Infectious side effects of cancer treatment in children

Poele , te, Esther

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Clinical and genetic aspects

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Stellingen behorend bij het proefschrift

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1. Met name bij kinderen met een verwacht goede uitkomst van de behandeling van hun acute lymfatische leukemie is het de vraag of het voordeel van langdurige en herhaalde toediening van dexamethason, zoals in de onderhoudsbehandeling van ALL-9, opweegt tegen het nadeel (verhoogd risico op infectieuze morbiditeit en mortaliteit). *Dit proefschrift*

2. Genotypen en/of haplotypen die resulteren in lagere MBL-spiegels veroorzaken geen toegenomen risico op koorts gedurende neutropenie bij kinderen met acute lymfatische leukemie. *Dit proefschrift*

3. Er is een verschuiving gaande van klinische behandeling naar poliklinische behandeling van kinderen met koorts gedurende neutropenie en een laag risico op ernstige infecties of infectieuze complicaties. *Dit proefschrift*

4. Momenteel is er slechts één gedegen onderzoek dat vervroegd ontslag van klinische behandeling met niet vervroegd ontslag vergelijkt bij kinderen met (een inschatting op) een laag risico op invasieve bacteriële infecties. Het is opmerkelijk dat vervroegd ontslag plaatsvindt zonder dat er evident bewijs is dat dit verantwoord is. *Dit proefschrift*

5. Vier SNPs in het *TLR4*-gen bepalen voor een deel het toegenomen risico op het ontwikkelen van door chemotherapie geïnduceerde neutropenie bij kinderen met acute lymfatische leukemie. *Dit proefschrift*

6. Twee polymorfismen in het *TLR6*-gen zijn geassocieerd met de omgekeerde associatie tussen atopie en ALL bij kinderen. *Dit proefschrift*

7. Een gezonde mens heeft duizend wensen, een zieke mens maar één. *Jan Mens*

8. In deze tijd van internationalisering is het door de overheid niet meer vergoeden van de tolkentelefoon voor zieke, en dus kwetsbare, mensen een vorm van grensoverschrijdend gedrag.

9. De nacht is geen vriend van het optimisme. *Harry Mulisch*

10. Of men het nu huilend of lachend aflegt, het levenspad is hetzelfde. *Chinees gezegde*

11. Als een rommelig bureau staat voor een rommelige geest, waar staat dan een leeg bureau voor? *Albert Einstein*

12. Samuel Beckett said it is one's moral duty to support artists and if one has the opportunity to acquire any paintings, one should buy off living artists rather than dead artists. *Vrij naar Peggy Guggenheim*

13. Er zijn geen feiten, alleen interpretaties. *Vrij naar Friedrich Nietzsche*

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Contents

List of abbreviations

AB	antibiotics
ALL	acute lymphoblastic leukemia
ANC	absolute neutrophil count
BC	blood cultures
CI	confidence interval
CRP	C-reactive protein
DCOG	Dutch Childhood Oncology Group
FN	fever during neutropenia
HF	haplotype frequency
Ht	haplotype tagging
IL	interleukin
LD	linkage disequilibrium
MAF	minor allele frequency
MBL	mannose-binding lectin
MBL2	mannose-binding lectin gene
N	neutropenia episode
PAMPs	pathogen-associated molecular patterns
PICU	pediatric intensive care unit
PIM	pediatric index of mortality
PRISM	pediatric risk of mortality
RCTs	randomized controlled trials
SNP	single-nucleotide polymorphism
TLR	toll-like receptor
UTR	untranslated region

Chapter 1

General introduction and outline of the thesis

The survival of paediatric cancer patients has increased over the years. The prognosis of children with acute lymphoblastic leukaemia (ALL), the most common kind of cancer in children, improved with a five year event free survival of roughly 9% in patients starting their treatment in 1962 to 81% in patients starting their treatment in 2004.[1-5] Cancer treatment protocols are regularly revised to incorporate the latest insights and this contributes to the improved prognosis. Next to the better cure rates and avoiding relapse, minimizing unwanted side effects is another goal of the constant revision of cancer treatment protocols. The type and prevalence of these side effects depend on the type of cancer, the intensity of the cancer treatment regimens, and on host factors like the genetic constitution. These side effects can sometimes be life-threatening and patients might die of e.g. infections, not of cancer. Examples of side effects are neuropathy, nausea and vomiting, low numbers of thrombocytes, which can cause haemorrhage, low numbers of neutrophils, which predispose for a high risk of infection and also febrile neutropenia. This thesis is mainly focussed on the unwanted side effects of cancer treatment in children. The first part addresses clinical aspects of these side effects such as neutropenia, fever during neutropenia, and fatal infections. The second part covers genetic aspects of acute lymphoblastic leukaemia in children, and genetic aspects of side effects of cancer treatment, including neutropenia and fever during neutropenia.

Fever and neutropenia

Chemotherapy-induced neutropenia is one of the major side effects of cancer treatment. Both the duration and the depth of the neutropenia influence the risk for infections in these patients.[6] Due to an impaired inflammatory response, caused by the diminished number of neutrophils, the usual signs of inflammation such as local heat, swelling, exudate, fluctuation, ulceration and fever are often diminished in neutropenic cancer patients.[7] Fever can be the first sign of a bacterial infection and because of the high risk of bacterial infections and sometimes fulminant course, historically, all patients with fever (above 38.2 °C or 38.0 °C for several hours) during neutropenia are hospitalized and given intravenous antibiotics. However, in 70-89% of the cases of fever during neutropenia, no causative micro-organism is found.[8-12] Other causes of fever during neutropenia can be viruses, transfusion of blood products, mucositis and chemotherapeutic drugs. These patients are at low risk for invasive bacterial infections and/or serious complications. This implies that hospitalization and broad spectrum antibiotics are not necessary in these patients. However, it is difficult to distinguish these low risk patients from patients with fever during neutropenia and a high risk of complications. During the past decades several research groups studied parameters for risk stratification of patients with fever during neutropenia.[13-23] Moreover, new treatment strategies with less or no antibiotics or outpatient treatment were tested.[10,12,24-31] In this thesis we study these parameters, the

moments at which the risk assessments take place and the safety and feasibility of early discharge of cancer patients with fever during neutropenia.

