITPA | China | N/A | N/A | → |
| Moradveisi et al. | ITPA | Lebanon and Iran | N/A | ↑** | ↓ |
| Tanaka et al. | NUDT15 | Japan | ↑ | N/A | ↓ |
| Yang et al. | NUDT15 | USA | N/A | N/A | ↓ |
| Chiethong et al. | NUDT15 | Thailand | ↑ | N/A | ↓ |
| Liang et al. | NUDT15 | China | N/A | N/A | ↓ |
| Moriyama et al. | NUDT15 | Guatemala, Singapore and Japan | N/A | N/A | ↓ |
| Suzuki et al. | NUDT15 | Japan | → | N/A | ↓**** |
| Moriyama et al. | NUDT15 | Japan | N/A | N/A | ↓ |
| Zgheib et al. | NUDT15 | Lebanon | N/A | N/A | ↓* |
| Yi et al. | NUDT15 | Korea | ↑ ³ | ↑***** | ↓ |
| Zhou et al. | NUDT15 | China | ↑ | N/A | ↓ |
| Moradveisi et al. | NUDT15 | Lebanon and Iran | N/A | →* | ↓* |

↑: Increase/higher levels, ↓ Decrease/lower levels, → No association

*Few deficient patients, ** combined ITPA and TPMT and increased onset of FN, *** wild-type alleles higher risk as compared to heterozygous, ****<7years of age, *****In children with two defect alleles

¹Neutropenia, ²Myelosuppression, ³Leukopenia

^ars 7270101, ^b rs1127354

Abbreviations: FN, Febrile neutropenia; ITPA, inosine triphosphate pyrophosphatase, N/A, not investigated/reported; NUDT15, Nudix hydrolase 15; TPMT, Thiopurine methyltransferase; WBC, White blood cells.

4.2.1.4 Combined sequence variants in *TPMT*, *NUDT15*, and *TPMT*

Hypothetically, would the presence of multiple sequence variants increase the risk of side effects even more? In **paper II**, eight children had >1 sequence variant in *TPMT*, *ITPA* and *NUDT15* and these tended to have lower 6-MP end doses (Figure 8). Interestingly, one child had both *TPMT* and *NUDT15* deficiency and a low 6-MP end dose of ~30 mg/m²/day. Liang *et al.* and Zhou *et al.* reported that children with both a deficiency in *TPMT* and *NUDT15* (n = 4 and n = 1, respectively) also had doses of 6-MP ~30 mg/m²/day. However, it was not possible to perform any statistical calculations based on this data due to the small sample sizes in each group in **paper II**.

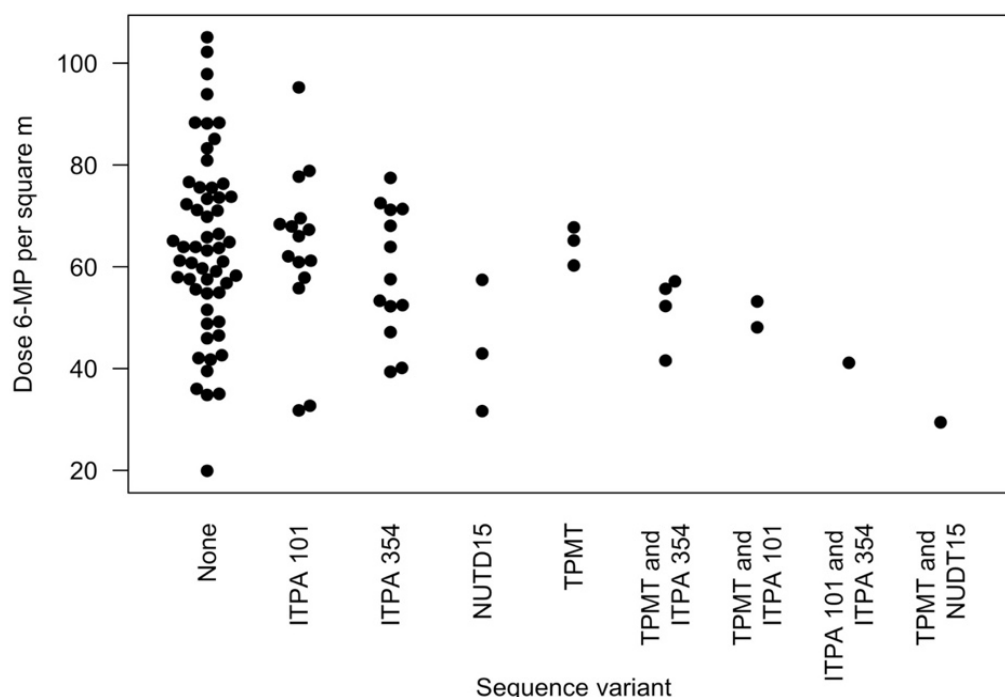


Figure 8: Genetic sequence variants in *TPMT*, *ITPA* and *NUDT15* and 6-MP end doses.

Abbreviations: *ITPA*, inosine triphosphate pyrophosphatase; *NUDT15*, Nudix hydrolase 15; *TPMT*, Thiopurine methyltransferase.

4.2.1.5 Limitations

Some limitations in **paper II** need to be addressed. First, the study was of retrospective design, and information was collected from medical records. However, strict pre-defined rules for the definition of neutropenia, febrile neutropenia, and end doses of 6-MP were used. Due to the retrospective design, the 6-MP metabolites (TGNs) were not available, although this would have provided valuable information. Another issue is that only the most common sequence variants in *TPMT*, *ITPA*, and *NUDT15* were investigated. Therefore, misclassification bias was possible, in which children with more uncommon variants were classified as “wild-type” by default (137). However, studies have determined that the three most prevalent *TPMT* sequence variants yield ~95% concordance between genotype and phenotype and therefore the risk of misclassification was relatively small (135, 137). In **paper II**, a few children without any of the sequence variants detected still had very low 6-MP doses (one patient with as low as ~20 mg/m²/day), and these children may have had another sequence variant not investigated in the study (Figure 8).

4.2.1.6 Summary

Paper II contributed to the field by investigating *TPMT*, *ITPA*, and *NUDT15* in a cohort of pediatric patients treated for ALL in Sweden. To the best of our knowledge, *ITPA* and *NUDT15* had not been previously investigated in this cohort within the Nordic countries. Most studies have concluded that *TPMT* and *NUDT15* play important roles in 6-MP metabolism and the risk of side effects (Table 3). However, the role of *TPMT* and *ITPA* deficiency for febrile neutropenia was not fully clarified in **paper II**. In addition, *NUDT15* seem to play a role for the treatment of 6-MP, resulting in lower end doses of 6-MP. However, due to the relatively small cohort and the low prevalence of some of the genetic variants investigated, additional larger studies are needed.

4.2.2 Paper III

In **paper III**, 122 children were included in the analysis (Figure 5, p. 25). A total of 380 episodes of febrile neutropenia were identified, which 64 represented solely viral infection (16.8%), 61 (16.1%) solely bacterial BSI, 2 (0.5%), or solely IFD, and the fever was defined as FOU in 240 episodes (one episode was undefined) (Figure 6). Nine episodes were mixed bacterial BSI and virus, six episodes had both Gram-positive and Gram-negative bacteria identified, and four episodes were IFD mixed with either bacterial BSI and/or virus. During the study period, two (2%) children died due to infection-related causes. The risk of febrile neutropenia and MDI during these episodes was associated with age, treatment protocol (NOPHO 2008 protocol), and treatment intensity (HR group, with exceptions for viral infections and Gram-positive bacteria). These factors were adjusted for in the final analysis. The results were similar to those of a study by Li *et al.*, in which both age and HR groups were associated with fever and MDI in children treated for ALL (55). When investigating sequence variants in genes coding for TLR4, MBL, and IL-1Ra, sequence variants in *TLR4* rs10759931 and rs11536889 were associated with an increased risk of viral infections. In addition, children with either the IL1RN*2 or IL1RN*3 genotype had a decreased risk of bacterial BSIs. A sub-analysis of children with bacterial BSI showed that genotype IL1RN*2 had a decreased risk of Gram-negative bacteria infection, while children with IL1RN*3 did not have any Gram-positive bacteria detected. The sub-analysis of the bacterial BSIs also revealed that children with sequence variants in *TLR4* rs4986791 had a decreased risk of infection with Gram-negative bacteria.

4.2.2.1 Microbiological findings during episodes of febrile neutropenia

There are still limited data on infection-related complications during ALL treatment. Only two studies investigating febrile neutropenia and infections during the entire ALL treatment could be identified (55, 56). These studies reached different conclusions for the most commonly detected agents; Inaba *et al.* reported respiratory tract infections to be the most prevalent, while Li *et al.* found that bacterial BSIs were the most common infections. However, one issue with these studies is that they reported episodes of febrile neutropenia and then infectious agents during treatment separately. This is one of the differences compared to **paper III**, in which the strength of the study is that all infectious agents detected during febrile neutropenia episodes were reported. In the clinic, febrile neutropenia is a dreaded complication since it can rapidly worsen into severe conditions if not immediately treated. The reported bacterial BSIs in **paper III** were in line with earlier studies reporting bacterial BSIs during cancer treatment, even though those studies did not follow the same children during the entire treatment period (Figure 6, p. 31) (52, 55, 65, 254). As discussed in **paper I**, bacterial BSIs do not

seem to be that common (<30%). Despite these low numbers, all children with febrile neutropenia receive broad-spectrum antibiotics ranging from a median/mean of 4–10 days and are treated at the hospital for a median of 4–8 days (52, 65-69). The viral infections reported in **paper III** were lower both compared to **paper I** and to earlier studies with reports of 44-52% (Figure 6, p. 31). The main reasons for this discrepancy is probably that the testing for respiratory viral infections in **paper III** was not as stringent and the testing was not performed in all episodes of febrile neutropenia as part of the clinical routine together with the fact immunofluorescence was the method used for detection of respiratory virus at the Karolinska laboratory during the first part of the study period. One major issue still remains: in ~40–70% of the episodes of febrile neutropenia, no cause of the fever could be identified wherefore those episodes were defined as FOU (Figure 6, p. 31) (52, 55, 56, 65, 66, 77). The results from both **paper I** and **paper III** highlight the importance of developing rapid and more sensitive methods for the detection and interpretation of microbiological agents during febrile neutropenia. This would facilitate the implementation of more individualized treatments, leading to a decrease in days of antibiotic treatment and in-hospital care.