Innate immunity

It is commonly accepted that the host response to microorganisms in patients with neutropenia results from the effector cells of the immune system. The immune system is divided into the innate immune system and the adaptive immune system. The innate immune system is the first line of defence against microbes. The cells of the innate immunity recognize and respond to a pathogen immediately in a non-specific way, but, unlike the adaptive immunity, it does not result in a long lasting or protective immunity. Innate immunity is the result of interactions between barriers, like skin and mucosa, and gatekeeper cells, like mast cells, natural killer cells and phagocytes, such as neutrophils and macrophages. Neutrophils and macrophages are the main executers of defence.[32] These effector cells of the innate immunity mediate the earliest phases of inflammatory response.[32,33] In more detail; effector cells recognize groups of pathogens by using a limited number of receptors. Molecular patterns on the bacterial cell wall, the so-called pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide, can be bound by these receptors, for example the Toll-like receptor family. Then this binding activates signal transduction pathways and the innate immune system resulting in phagocytosis ("eating and destroying" the pathogen), up-regulation of the production of cytokines (specialized chemical mediators), chemotaxis (recruitment of immune cells to the site of infection) and stimulation of the adaptive immune system in order to develop specific effector cells against the infectious micro-organism.[34,35] Recognition of PAMPs on a wide range of pathogens can be a function of proteins of the innate immunity, like Mannose Binding Lectin (MBL). When MBL recognizes and binds PAMPs it then activates the complement system[36] and mediates phagocytosis of the pathogen.[37,38] Moreover, pro-inflammatory cytokines, for example, interleukin (IL) 1β, tumor necrosis factor-α, IL-6 and their receptors,[39] cause local (e.g., redness and pain) and in the end systemic inflammation (e.g., fever and metabolic changes). This knowledge resulted in testing of various cytokines of the innate immunity system, such as lipopolysaccharide binding protein, IL-8 and procalcitonin, as potential decision makers to define a population at high risk for bacterial (gram negative) sepsis or a low risk population in whom antibiotics can be stopped after several days, or omitted at all.[10,17,40-48] Possibly genetic differences in the innate immunity also play a role in the risk for neutropenia, fever during neutropenia and even childhood ALL.

Genetic differences

Genetic polymorphisms are frequent (>1%) variations in genetic information. The polymorphisms analyzed in this thesis are single nucleotide polymorphisms (SNPs). A SNP is a genetic variation in a DNA sequence that occurs when a single nucleotide is altered. When SNPs occur in a gene, this can influence the amount of protein, the protein structure or the function. Thereby many SNPs have been described in relation with predisposition for certain diseases, like atopy, ulcerative colitis, meningococcal disease, and severe malaria.[49-56] For example, Emonts et al reported several SNPs involved in susceptibility, severity, and outcome of meningococcal disease. SNPs can also be associated with for example the functional life span of neutrophils and endotoxin hyporesponsiveness.[50,53,57-59] Moreover, SNPs can be associated with side effects of cancer treatment, like SNPs in genes encoding for thiopurine S-methyltransferase (*TPMT*) and catechol O-methyltransferase (*COMT*), which are associated with hearing loss in children receiving cisplatin chemotherapy.[60] Furthermore, SNPs can also be associated with childhood acute lymphoblastic leukaemia; Yang et al found a SNP associated with leukaemia in blacks; this finding contributes to the explanation of racial differences in leukaemia incidence.[61] In this thesis we investigate SNPs of important players in the innate immunity as a possible predictor for neutropenia, fever during neutropenia and childhood ALL.

Outline of the thesis

This thesis consists of two parts.

Part 1: Clinical aspects

Chapter 2 reviews the literature on parameters in risk assessment for early discharge in fever during neutropenia in children with cancer. Additionally it gives a historical overview of the changing approach to fever during neutropenia with a low risk for invasive bacterial infection. Based on the results of **chapter 2** we wondered whether early discharge in paediatric cancer patients with fever during neutropenia and a low risk for invasive bacterial infections was as safe as inhospital treatment of this patient-group. To address this question we performed a Cochrane review on very early versus early versus non-early discharge in paediatric cancer patients with fever during neutropenia and a low risk for invasive bacterial infection in **chapter 3**.

To improve the outcome of paediatric cancer patients, treatment protocols are revised every several years. An important characteristic of chemotherapeutic drugs is that they can all have serious side effects, for instance pancytopenia, polyneuropathy, allergic reactions and kidney failure. Since the start of the ninth protocol for acute lymphoblastic leukaemia treatment of the Dutch Childhood Oncology Group (DCOG-ALL-9), an

increase in lethal infections in children with acute lymphoblastic leukaemia during the maintenance treatment was noticed. We hypothesized that an increase of infectious deaths during maintenance treatment might be caused by the repeated administration of dexamethasone during this part of the treatment. Therefore, in **chapter 4**, we investigated the incidence of infectious deaths during the maintenance phase of the DCOG-ALL-9 protocol (consisting of a treatment scheme with methotrexate, 6-mercaptopurine, vincristine and dexamethasone) and compared it with the incidence of infectious deaths during the maintenance phase of the DCOG-ALL-7 and 8 (consisting of a treatment scheme with only methotrexate and 6-mercaptopurine, thus without vincristine and dexamethasone). Moreover we studied the patient and the episode characteristics of the infectious deaths.

Various side effects caused by cancer treatment can be life-threatening, leading to admissions to a paediatric intensive care unit (PICU). In the past mortality rates of paediatric oncology patients admitted to the PICU have been reported to be more than 84% in patients needing respiratory and circulatory support.[62,63] More recently a decreased mortality rate in paediatric oncology patients admitted to the PICU has been demonstrated.[64] In **chapter 5** we studied PICU survival in non-elective PICU admissions in oncology patients. Moreover we identified variables associated with mortality and evaluated predictive mortality risk scores (Paediatric Index of Mortality and Paediatric Risk of Mortality (PIM and PRISM)) in this PICU population.

Chemotherapy-induced neutropenia is one of the major dose limiting side effect of intensive chemotherapy in paediatric cancer patients. To diminish or even prevent neutropenia Pegfilgrastim, a (once-per-cycle dosage) granulocyte colony stimulating factor, is available for adults, but not registered for children. For this reason we reported 32 episodes of pegfilgrastim use in seven paediatric cancer patients in **chapter 6** and we studied its feasibility and safety in paediatric cancer patients.

Part 2: Genetic aspects

The chapters of the first part of the thesis concerned clinical aspects of side effects of cancer treatment in children. In the second part we study genetic aspects of the innate immunity in relation to side effects of cancer treatment and to leukaemia in children. We describe three different studies on a possible relation between gene polymorphisms of the innate immunity and the risk of developing neutropenia, fever during neutropenia during cancer treatment, and the risk of developing childhood ALL.

As mentioned in the general introduction, susceptibility to infections increases as the neutrophil count decreases. Patients vary considerably in the number of neutropenic episodes, despite receiving identical treatment. Toll-Like Receptor 4 (TLR4) has been shown to play a role in the functional life-span of neutrophils. In **chapter 7** we studied

the possible association of SNPs in the TLR4 gene with the risk of developing neutropenia in children with ALL.

In paediatric cancer patients the risk for fever during neutropenia is even more important than the risk for neutropenia. SNPs in genes involved in immunity defence might influence the risk for fever during neutropenia episodes. Mannose binding lectin (coded by *MBL2*) levels play a central role in innate immunity defence. We hypothesized that genotypes and haplotypes associated with low MBL2 production would increase the risk of fever during neutropenia. Therefore, in **chapter 8**, we studied the role of *MBL2* low producing genotypes and haplotypes and the association with fever during neutropenia in the maintenance treatment of children with ALL.

The presence of atopic disease has been shown to protect against developing childhood ALL. In **chapter 9** we examine whether SNPs in innate immunity genes that have previously been associated with atopic disease might explain the inverse association between childhood ALL and atopic disease.

The summary and discussion of the findings of this thesis are shown in **chapter 10** (English) and **chapter 11** (Dutch), these are supplemented with ideas on future perspectives.