4.2.2.2 *The role of TLR4 in children treated for ALL*

Studies investigating *TLR4* sequence variants have mainly been conducted in adults treated for malignancies (181, 202). These studies have identified sequence variants in *TLR4* as a potential risk factor of Gram-negative BSIs and as carrying increased risks of sepsis and pneumonia (Table 4) (181, 202). However, similar data for the role of *TLR4* in immunosuppressed children is scarce. Miedema *et al.* investigated a pediatric cohort and found four different *TLR4* sequence variants that independently increase the risk of neutropenia during ALL treatment and concluded that additional studies were needed to evaluate the risk of febrile neutropenia in *TLR4*-deficient children. In **paper III**, two sequence variants in *TLR4* were associated with an increased risk of viral infections, and these two were the same as identified in Miedema *et al.* This raises the following questions: does the viral infection increase the risk of neutropenia, or vice-versa? Or, are the two conditions not correlated? To investigate neutropenia episodes in our cohort to address these questions would have been interesting. *TLR4* is known to bind LPS present on Gram-negative bacteria, even though it is also an important receptor for recognizing specific viruses (217, 255). However, the role of all the sequence variants and the effect on phenotype and loss of *TLR4* receptor function is not fully understood, and studies in this area have been difficult to identify. In addition, the different sequence variants of *TLR4* in **paper III** included sequent variants in promoter, exons, and intronic elements of the genes. Therefore, challenges remain in interpreting the results regarding *TLR4* in a clinical setting.

4.2.2.3 *The role of MBL in children treated for ALL*

MBL deficiency has been investigated in several different studies during pediatric cancer treatment. However, due to inconsistent results, there are still no conclusions of whether this protein plays a role during episodes of febrile neutropenia (Table 4) (181-184, 186, 256, 257). Dommett *et al.* and Pana *et al.* reported that MBL-deficient children have an increased risk of multiple episodes of febrile neutropenia and infections (186, 257). These results were not reproduced in **paper III**, and no associations between MBL deficiency and episodes of febrile neutropenia or MDI during these episodes were identified. The results in **paper III** were in line with studies by Neth *et al.*, Frakking *et al.*, and Lausen *et al.* in that neither identified a correlation to febrile neutropenia nor infectious

episodes (183, 184, 256). However, none of those studies followed the children during the entire treatment period, and with the exceptions of Pana *et al.* and Lausen *et al.*, studies have used heterogeneous cohorts of children treated for different types of malignancies (181-186). The inconsistent follow-up time and heterogeneous cohorts have hampered the interpretation of MBL's role during febrile neutropenia and infectious episodes. Therefore, the results in **paper III**, with both a homogeneous cohort of children and follow-up during the entire ALL treatment period, contributes to the field. The findings suggest that MBL does not play a major role during episodes of febrile neutropenia and specific infections during these episodes.

4.2.2.4 The role of IL-1Ra in children treated for ALL

There are limited data on the role of IL-1Ra in children with cancer and risk of febrile neutropenia, and a study conducted by Zapata Tarres *et al.* was one of the few identified. Children treated for ALL were included, and the authors identified associations with the genotype IL1RN*2 and the risk of septic shock. This finding was contrary to the results from **paper III**, in which IL1RN*2 and IL1RN*3 were associated with a decreased risk of bacterial BSIs. These findings were not expected and opposed our hypothesis. However, when starting to thoroughly review the literature, an interesting study by Danis *et al.* was identified. They investigated IL-1Ra immunocompetent individuals and suggested that sequence variants in the IL-1Ra gene not only affect the IL-1Ra protein, but also the IL-1 α protein, and proposed that either the gene directly affects IL-1 α production or the two alleles

Table 4: Summary of studies investigating genetic sequence variants in MBL, TLR4, and IL-1Ra and the risk of neutropenia, febrile neutropenia and MDI during pediatric cancer treatment

| | Sequence variant | Included patients | Study population | Diagnose | Follow up | Results |
|-----------------------------|------------------|-------------------|------------------|----------|-----------|--|
| Lausen <i>et al.</i> | MBL | 136 | P | ALL | 50d | No association* |
| Neth <i>et al.</i> | MBL | 100 | P | Mixed** | 6m | Increased days of febrile neutropenia |
| Frakking <i>et al.</i> | MBL | 110 | P | Mixed** | 13m | No association* |
| Pana <i>et al.</i> | MBL | 44 | P | ALL | I-MT | Increased risk of bacterial infection Longer duration of febrile neutropenia |
| Dommett <i>et al.</i> | MBL | 269 | P | Mixed** | 12m | Multiple episodes of febrile neutropenia Multiple infections during febrile neutropenia |
| Fekete <i>et al.</i> | MBL | 107 | P | Mixed** | 2y | Shorter period of fever |
| Pehlivan <i>et al.</i> | MBL | 120 | A | ALL/AML | N/A | Increased risk of BSI and Gram-neg bacteria Higher mortality during febrile neutropenia |
| Miedema <i>et al.</i> | TLR4 | 194 | P | ALL | MT | Increased risk of neutropenia*** |
| Schnetzke <i>et al.</i> | TLR4 | 155 | A | AML | I | Increased risk of sepsis and pneumonia |
| Pehlivan <i>et al.</i> | TLR4 | 120 | A | ALL/AML | N/A | Increased risk of Gram-negative BSI |
| Pehlivan <i>et al.</i> | IL-1Ra | 120 | A | ALL/AML | N/A | No association* |
| Zapata Tarres <i>et al.</i> | IL-1Ra | 57 | P | ALL | 2.5y | Increased risk of septic shock**** |

* No significant differences between sequence variants and the investigated risk events were identified., **Refers to different hematological- and/or solid malignancies, ***Associated with rs10759931, rs11536889, rs1927911 and rs6478317, **** Associated with the IL1RN*2 variant.

Abbreviations: A; adults, ALL, acute lymphoblastic leukemia, AML; Acute myeloid leukemia, BSI, bloodstream infection; d; days, I; induction, IL-1Ra; interleukin-1 receptor antagonist, m; months, MBL; mannose binding lectin, MT; maintenance, N/A; Not reported, P; pediatrics, TLR4; Toll-like receptor 4, y; years

occur in linkage disequilibrium (*i.e.*, often inherited together). Similar results were also presented by Hurme *et al.* (2009). If these two ILs are both affected in IL-1Ra deficiency, does this increase or decrease the host's inflammatory response? Is an increased inflammatory response positive or negative for the host, as sepsis is not only induced by the bacteria itself but also by the massive inflammatory immune response? And is this response the same in immunosuppressed patients? Was our hypothesis correct? When further reviewing the literature, another issue emerged. Contradictory results regarding the effect on the phenotype (production of IL-1Ra) was identified for the IL1RN*2 allele. Danis *et al.* reported that healthy controls had higher levels of IL-1Ra than patients carrying IL1RN*2, while Arnalich *et al.* reported the opposite in a cohort of patients with sepsis. The immune response is very active during sepsis, and one explanation of the different results may be that because IL-1Ra is an antagonist, the production of this protein is suppressed during sepsis, resulting in its lower levels. As **paper III** only focused on the genotypes of the IL-1Ra gene and not levels of protein, these questions are difficult to address.

4.2.2.5 Limitations

Some obstacles must be overcome when obtaining clinical data retrospectively from medical records. Important data may not be documented. An optimal study design would have been prospective; however, to be able to include as many patients in Stockholm for **paper III**, the study would have needed to proceed for at least 12 years. To optimize our retrospective study design, we developed strict pre-defined criteria to determine whether episodes of febrile neutropenia and microbiological findings should be included. In addition, two independent clinicians controlled the included episodes and microbiological findings, to ensure that the same criteria were used. Another limitation was that the genotype for *TLR4* was not assessed because seven different sequence variants were investigated, and pyrosequencing could not determine the specific haplotype. Also, as previously mentioned, the phenotypes for many investigated *TLR4* sequence variants are not known, making it difficult to assess if all the investigated sequence variants in *TLR4* affect the receptor equally. As in **paper II**, only the most common and earlier reported sequence variants in the *TLR4*, *MBL*, and *IL-1Ra* genes were investigated. Therefore, misclassification bias was possible, in which children with more uncommon variants were incorrectly classified as “wild-type”. Ultimately, protein levels of *MBL* and *IL-1Ra* would have been ideal to compare to their genotypes, but this was not possible due to the study design.

4.2.2.6 Summary

Viral infections were the most commonly detected infectious agent. However, the majority of the episodes were still defined as FOU. To correctly address the etiology of febrile neutropenia episodes, a prospective study with samples for bacterial, viral, and fungal infections at every episode is needed. **Paper III** is one of the first studies investigating *TLR4*, *MBL*, and *IL-1Ra* in a homogeneous cohort and that follows children treated for ALL during the entire treatment period. *MBL* appears to play a minor role during episodes of febrile neutropenia during pediatric ALL treatment. However, the roles of *TLR4* and *IL-1Ra* need to be further investigated due to the complexity of the proteins and limited knowledge of how sequence variants affect phenotype. Therefore, additional prospective studies in larger cohorts that measure protein levels at the time of the fever would be of great interest before drawing any major conclusions.

4.3 ETIOLOGY AND RISK FACTORS FROM A CLINICAL PERSPECTIVE

4.3.1 How to manage children presenting with febrile neutropenia

Febrile neutropenia is a potentially lethal condition and often requires rapid assessment when suspected in the emergency room. Bacterial BSIs and IFDs are dreaded causes of the condition and sepsis can develop within hours from symptom onset in neutropenic patients. However, relatively few of these episodes develop into severe sepsis, and today the mortality rate due to infections is ~1–2% (56). The initial management with rapid administration of broad-spectrum antibiotics is probably why the outcome has improved for this condition. Respiratory viruses are the most common microbiological findings during febrile neutropenia episodes, often causing mild disease. Notably, there are still many episodes for which no cause of the fever can be identified. Despite this fact, all children receive many days of treatment with broad-spectrum antibiotics and days of in-hospital care. Hence a more individualized risk prediction model for the febrile neutropenia episodes is needed. The question is: what is a safe approach to predict the risk of severe episodes while decreasing the days of broad-spectrum antibiotics and in-hospital care? After approximately 48 hours (when results from blood cultures usually are received), changing the management of these episodes might be possible. Many different prediction models have been developed to try to risk stratify febrile neutropenia episodes (60, 258-261). Such models have not yet been thoroughly investigated and implemented in a safe manner in a clinical setting (62). Implementation of models would improve the management of this frequent complication during childhood cancer treatment. There are several different predictive variables that can be included in these models, including cancer diagnosis, treatment intensity phase and acute phase proteins. Microbiological findings and sequence variants in genes coding for different immune proteins/enzymes could also be considered in such prediction models to identify the children at high risk of severe infection who need to continue antibiotic treatment.