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Part 1

Clinical aspects

Chapter 2

Risk assessment in fever and neutropenia in children with cancer: What did we learn?

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Abstract

Children with cancer treated with chemotherapy are susceptible to bacterial infections and serious infectious complications. However, fever and neutropenia can also result from other causes, for which no antibiotic treatment is needed. In the past decades attempts have been made to stratify the heterogeneous group of pediatric cancer patients with fever and neutropenia into high- and low-risk groups for bacterial infections or infectious complications. Strategies for risk assessment have resulted in treatment regimens with early discharge or even no hospital admission at all, and/or treatment with oral or no antibiotics. We will provide a historical overview of the changing approach to low-risk fever and neutropenia, and we will also try to identify clear and objective parameters for risk assessment strategies and illustrate their relationship to innate immunity. In the future, new insights into genetic susceptibility on neutropenic fever might be of use in children with cancer with fever and neutropenia.

Keywords: Child, Cancer, Assessment, Neutropenia, Fever, Antibiotics

1. Introduction

Historically, subjective and objective parameters have been used in various attempts to divide children with cancer and chemotherapy-induced fever and neutropenia (FN) into high- and low-risk groups in order to customize their treatment and provide a better quality of life. This review will focus on clear and objective prognostic parameters for risk assessment in separating pediatric cancer patients with FN into high- and low-risk groups.

1.1. Chemotherapy-induced neutropenia

Chemotherapy-induced neutropenia is one of the major side effects of cancer treatment. In 1966 Bodey et al. published some calculated relative risks as follows. In patients with less than 1000 granulocytes/mm^3, the risk of infection increased from about 38% to 63% from the onset of the neutropenia to 3 weeks after onset.[1] In patients with less than 100 granulocytes/mm^3, the infectious risk after 3 weeks of neutropenia even increased as much as 100%. In severe infections the mortality risk was approximately 46% when the initial granulocyte count was less than 1000 /mm^3, and when the granulocyte count decreased below 100 /mm^3, the mortality risk increased as much as 80%. These results underscore the importance of both the duration and depth of the neutropenia for an increased risk for and fatal outcome of infectious diseases.[1] Internationally, the most frequently used definition of neutropenia in relation to fever is an absolute neutrophil count $<0.5 \times 10^9$/l (or $<1.0 \times 10^9$/l and expected to fall, or a leukocyte count $<1.0 \times 10^9$/l).

1.2. Fever and neutropenia

Fever (defined as a single body temperature of >38.5 ºC or two repeated readings of >38.0 ºC [2-5]) can be the first sign of bacterial infection. Other signs of inflammation such as local heat, swelling, exudate, fluctuation and ulceration are often diminished in neutropenic cancer patients due to an impaired inflammatory response.[6]

Historically, standard care for pediatric cancer patients with FN consists of routine hospitalization and empirical treatment with parental administration of broad-spectrum antibiotics (AB), in the light of the relative risk for infectious complications. These episodes of FN might be the result of bloodstream infections with pathogenic micro-organisms. However, 70-89% of the episodes of FN have no causative micro-organisms demonstrated in their blood cultures (BC).[7-11] It can be hypothesized that many episodes of FN can be the result of inflammatory responses to, for example viruses, transfusions of blood products, malignancy itself, chemotherapeutic drugs or mucosal damage.

Historically, patients are considered to be eligible for discharge when they have completed their antibiotic course, are afebrile and when the absolute neutrophil count has recovered to at least 0.5×10^9/l.[12-14] This approach results in overtreatment of the

subgroup of patients without a proven bacterial infection, in a risk of increased bacterial resistance, and in a decrease in quality of life and increase in health care costs. In 2002 the Infectious Diseases Society of America (IDSA) published a clear protocol for adult cancer patients with FN.[15] Early discharge of pediatric patients was a yet more delicate question; after ≥48 h of in-hospital treatment with parental AB and observation, early discharge with oral cefixime may be considered for selected children at low risk for bacterial infections[15] (Figure 1). Low-risk selection criteria were defined as: absence of severe comorbidity, good clinical condition, negative BC, no MRSA in cultures in the last 12 weeks, control of local infection and afebrile for the past 24 h.[16,17] In these guidelines, initial therapy with oral AB alone or without AB is not recommended for children. Therefore, it is appropriate to use less intensive antibiotic treatment regimens in children with cancer at low risk. How can we define low risk using objective parameters? To answer this question we will first discuss the mechanisms of the innate immunity in

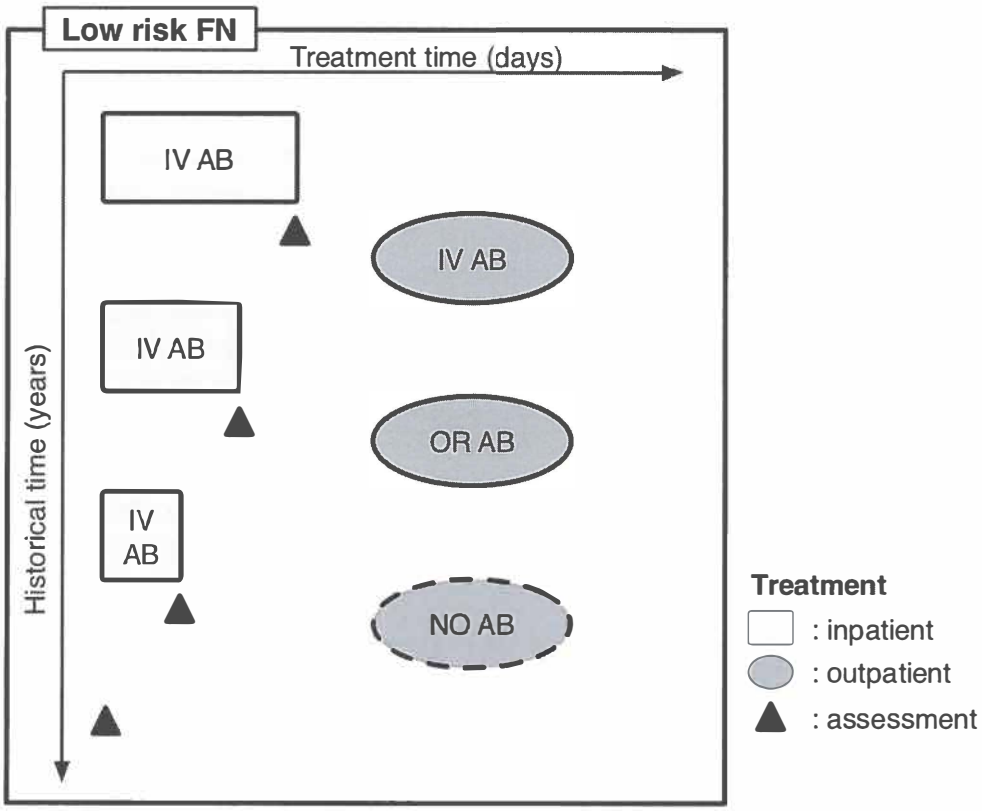

Figure 1: Historical overview of the changing approach to low-risk FN. This figure illustrates the overall changes in progressive treatment regimens for FN in pediatric cancer patients, with treatment time in days on the horizontal axis and historical time in years on the vertical axis. Almost all combinations of treatment have been used. The oval with the dashed line indicates a possible future approach. Abbreviations: IV, intravenous; AB, antibiotics; OR, oral; FN, fever and neutropenia.

oncology patients. Second, a summary of possible prognostic parameters found in various studies will be provided. Finally, clinical studies will be discussed, along with the possible ways to handle the dilemma of "low risk."