One of the challenges during my doctoral studies has been to answer the following questions: How does one interpret respiratory viral findings during febrile neutropenia episodes? Is it safe to withdraw antibiotics if a respiratory virus is detected during an episode? The methods used for respiratory virus detection in **paper I** are the same used in the clinic today. Still, difficulties remain in interpreting the results from positive respiratory viral findings, especially RV, EV, HCoV, HBoV, and in some cases, HAdV. In addition, all the episodes of FOU are in need of further investigation; what is the actual cause of the fever? We hoped that the results from **paper IV** could bring us closer to the answers. Unfortunately, **paper IV** could not address the outstanding issues, as the results indicated that these children did not have the same innate-immune-response signature as otherwise healthy children. However, we only investigated the immune response in blood. Yu *et al.* compared the immune response in blood and in the nasal compartment in children with respiratory viral infection and asymptomatic children (262). The authors were able to identify a local innate response in symptomatic children that were not present in the asymptomatic groups and identified both in blood and nose. However, the response also differed between the nose and blood, which the authors explained as the epithelial cells activate the immune response when they are infected themselves, whilst immune cells in blood are activated as a response to an infection. Nevertheless, all this indicates that by using gene-expression profiling it is possible to differentiate symptomatic from asymptomatic respiratory infections and that samples from relevant mucosal sites would be of value.

So, how can we improve management of an episode of febrile neutropenia today? One initial suggestion is that respiratory viral testing should be considered as part of the clinical routine when a child presents with febrile neutropenia, especially if any signs of respiratory symptoms are present.

Results positive for influenza, PIV, HMPV or RSV, together with respiratory symptoms, a negative blood culture, diagnosis and treatment phase of the patient, a low CRP level and a good general condition could be helpful in the decision to withdraw antibiotic treatment and discharge the patient after 48 hours. This is supported by a study conducted by Santolaya *et al.* (263). The authors stratified children after 48 hours with a positive respiratory viral finding, a negative blood culture, and favorable clinical evaluation (children excluded if they had a persistent fever, CRP ≥ 90 mg/L or hemodynamic instability) and then randomized these children to either continue the antimicrobials until the end of the febrile episode or withdrawal of treatment. The episodes resolved uneventfully in 95% of cases in which the antimicrobial treatment was withdrawn. In four cases in the withdrawal group, antimicrobial treatment was reinstated due to repeated fever, but no new bacterial infection or sepsis developed. The respiratory pathogens RV, EV, HCoV, HBoV, and HAdV could also be included in such a stratification but should be more cautiously interpreted as causative agents until more sensitive methods have been developed and hopefully introduced in the clinic. Similar studies on discontinuation of antibiotic treatment and early discharge have also been conducted; however, respiratory viral infections were not included in those stratifications (264, 265).

In addition, it would strengthen the risk prediction model of febrile neutropenia if we could identify genetic defects in proteins/enzymes important in the immune system that affect the risk of developing febrile neutropenia and MDI during these episodes. Unfortunately, in **paper III**, the results regarding MBL, TLR4, and IL-1Ra were not sufficient for model inclusion. However, additional investigations are requested, such as genome-wide association studies to identify novel proteins/enzymes associated with the risk of febrile neutropenia that later might be implemented as model predictors.

4.3.2 How to evaluate a child with repeated or prolonged neutropenia and febrile neutropenia during cancer treatment

When treating children with cancer, there is a fine balance between giving sufficient amounts of chemotherapy to eliminate the malignant cells without increasing the risks of severe side effects and secondary cancer (24). Neutropenia is a common condition during cancer treatment. However, how should children presenting with this condition be managed? The most common reason for neutropenia during treatment is as a chemotherapy side effect, however, only to a certain extent and it is not expected to be persistent. Other reasons for unexpected or prolonged neutropenia that need to be investigated are relapse of the disease or different infections (*e.g.*, B19V) (85). After ensuring that relapse or infection is not the cause, other reasons, such as pharmacogenetic variables, are of great interest and need to be investigated further.

In this thesis, sequence variants in enzymes important for drug metabolism were investigated, with 6-MP as a main focus. Importantly, signs of prolonged and otherwise unexplained neutropenia in children treated with 6-MP could be due to sequence variants in genes important for 6-MP metabolism. Evidence that both sequence variants in *TPMT* and *NUDT15* affect the prescribed doses of 6-MP and increase the risk of myelosuppression were reported in several studies, but the role of *ITPA* is still not fully understood (Table 3). Dose adjustments according to *TPMT* genotype have been implemented during the last decade in many of the clinical treatment protocols for ALL but were only recently introduced for *NUDT15* (162). Moreover, children with the combination of *TPMT*- and *NUDT15*-deficient genotypes seem to be more sensitive to 6-MP treatment with increased risk of myelosuppression (126, 266). In **paper II**, our results indicated that *TPMT*-deficient children had decreased risks of both neutropenia and febrile neutropenia during the maintenance II phase, which

was opposed to our hypothesis. However, we cannot fully explain these results and due to the small study cohort, future studies are needed to confirm these results. In addition, follow-up studies should also include evaluation of the relapse risk in deficient patients, as an increased risk of relapse has been reported after dose-adjustments for *TPMT* was implemented (148).

Why some children suffer from more frequent febrile neutropenia episodes has not yet to be fully understood. The sequence variants mentioned above could lead to an increased risk of neutropenia, and thereby increase the risk of febrile neutropenia. However, it could also be due to genetic variants in immune genes, making some patients more susceptible to infections. In **paper III**, we could identify that TLR4 deficient children had an increased risk of viral infections. However, the small and heterogeneous cohort made it difficult to draw any major conclusions. Nevertheless, it highlights that specific immune proteins or receptors probably play important roles in the development of different infections. The risk of developing both neutropenia and febrile neutropenia episodes during cancer treatment is probably due to multiple factors, both pharmacogenetic and immunological and the complexity of these factors need to be further investigated in order to properly evaluate children with frequent and prolonged neutropenia and febrile neutropenia episodes.

5 CONCLUSIONS

Overall conclusions:

- (i) In many of the episodes of febrile neutropenia, the cause of the fever is still unknown. Even though respiratory viruses are common during febrile neutropenia episodes there is a need for development of additional more sensitive methods to be able to identify and prove causality between the microbiological findings and the episode.
- (ii) Genetic variants in specific enzymes and proteins involved in drug metabolism and the immune system seem to play a role in the development of neutropenia, febrile neutropenia and infections during cancer treatment. However, some of these need to be further evaluated before modifications of the management of febrile neutropenia could be recommended.

Study specific conclusions:

Paper I

- Respiratory viral infections are common during episodes of febrile neutropenia, and most virus are cleared within a few weeks after the initial finding. This strengthens the evidence that respiratory viruses are the cause of febrile neutropenia episodes, however causality could not be proven in paper I.

Paper II

- Sequence variants in TPMT and ITPA (rs1127345) were identified to decrease the risk of febrile neutropenia in our study. However, due to the complexity of 6-MP metabolism and the small study cohort, it is still not fully understood how these genetic variants affect the risk of febrile neutropenia.
- Children with NUDT15 genetic variants received lower 6-MP end doses in our study. NUDT15 deficiency seem to play a role during 6-MP treatment. However, the analysis and conclusions of our study is limited by the low prevalence of NUDT15 in the cohort.

Paper III

- Viral infections are the most common microbiological findings during febrile neutropenia episodes in pediatric ALL patients. However, the majority of the episodes were defined as FOU.
- Sequence variants in the genes encoding TLR4 and IL-1Ra seem to alter the infection risk during febrile neutropenia episodes during pediatric ALL treatment. However, further investigation to evaluate these findings is needed.

Paper IV

- Based on our results, gene-expression profiling is not a suitable diagnostic tool for detection of pathogens in children treated for cancer. The low number of immune cells in these children, and the corresponding low amount of RNA for analysis, hinder the assessment of specific innate transcriptional pathways in blood.
- Respiratory viruses' role as an etiological agent could not be directly correlated to febrile neutropenia episodes by using gene-expression profiling from blood.

6 FUTURE PERSPECTIVES

As the survival rate continues to increase during cancer treatment, it is important to develop a proper strategy for the severe side effects sometimes related to treatment, such as febrile neutropenia. Some challenges remain in the management of children presenting with febrile neutropenia. First, all the episodes during treatment are not fully characterized with all the different microbiological agents in focus. Therefore, a proper prospective study investigating bacteria, viruses, and fungi with microbiology testing and clinical evaluation of the detected pathogens would be of great interest.

Antibiotic treatment is definitely essential during febrile neutropenia episodes; however, the issue of antibiotic resistance has emerged in the past decade (35, 36). As with all drugs, antibiotic treatment has side effects such as an increased risk of subsequent infections with resistant bacteria and complicated IFD (71, 72). In addition, intravenous antibiotic treatment necessitates in-hospital care in most cases. This highlights the second challenge during febrile neutropenia: the current methods for pathogen detection need improvement due to both low sensitivity (blood cultures in pediatric patients) and specificity (PCR) that make it difficult to rely on these results in a clinical setting. If a prospective study investigating the different microbiological agents during febrile neutropenia were to be performed, the method used in **Paper IV** (RNA sequencing) should be included. However, samples from relevant mucosal sites should be collected to further understand the immune response to pathogens in this patient cohort, with the main focus on respiratory viruses. Then, if specific signatures for the local response could be identified, this could help guide the management of these patients. Still, RNA sequencing is a rather time consuming and costly method, and more rapid and cost-effective methods are needed to have an impact on clinical management. PCR has been presented to validate the results from sequencing methods, and it is both time saving and can be performed at a relatively low cost (231, 267). Gómez-Carabella *et al.* developed a reverse transcription-PCR to test and validate the two structures (IFI44L and FAM89A) identified by Herberg *et al.* to distinguish bacterial from viral infections in febrile children and healthy controls (230, 267). However, the local response in immunosuppressed patients need to be evaluated first before similar studies could be performed.