1.3. Innate immunity

It is commonly accepted that the host response to fever in neutropenia patients results from the effector cells of the immune system. The immune system is divided into the nonspecific innate immune system and the specific adaptive immune system. The adaptive immune system consists of components that are acquired after birth; it is the response of antigen-specific lymphocytes to antigens and includes the development of immunological memory. The innate immune system (including the mucocutaneous barriers) is the first line of defense against microbes. In healthy people all the components of innate immunity are already present prior to exposure to microbes and will act immediately against them. The complement system and its' lectin pathway are essential in the opsonization of pathogens. Granulocytes and macrophages are the main executers of defense[18] (see Figure 2). These effector cells of innate immunity mediate the earliest phases of inflammatory response.[18,19] The effector cells recognize groups of pathogens by using a limited number of receptors. Molecular patterns on the bacterial cell wall, the so-called pathogen-associated molecular patterns such as lipopolysaccharide, are bound by these receptors, for example the Toll-like receptor family. This binding induces signal transduction pathways, resulting in up-regulation of the production of cytokines and stimulation of the adaptive immune system in order to develop specific effector cells against the infectious micro-organism.[20,21] Moreover, proinflammatory cytokines, for example, interleukin (IL) 1ß (IL-1ß), tumor necrosis factor-α (TNF-α), IL-6 and their receptors,[22] cause local (e.g., redness and pain) and in the end systemic inflammation (e.g., fever and metabolic changes) (see Figure 2). The innate immunity system might therefore provide objective parameters for risk assessment of FN.

2. Prognostic parameters

2.1. Search strategy

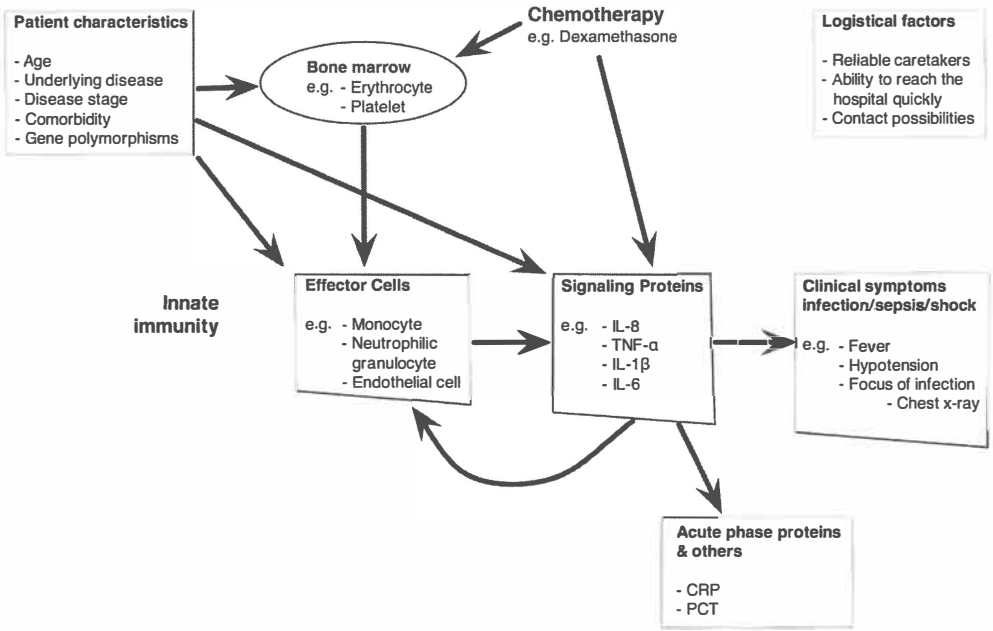

Figure 2: Innate immunity and parameters for risk assessment in FN. This figure shows parameters for risk assessment strategies for pediatric cancer patients with FN. In addition, the influence of various factors on innate immunity and clinical presentation of pediatric cancer patients with FN is illustrated. Abbreviations: IL, interleukine; TNF-α, tumor necrosis factor-α; CRP, C-reactive protein; PCT, procalcitonin.

We performed a search for possible clear and objective prognostic parameters for such a risk assessment in children with cancer by using Pubmed screening for papers written in English. We utilized the search terms "fever" and "neutropenia" or "febrile neutropenia" combined with "child", and "predictive value of tests" or "risk assessment" or "biological markers" or "protein precursors." Studies with clear definitions of fever and neutropenia (ANC $<0.5 \times 10^9$ or $<1.0 \times 10^9/1$ and that were expected to fall, or a leucocyte count $<1.0 \times 10^9/1$) were included. Studies including patients >20 years of age or studies in which the results of pediatric and adult patients could not be separated were excluded. We tried an analysis according to levels of evidence, but this was problematic because much of the literature was not advanced enough in this respect. For this reason we chose not to use levels of evidence in this review. Additional relevant articles were identified from the references found in the initial search. This search resulted in 21 retro- and prospective studies up until and including September 2008. The intervention studies will be evaluated in the next section.

Variable combinations of parameters were used to arrive at various risk assessment strategies. The parameters were divided into four groups: patient characteristics, physical examination parameters, radiological findings and laboratory findings (see Table 1).

2.2. Patient characteristics

Most of these patient characteristics are clear and objective, for instance age, underlying malignancy, disease stage, and number of previous FN episodes, but some can be interpreted in more than one way. For example, anticipated neutropenia of less than 7 days is a semi-objective criterion. This latter parameter was used by Alexander et al. for patients not having acute myeloid leukaemia, Burkitt lymphoma, acute lymphoblastic leukemia in the induction phase, and progressive or relapsed disease with bone marrow involvement.[29] On that basis, Alexander et al. used this parameter to divide solid malignancies without bone marrow involvement from hematological malignancies and solid tumors with bone marrow involvement.

Table 1: Possible prognostic parameters to distinguish between pediatric cancer patients with FN at high or low risk for bacterial infection or complications

Type parameter	Parameter	Reference
Patient characteristics	Age	[23,24]
	Underlying malignancy	[25,26]
	Disease stage	[4,5,25,27,28]
	Number of previous FN episodes	[27]
	Anticipated time period of N <7 days	[29]
	≤7 days since last chemotherapy	[4,5]
Physical examination	Blood pressure	[4,5,26,29]
	Chills	[26]
	Focus of infection	[23,25,29]
	Comorbidity	[2,27–29]
	Temperature	[23,27,30]
	In-dwelling catheter	[23,25]
Radiological findings	Chest X-ray	[2,29]
Laboratory findings	AMC	[2,5,30–32]
	ALC	[25,27]
	Platelet count	[4,5]
	CRP	[4,5,24,25,33,34]
	PCT	[35–37]
	Serum adenosine deaminase	[37]
	sIL-2R	[38]
	IL-6	[35,38--40]
	IL-8	[24,34,36,38,40]
	Hb	[23,25]

Abbreviations: FN: fever and neutropenia; N: neutropenia; AMC: absolute monocyte count; ALC: absolute leukocyte count; CRP: C-reactive protein; PCT: procalcitonine; IL-6: interleukine-6; IL-8: interleukine-8; Hb: hemoglobin.

2.3. Physical examination parameters

In the group with physical examination parameters, several parameters can be defined strictly, whereas the focus of infection might result in a variable definition and interpretation. Rondinelli et al. differentiated between upper respiratory tract infections and other clinical foci of infection.[23] Rondinelli et al. and Amman et al. interpreted the

presence of a (viral) upper respiratory tract infection as one of the low-risk criteria for severe infectious complications and bacteremia.[23,25] Rondinelli et al. showed infectious foci such as mucositis, pneumonia and urinary tract infections to be high-risk predictors,[23] whereas others only counted tunnel infections, perirectal abscesses and cellulitis as a focus of infection contributing to a high risk of bacterial infection.[29]

The definition of comorbidity is ambiguous. Alexander et al., Paganini et al., and Klaassen et al. used different, but detailed descriptions of comorbidity.[2,28,29] These descriptions may coincide with other prognostic parameters, such as blood pressure, focus of infection and chest X-ray abnormalities. Ammann et al. mentioned the covariate as comorbidity requiring hospitalization independent of FN, without giving a more detailed definition.[27] Whether these different definitions result in largely identical classifications remains unclear.

2.4. Radiological findings

In the group with radiological findings only the chest X- ray was selected to be a possible prognostic parameter. Chest X-ray abnormalities were mentioned as a high-risk feature.[2,29] However, chest X-rays at presentation were of limited value, since abnormalities can be delayed as compared to the onset of clinical infections. This implies that normal chest X-rays cannot exclude a pulmonary infection. Moreover, it remains disputable whether chest X-ray abnormalities are uniformly the result of infection.[41] This would be in concordance with studies on adult febrile neutropenic cancer patients.[3]

2.5. Laboratory findings

Laboratory parameters for inflammation, such as acute phase proteins and cytokines, were used for the risk assessment as well. At admission, CRP was shown to be one of the discriminating factors between children with low-risk and high-risk FN.[4,5,25,33,34] In a later study Santolaya et al. an increased predictive value for their model was revealed by incorporating an additional measurement of CRP on day two,[5] suggesting that CRP in their patients can rise slowly. The group of high-risk patients was split by using CRP and IL-8 on admission and after 24 h combined with age, in a group with and without an increased risk for severe sepsis not clinically apparent during the first 24 h of hospitalization.[24]

On admission, IL-6 was also shown to be a sensitive marker for disease severity, and it may help in the differentiation.[35,38-40] Furthermore, in the studies performed by Kitanovski et al., and Diepold et al., IL-6 concentrations appeared to be of value on day two and three as well.[35,40] Due to rapid declination of IL-6 plasma levels, sequential determination did not further improve diagnostic accuracy.[35,39] In addition, IL-8 was shown to be of discriminating value on admission,[34,36,38,40] as well as at later time points.[36,40] PCT was shown to be a discriminatory parameter on admission, and also on day two and three, and

it discriminated more precisely when a serial measurement was used.[35-37] The merit of these individual laboratory parameters at the moment of presentation might increase when different parameters are combined, thus resulting in sensitive and specific markers for bacterial sepsis in FN patients, which has already been shown using the combination of PCT and IL-8.[36] Furthermore, sequential determination of CRP and PCT improved diagnostic accuracy.[5,35]

In conclusion, parameters such as age, underlying malignancy, disease stage, and hypotension appear to be objective and clear parameters. The combination of various laboratory parameters and their course over time might improve discrimination between patients at high and low risk for bacterial infections or serious complications.

3. Moments of assessment

As a result of the investigations for prognostic parameters, intervention studies in pediatric cancer patients were carried out in which various subjective and objective prognostic parameters were used to identify patients for a more liberal treatment regimen for expected low-risk patients with, for example, short duration of IV AB, early discharge, complete outpatient treatment or even no treatment with AB at all (see Figure 1). At this point, we discuss the moment of assessment, along with the assessment items on which the treatment interventions are based.

3.1. Search strategy

A search was performed looking for papers written in English with Pubmed, utilizing the search terms "fever" and "neutropenia" or "febrile neutropenia" combined with "early discharge", "home therapy" or "outpatient". Studies including less than 20 episodes of FN in the low-risk group and studies including patients >20 years of age or studies in which the results of pediatric and adult patients could not be separated were excluded. Additional relevant articles were identified from the references found in the initial search. This resulted in 24 pro- and retrospective studies.

We divided these studies in terms of their moment of assessment. In Group I patients were assessed after a minimum of 48 h of inpatient treatment with parental AB.[10-14,16,17,42-48] Group II assessed after at least 24 h of hospital admission,[8,49-51] and in Group III patients were assessed within 24 h after the first parental AB[7,52-56] (see Table 2). After the respective assessments, patients were either given oral or no AB and/or discharged from the hospital.

3.2. Group I

The studies included in Group I, that is, assessment after a minimum of 48 h, showed that treatment was altered in oral or no AB and/or hospital discharge before recovery from

neutropenia. The first of the studies was published in 1990. The safety of the moment of assessment and of the assessment items was measured by the success percentage of the various treatment regimens. The total group of 923 patients had an average treatment failure of 9%. Treatment failure was defined as hospitalization in outpatients or antibiotic switch in inpatients. In the 82 treatment failures, a total of seven positive BC were noted. One patient died of infection and resistant leukemia[42]; all other patients recovered from FN.

Table 2: Studies concerning early discharge and/or cessation of IV AB in FN

Moment of assessment	Reference	Year of publication	Percentage of LR of all evaluable FN	Percentage of LR with early discharge and/or cessation of IV AB of all LR	LR with early discharge and/or cessation of IV AB	Treatment failure N (%)	Outcome treatment failures
Group 1 ≥48 - 72 h	[12]	1990	68	100	77	3 (4)	Good
	[13]	1992	65	100	70	1 (1)	Good
	[42]	1994	62	62	83	18 (17)	1 death; inf + resist leukemia
	[43]	1994	53	100	70	1 (1)	Good
	[14]	1994	NAV	79	23	3 (13)	Good
	[44]	1995	NAV	NAV	32	6 (19)	Good
	[45]	1997	38	100	33	2 (6)	Good
	[11]	1997	37	40	36	2 (6)	Good
	[16]	2000	44	100	154	3 (2)	Good
	[46]	2000	17	97	71	7 (10)	Good
	[17]	2001	NAV	50	100	28 (28)	Good
	[47]	2003	11	77	30	6 (20)	Good
	[10]	2005	41	100	112	0 (0)	NAP
	[48]	2007	12	100	32	2 (6)	Good
Total					**923**	**82 (9)**	
Group 2 ≥24 h	[49]	1997	NAV	NAV	315	6 (2)	NAV
	[50]	2001	39	100	93	1 (1)	Good
	[51]	2004	41	48	78	4 (5)	Good
	[8]	2005	19	100	20	0 (0)	NAP
Total					**506**	**11 (2)**	
Group 3 <24 h	[7]	1993	50	71	86	0 (0)	Good
	[52]	1994	NAV	NAV	50	9 (18)	Good
	[53]	1999	NAV	NAV	73	10 (14)	Good
	[54]	2000	NAV	84	116	6 (5)	Good
	[55]	2000	28	69	45	5 (11)	Good
	[56]	2003	32	100	177	10 (6)	Good
Total					**547**	**40 (7)**	**Good**

Abbreviations: LR: low-risk fever and neutropenia episodes; FN: fever and neutropenia episodes; IV: intravenous; AB: antibiotics; h: hours; inf: infection; resist: resistant; NAV: not available; NAP: not applicable.