With the new emerging SARS-CoV-2, there has been an increased interest in the role of respiratory viruses. For how long are respiratory viruses abundant in the host after primary infection? Is there such an entity as asymptomatic infections? For how long are the viruses contagious? Because of this new virus, I think and hope that the role of respiratory viruses will be better characterized and investigated to establish their role as infectious pathogens. Gene-expression analysis for respiratory viruses will be of great interest, both samples taken from the nasal cavity as well as the blood in order to be able to differentiate an active infection from a viable virus from viral remnants or asymptomatic infections. SARS-CoV-2 has not yet been reported as a major risk in children treated for cancer. However, to date, the role of SARS-Cov-2 is not comprehensively evaluated in this patient cohort.

The third challenge is that additional information regarding the risk of developing febrile neutropenia needs to be further evaluated. Within the pharmacogenetic field, certain enzymes increase the risk of side effects (e.g., *TPMT* and *NUDT15*) and therefore have been implemented in treatment protocols. However, similar proteins in the immune system have not been discovered and implemented to the same extent, but hypothetically, there are factors that indeed affect the child's risk of febrile neutropenia. Genome-wide association studies could be useful here, but only one such study, by Lund *et al.*, has been performed in a smaller cohort of children with febrile neutropenia in the

Nordic countries (185). However, as the results are difficult to interpret and confounders difficult to properly control for, larger cohorts with sufficient clinical data during the febrile neutropenia episodes are needed for such studies. If genetic defects in important immune proteins could be identified as risk factors, they could be used as predictive values for a better risk stratification model during febrile neutropenia. Such an algorithm could be evaluated in a study similar to Santolaya *et al.* but in a Nordic context to assess the safety of withdrawing antibiotic treatment during febrile neutropenia episodes (263). If microbiological pathogen detection, together with a proper febrile neutropenia risk stratification could be developed, this information would be of great clinical use to decrease broad-spectrum antibiotic use and time of in-hospital care. This in turn could lead to decreased risk of infections with resistant bacteria and improved quality of life in this patient cohort.

In summary, treatment of the different cancer diagnoses is increasingly individualized, with the goal to give patients just the “right amount” of chemotherapy — sufficient to cure the disease while causing minimal side effects. The same approach should also be applied during febrile neutropenia episodes in that all children should be stratified according to a febrile neutropenia risk group and managed in a more individualized manner. Hopefully, this scenario is clinically implemented within a few years. To reach this goal, more research within the field of febrile neutropenia during cancer treatment is needed.

7 POPULÄRVETENSKAPLIG SAMMANFATTNING

Överlevnaden för barn som behandlas för cancer har ökat under de senaste decennierna och är idag över 80%. Detta beror bland annat på nya läkemedel och en mer individualiserad behandling. Många barn upplever dock fortfarande biverkningar p.g.a. behandlingen, där ett lågt antal vita blodkroppar (så kallad neutropeni) och neutropen feber (neutropeni tillsammans med feber) är en allvarlig biverkning från cellgifter. När neutrofilema sjunker till under $0,5 \times 10^9/L$, ökar risken för infektioner kraftigt. Trots att infektionsdödligheten har sjunkit i den här gruppen orsakar den långa inläggande sjukhusperioder och många dagars behandling med bredspektrumantibiotika. Att kunna identifiera vilka agens som orsakar neutropen feber och vilka riskfaktorer det finns för att drabbas av neutropen feber skulle kunna användas i kliniken för att individualisera behandlingen för barn som behandlas för cancer. Det skulle i längden kunna leda till minskad tid på sjukhus och kortare behandling med bredspektrumantibiotika, vilket i sin tur skulle ha positiva sociala effekter på barnen och deras familjer såväl som minska risken för att drabbas av resistent bakterier. Min avhandling har därför haft två huvudfokus: Att undersöka etiologin till neutropen feber hos barn med cancer (**delstudie I och IV**) och att undersöka genetiska riskfaktorer för att drabbas av neutropeni och neutropen feber (**delstudie II och III**).

Etiologi till neutropen feber hos barn som behandlas för cancer

Luftvägsvirus är ett av de vanligaste mikrobiologiska fynden vid neutropena feberepisoder hos barn som behandlas för cancer vid användning av molekylärbiologiska metoder (så kallad ”*polymerase chain reaction*” (PCR)). Luftvägsvirus hittas i 44-52% av alla episoder, vilket också kunde bekräftas i vår **delstudie I** där luftvägsvirus identifierades som det vanligaste fyndet vid neutropen feber (45%). Det som skiljer vår studie från tidigare studier var att vi också tog uppföljningsprov på alla viruspositiva barn och vi kunde då se att de flesta hade icke-detekterbara nivåer av virus efter ett par veckor. De virus som fortfarande hittades vid uppföljning var rhinovirus och coronavirus, två av de vanligaste förkylningsvirusen. Resultaten styrkte vår tes att luftvägsvirus är en vanlig orsak till neutropen feber i denna patientgrupp, men vi hade fortfarande svårt att fastslå att det förelåg ett orsakssamband.