Evaluation of FN episodes in Group I resulted in important assessment items. In at least two studies the following items were used: no comorbidity needing hospitalization, good clinical condition, negative BC for a minimum of 24, but most of the time 48 h, evidence of bone marrow recovery and control of local infection (if identified). In most studies, patients had to be afebrile for a minimum of 24 h (Figures 1 and 2). Interestingly, in two studies, patients were not obliged to be 24 h afebrile before discharge; a total of only two positive BC were reported in the 136 patients included.[11,17] The treatment failure in these three studies was 22%. This failure came from one study only, that performed by Shenep et al., in which the treatment failure was far higher than the average in Group I (28% in his study versus 9%). In this study, patients did not have to show any evidence of bone marrow recovery and the treatment failure was higher in patients with an ANC $<0.1 \times 10^9/l$.[17] These objective parameters and a monocyte count $<0.1 \times 10^9/l$ have been shown to significantly correlate with readmission and might explain the difference .[46]

In the studies in which the observation periods were described, patients were discharged from the hospital within an average or median of 5 days.[16,44,46-48] Thus, this represents only a moderate change as compared to the standard treatment schedule of 7 days IV AB or until neutropenia recovery.

3.3. *Group II*

After the successful initial studies of Group I, an earlier moment of assessment seemed desirable. From 1997 onwards, such studies have been published. The studies included in Group II, assessment after a minimum of 24 h, showed hospital discharge and treatment without or with AB, and/or before recovery from the neutropenia. In the total group of 506 patients, the treatments resulted in an average treatment failure of 2%. Treatment failure occurred eleven times, but none of the children died.

The evaluation of Group II resulted in the following important assessment items for low-risk patients: good clinical condition, low host inflammation response as indicated by laboratory parameters (IL-8, CRP), afebrile ≥12 h, having a negative BC ≥24 h, no sign of or control of local infection, indication or evidence of bone marrow recovery, chemotherapy ≥7 days previous, neutropenia expected to prolong <10 days, or an absolute platelet count >50,000/µl (Figure 2).

In the prospective study by Oude Nijhuis et al., patients were assessed at the moment of admittance and after 24 h. Twenty episodes in 17 children (age <18 years) were classified as low-risk for bacterial infection and discharged from the hospital after a minimum of 12h of afebrile observation; these low-risk patients were not treated with AB at all ([8] and personal remarks from the author).

The hospital observation period for the low-risk patients in Group II varied from 24 to 36h,[51] with a mean and median duration of 3 days (range: 2-5 days) in the study performed

by Oude Nijhuis et al. ([8] and personal remarks from the author), 3 days[50] and a mean duration of 5.4 days in the study done by Aquino et al.[49]

These four studies had earlier moments of assessment, different treatment designs and different aims. New in this group, in comparison with the studies in Group I, was the introduction of objective laboratory parameters indicating inflammation (CRP, IL-8) (Figure 2). Nevertheless, these studies had shorter observation periods than most studies in Group I and a very low treatment failure rate.

3.4. Group III

In 1993, the first study with an in-hospital observation period of less than 24 h was published; these studies have been assembled in Group III. As the moment of assessment comes closer to the time of presentation and patients are discharged early, theoretically the chance of treatment failure should increase. However, treatment failure appeared in only 40 of the 547 patients leading to a success percentage of 93%, in which patients were treated with oral or IV AB on an outpatient basis. All of the treatment failures had uneventful hospital admissions. Treatment modifications in the outpatient setting were reported in 5% of the FN episodes.

These studies all still depend more on subjective, than on objective parameters. Because of the earlier moments of assessment, previously cited objective assessment parameters, such as evidence of bone marrow recovery, being afebrile ≥24 h and control of local infection, cannot be used. The assessment items used most frequently were: being clinically well, stable disease or remission, no comorbidity needing hospitalization, reliable family/caretakers, and the possibility of being able to contact and come to the hospital within a short period of time (Figure 2). Still, a large part of the parameters were subjective. Patients were seen daily in the outpatient ward when IV AB were administered. There was one exceptional study: after the first administration of IV AB in the outpatient department, IV AB were administered at home and the patients were not seen on a daily basis.[52]

Although the moment of assessment was set within the first 24 h three studies, used positive BC at 48 h as an exclusion criterion. In two studies these positive BC are mentioned as treatment failures,[52,55] in the study by Paganini et al it remains unclear how many patients had a positive BC.[56] This might introduce a selection bias and implies that treatment failure might be underestimated in the last study.

4. Discussion

Due to chemotherapy-induced neutropenia children with cancer are at increased risk for FN. When a trigger stimulates effector cells of the innate immune system, signaling proteins are produced, resulting in symptoms of infection, sepsis and shock (Figure 2).

The eventual clinical symptoms which a patient presents are influenced by patient characteristics and chemotherapy schedule, but also by logistical factors (the time between falling ill and presentation in the hospital).

The trigger stimulating these symptoms might be due to a bacterial infection, but could also be due to other inflammatory causes such as a viral infection, chemotherapeutic drugs, or blood products. All patients with FN undergo broad-spectrum antibiotic treatment and hospitalization, although this is only necessary in patients with a bacterial infection. There are reports concerning risk assessment of defining high-risk patients. These studies include items defining the risk of a severe sepsis not apparent in the first 24 h[24] as well as assessments of predicting mortality[28] in children with cancer presenting with FN. However, in this review we want to clarify the definition of a low-risk population with a good outcome. To be able to define these patients at an earlier stage, many retrospective studies have been done to discover new parameters in order to optimize risk assessment strategies and divide patients safely into low and high risk for infection or infectious complications. However, only a selection of parameters have proven to have value in the various prospective intervention studies: good clinical condition, no comorbidity needing hospitalization, indication or evidence of bone marrow recovery/activity, being afebrile, control of local infection, negative BC, low inflammation laboratory parameters (IL-8, CRP), chemotherapy ≥7 days previous, neutropenia expected to prolong <10 days, stable disease or remission, reliable family/caretakers, and the possibility of being able to contact and come to the hospital within a short period of time (e.g. residence within 1 h or within 100 miles from the hospital).

Over the years there has been a shift to earlier assessment and a shift away from inpatient towards outpatient treatment (Figure 1). This has led to an improvement in quality of life and a reduction in health care costs; Santolaya et al. reported a mean of $903 per episode in the hospital-treated and $638 in the ambulatory-treated group[51] and Ahmed et al. stated $1783 per episode in the control and $1208 in the early hospital discharge group.[48] Some groups dared to execute more progressive studies than did others. This changed the emphasis of the assessment items from those based on laboratory parameters and negative BC, towards a more important role for logistical variables such as contact possibilities and being able to come to the hospital quickly (Figure 2). Being clinically well remains a central assessment item in all hospital assessment strategies, however. When the moment of assessment tends to take place at an earlier stage, the role of determining biochemical parameters sequentially diminishes. The merit of using biochemical parameters at the moment of presentation might increase in validity as different parameters are combined. Some patients continue to present early in the inflammatory process, accompanied by still rising plasma levels for the biochemical parameters, while others continue to present late with peak plasma levels already attained. This limits the value of biochemical parameters as an optimal assessment item.

The studies found in the previous section are quite heterogeneous with regard to their quality and aim, as well as with regard to the size and composition of the study population. This makes it a challenge to weigh the different assessment items. Because of this, we did not use statistical analyses. However, when the treatment failures in the different groups are looked at roughly, one can see that when the moment of assessment comes earlier, no increase in treatment failures is seen (Table 2). For a select group of patients, then, early discharge can be a valuable option. Of course, an experienced medical team that can assure close follow-up of patients and rapid response for those patients who are not doing well at home is obligatory, especially in ambulatory treatment. In conclusion, the risk assessment strategies for a large part depend upon subjective parameters. More objective parameters are still needed.

5. Future perspective

The number of previous FN episodes was mentioned as being a possible prognostic parameter.[27] This would imply that there might be a genetic predisposition for the development of FN (Figure 2). The literature supports the potential importance of genetic differences (such as single nucleotide polymorphisms (SNPs)) influencing the risk for infection and infectious complications; this might provide objective parameters.

Genetic differences are called gene-polymorphisms when they appear in ≥1% of the general population and the frequency of heterozygotes is ≥2%. Ninety percent of the polymorphisms are differences in nucleotides; SNPs. Recently articles have been published on SNPs of, for example, Toll-like Receptor 4 and the increased susceptibility for and severity of meningococcal and malarial infections.[57,58] Moreover, the depth and duration of neutropenia might well be determined not only by the intensity of the chemotherapy regimen, but also by individual genetic differences (Figure 2). Furthermore, deficiency of mannose-binding lectin-associated serine protease-2 (MASP-2) and mannose-binding lectin (MBL) are common due to SNPs. Deficiency of MASP-2 has shown to give a twofold increased risk for FN[59] and sufficient levels of MBL increase the risk for intensive care unit admissions in children with FN.[60] One can easily imagine that more extensive information concerning the influence of genetic polymorphisms on FN in pediatric cancer patients could lead to a genetic fingerprint in the future. This fingerprint would be taken at the start of cancer treatment and might help us to distinguish between patients at high risk for infectious complications, on the one hand, and patients easily developing fever due to minor non-microbial or microbial triggers who have a very low risk for serious infections, on the other. Moreover, this might possibly enable us to create tailor-made supportive care based on the risk profile for the individual patient. Eventually this could result in a decrease in morbidity, a higher quality of life and reduction in health care costs.

6. Concluding remarks

The current standards for empirical broad-spectrum intravenous antibiotic treatment combined with hospitalization[15] are cautious and safe, but most probably lead to the overtreatment of a substantial group of patients. Large randomized controlled trials are needed to find and validate parameters in order to optimize assessment strategies and safely enlarge the group of patients with low-risk FN. This select group of low-risk patients could then be safely treated in an outpatient setting with minimal AB treatment or none at all.

Upon this review the following general recommendations can be given. Critically ill pediatric oncology patients with FN need to have maximal life support and immediately given intravenous broad-spectrum antibiotics. Children presenting with fever and neutropenia and no others signs of severe sepsis deserve treatment with IV AB for at least 3 days. When BC are negative and patients are afebrile and clinically well, IV AB might be discontinued. Withholding AB cannot be proclaimed yet as standard care and can only be supported in a trial with good standardized outpatient care.

Over the years there has been a shift towards earlier assessment and a shift away from inpatient towards outpatient treatment (Figure 1). This has led to an improvement in quality of life for the patients and a reduction in health care costs. This also has changed the emphasis from the assessment items based on laboratory parameters and negative BC, to a more important role for logistical variables such as contact possibilities and being able to come to the hospital quickly. The subjective parameter of being clinically well remains a central assessment item. Moreover, interesting new developments have appeared in the field of the genetic influence on infection and infectious complications, which might contribute to clear and objective parameters, and prove to be of importance for pediatric cancer patients with FN.

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Conflict of interest statement

None declared.

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Chapter 3

Very early discharge versus early discharge versus non-early discharge in pediatric cancer patients with febrile neutropenia

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Abstract

Background

Chemotherapy induced neutropenia is a common side effect in pediatric cancer patients. Due to the high relative risk of infections and infectious complications, standard care for pediatric cancer patients with febrile neutropenia consists of routine hospitalization and parenteral administration of broad spectrum antibiotics. However, there are less serious causes of febrile neutropenia; in a subgroup of these patients lengthy in-hospital treatment might be unnecessary. Various research groups have studied the adjustment of standard care to shorten in-hospital treatment for patients with febrile neutropenia at low risk for bacterial infections. However, most of these studies were not done in randomized controlled trials.

Objectives

To evaluate whether (very) early discharge from in-hospital treatment (within 24 hours and less than five days respectively), for a selected group of patients, is not inferior to non-early discharge from in-hospital treatment (more than five days) in pediatric cancer patients with febrile neutropenia. Furthermore, to evaluate whether very early discharge (within 24 hours) is not inferior to early discharge (less than five days) from in-hospital treatment.

Search methods

Searches were made of the MEDLINE/PubMed (from 1945 to December 2009), EMBASE/Ovid (from 1980 to December 2009) and the Cochrane Central Register of Controlled Trials (CENTRAL) (The Cochrane Library 2009, issue 4), conference proceedings of the International Society for Paediatric Oncology (SIOP) (from 2005 to 2009), the American Society of Clinical Oncology (ASCO) (from 2005 to 2009), and the Multinational Association for Supportive Care in Cancer (MASCC) (from 2005 to 2009). We scanned the ISRCTN Register and the National Institute of Health (NIH) Register for ongoing trials.

Selection criteria

We included all randomized controlled trials and controlled clinical trials in which pediatric cancer patients with febrile neutropenia were divided in groups with different moments of discharge (within 24 hours and/or within five days and/or more than five days).

Data collection and analysis

We used standard methods of The Cochrane Collaboration and its Childhood Cancer Group.