Delstudie IV var därför en uppföljningsstudie på **delstudie I**, med samma grupp av patienter, men där vi tittade på det immunologiska svaret i blod istället för de vanliga diagnostiska metoderna. Genom att använda RNA-sekvensering, en metod som gör det möjligt att mäta allt RNA vid ett specifikt tillfälle, kunde vi indirekt studera vilka gener som var aktiverade vid en specifik tidpunkt. Detta gjordes för att undersöka om vi kunde hitta specifika immunsignaturer i blodet vid olika typer av infektioner. Sådana specifika signaturer finns beskrivna hos immunfriska barn med feber, men det var fortfarande oklart om barn med cancer som genomgår immunosupprimerande behandling också uttrycker liknande svar. Tyvärr upptäcktes två problem med att använda denna metod i **delstudie IV**: Det första var att i över 30% av alla episoder med neutropen feber fanns det för få celler i blodet för att kunna generera något mätbart RNA. Det andra var att i de fall där det faktiskt fanns tillräckligt med RNA i blodet så kunde vi inte identifiera några specifika immunsignaturvägar vid olika typer av infektioner. Slutsatsen blev därför att det i nuläget är svårt att använda en sådan metod hos barn som behandlas för cancer.

Risikfaktorer till neutropeni och neutropen feber hos barn som behandlas för akut leukemi

Alla barn som behandlas för akut lymfatisk leukemi (ALL) i Sverige följer samma behandlingsprotokoll, vilket betyder att de får ungefär samma mängder med cellgifter. Trots detta drabbas vissa barn av både fler och allvarigare biverkningar och infektioner än andra, vilket gör att man tror att vissa barn har en predisponerad benägenhet att drabbas. Orsaken till detta skulle kunna vara genetiska varianter i viktiga enzymer eller proteiner i läkemedelsmetabolismen och/eller immunförsvaret. Läkemedlet 6-Mercaptopurine (6-MP) är ett av de vanligaste immunosupprimerande läkemedlen som används för behandling av ALL och ges nästan varje dag under 2,5 års behandling. I **delstudie II** undersöktes genetiska förändringar i gener viktiga i 6-MP läkemedelsmetabolismen såsom TPMT, NUDT15 och ITPA och deras roll för neutropeni, neutropen feber och slutdoser av 6-MP. Vi kunde identifiera att barn med varianter i TPMT-genen hade en minskad risk att drabbas av neutropeni och neutropen feber under den sista delen av ALL behandlingen och barn med genetiska förändringar i ITPA (en av de två undersökta varianterna) hade en minskad risk att drabbas av neutropen feber under hela behandlingstiden. Vi upptäckte också att barn med genetiska förändringar i NUDT15 hade lägre slutdoser av 6-MP. Trots våra resultat är det fortfarande oklart vad genetiska förändringar i TPMT och ITPA spelar för roll i utvecklingen av neutropen feber hos barn som behandlas för cancer. Däremot verkar NUDT15 ha en viktig roll vid behandling med 6-MP. Det behövs mer studier inom ämnet med större patientgrupper för att kunna dra säkrare slutsatser.

Att hitta riskfaktorer för att drabbas av neutropen feber och infektioner såsom virus, bakterier eller svamp skulle kunna leda till att behandlingen blir mer individualiserad. I **delstudie III** karaktäriserade vi de mikrobiologiska fynd som rapporterats under episoderna av neutropen feber under hela ALL-behandlingen och undersökte om genetiska förändringar i viktiga proteiner i det medfödda immunsystemet, såsom MBL, TLR4 och IL-1Ra påverkade risken för att drabbas. Vi kunde rapportera att virusinfektioner var det vanligaste fyndet under neutropena feberepisoder. Det slående var dock att i ca 60% av alla episoder rapporterades ingen infektiöst orsak under episoden. I de genetiska analyserna identifierades att två specifika förändringar i TLR4 genen ökade risken för att drabbas av virusinfektioner och att genetiska förändringar i IL-1Ra minskade risken att drabbas av bakteriella infektioner. Sammanfattningsvis är virusinfektioner vanliga vid neutropena feberepisoder, men i majoriteten av episoderna hittas ingen orsak till febern. Även om det i vår studie pekade på att TLR4 och IL-1Ra påverkar risken att drabbas av olika infektioner, är det fortfarande inte tillräckligt för att ändra några rekommendationer och hitta patienter som är specifikt i riskzon för att drabbas. Ytterligare studier, både med fokus på diagnostik för att hitta orsaken till febern samt andra riskfaktorer för att drabbas behövs.

Slutsats

Ett av problemen som kvarstår är att det fortfarande i många episoder inte hittas någon orsak till neutropena feberepisoden. Luftvägsvirus är ett vanligt fynd vid neutropen feber, men vi kunde inte genom våra metoder visa att det fanns ett orsakssamband mellan virusfyndet och neutropena feberepisoden. Därför behövs det fler och mer känsliga metoder för att kunna sammankoppla mikrobiologiska fynden med den aktuella episoden. Genetiska varianter i enzymer och proteiner i läkemedelsmetabolismen och immunförsvaret verkar spela roll i utvecklingen av neutropen feber och infektioner. Men dessa behöver utredas vidare innan man kan använda den informationen i kliniken för att guida handläggningen av neutropena feberepisoder.

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