Results

We identified one eligible study. This study showed that early discharge of pediatric cancer patients with febrile neutropenia and a low risk for invasive bacterial infection is as safe as non-early discharge; there was no significant difference in treatment failure between the two groups. Moreover, the mean treatment costs in the early discharge group were significantly lower than in the non-early discharge group. There was no significant difference in the duration of antibiotic treatment between the early discharge and the non-early discharge group. We identified no studies in which very early discharge was compared to early or non-early discharge.

Authors' conclusions

Only one study compared early discharge to non-early discharge in pediatric cancer patients with febrile neutropenia and low risk for invasive bacterial infection. Early discharge was as safe as non-early discharge. Future trials are needed to confirm or contradict these results.

Plain language summary

Very early discharge versus early discharge versus non-early discharge in children with cancer and fever during neutropenia

Treatment with chemotherapy can cause a low white blood cell count (neutropenia) in children with cancer. Due to the high relative risk of bacterial infections and of a fulminant course of infections, standard care for children with cancer and fever during neutropenia consists of routine hospitalization and intravenous administration of broad spectrum antibiotics. However, causes of fever during neutropenia can be less serious; in a subgroup of these patients lengthy in-hospital treatment might be unnecessary. Various research groups have studied the adjustment of standard care to shorten in-hospital treatment for patients with fever during neutropenia and a low risk for bacterial infections. However, most of these studies were not done in randomized controlled trials.

In this review of the literature we aimed to determine whether (very) early discharge from in-hospital treatment, for a selected group of patients, is not inferior to non-early discharge from in-hospital treatment in children with cancer and fever during neutropenia. Furthermore, we wanted to evaluate whether very early discharge is not inferior to early discharge from in-hospital treatment. The current study identified one study in which early discharge was compared to non-early discharge in this group of

patients. Early discharge appeared to be as safe as non-early discharge in children with cancer and fever during neutropenia with a low risk for bacterial infections; there was no significant difference in treatment failure between the two groups. Moreover the treatment costs in the early discharge group were lower than in the non-early discharge group. There was no difference in the total duration of antibiotic treatment between the early and the non-early discharge group. We identified no studies in which very early discharge was compared to early or non-early discharge.

In conclusion; in this study early discharge was as safe as non-early discharge. Future trials are needed to confirm or contradict these results.

Background

Survival rates for children with cancer have improved substantially in recent decades.[1] This has been attributed to better understanding of the disease, optimized supportive care, and improved treatment protocols. However, cancer treatment also has unwanted side effects. One of the most important side effects in pediatric cancer patients is chemotherapy-induced neutropenia, a haematological disorder characterized by an abnormally low number of neutrophils (type of white blood cells). In 1966 it was shown that low numbers of granulocytes was associated with an increased risk of severe infections.[2] Due to the high relative risk of infections and infectious complications, standard care for pediatric cancer patients with febrile neutropenia (neutropenia with fever) consists of routine hospitalization and parenteral administration of broad spectrum antibiotics. Patients are considered eligible for discharge from in-hospital treatment when they are afebrile, have completed their antibiotic course, and/or their absolute neutrophil count is recovering or has recovered. However, in 70% to 89% of febrile neutropenia episodes no causative organism is found.[3-7] This implies that patients with febrile neutropenia are a heterogenous group, in which fever can be caused by a bacterial infection, but also, for example, by viruses, transfusion of blood products, or chemotherapeutics. As a consequence a large proportion of patients are admitted to hospital and receive standard care unnecessarily. This leads, for example, to unnecessary occupation of hospital beds, increased bacterial resistance, reduced quality of life and increased hospital costs.

In 2002, the Infectious Diseases Society of America published a clear protocol for early discharge in adult cancer patients with febrile neutropenia.[8] Early discharge of pediatric patients was a more delicate topic; it was stated that after a minimum of 48 hours of in-hospital treatment with parental antibiotics and observation, early discharge with oral antibiotics might be considered for selected children at low risk of bacterial infections.[8] In this protocol low risk for bacterial infection was defined as proposed by Paganini et al and Shenep et al; absence of severe comorbidity, good clinical condition, negative blood

cultures, no Pseudomonas aeruginosa or methicillin-resistant Staphylococcus Aureus (MRSA) in cultures in the last 12 weeks, control of local infection, and afebrile for the last 24 hours.[9,10]

Various research groups have studied the adjustment of standard care to shorten in-hospital treatment for patients with low-risk febrile neutropenia, however, not in randomized controlled studies.[5,9,10] In this review we will compare early discharge from in-hospital treatment to non-early discharge from in-hospital treatment in pediatric cancer patients with febrile neutropenia, and evaluate the effects on treatment failure.

Objectives

The primary aim of the review is to evaluate whether (very) early discharge from in-hospital treatment, for a selected group of patients, is not inferior to non-early discharge from in-hospital treatment in pediatric cancer patients with febrile neutropenia, with regard to rehospitalization and/or adjustment of antimicrobial treatment and death. And moreover, to evaluate whether very early discharge is not inferior to early discharge from in-hospital treatment. Furthermore, we will look at quality of life, costs, and duration of treatment as secondary outcomes. We will analyze these outcomes in all pediatric cancer patients as well as in various subgroups.

Methods

Criteria for considering studies for this review

Types of studies

Randomized controlled trials (RCTs) and controlled clinical trials.

Types of participants

Pediatric cancer patients less than 21 years of age, presenting with febrile neutropenia. Neutropenia is defined as an absolute neutrophil count of less than 0.5 x 10^9 cells/l, or a leucocyte count < 1.0 x 10^9 cells/l (when an absolute neutrophil count is not available). Fever is defined as a single oral reading of > 38.2 °C or two readings of a temperature > 37.9 °C within 24 hours.

The intervention group will be the group of patients selected for (very) early discharge by the trial researchers.

Types of interventions

- Early discharge from in-hospital treatment versus non-early discharge from hospital treatment.
- Very early discharge from in-hospital treatment versus non-early discharge from hospital treatment.

- Very early discharge from in-hospital treatment versus early discharge from hospital treatment.

Non-early discharge is defined as discharge from in-hospital treatment after at least five days. Early discharge from hospital treatment is defined as discharge from the hospital before patients had received five days of in-hospital treatment. Very early discharge is defined as discharge from in-hospital treatment within 24 hours after presentation with fever and neutropenia.

Types of outcome measures

Primary outcomes

Treatment failure:

1. Rehospitalization and/or adjustment of antimicrobial treatment related to febrile neutropenia (patient deterioration or other febrile neutropenia-related causes) within one week or within the same neutropenic episode.
2. All death and death due to (complications of) febrile neutropenia within one week after hospital discharge or within the same neutropenic episode after hospital discharge.

Secondary outcomes

1. Quality of life.
2. Costs.
3. Duration of treatment.

Search methods for identification of studies

See: Cochrane Childhood Cancer Group methods used in reviews (Module CCG)[11].

We will search the following electronic databases: MEDLINE/PubMed (from 1945 to present), EMBASE/Ovid (from 1980 to present) and the Cochrane Central Register of Controlled Trials (CENTRAL) (The Cochrane Library, latest issue). The search strategies for the different electronic databases (using a combination of controlled vocabulary and text words) are shown in the appendices (Appendix 1; Appendix 2; Appendix 3)

We will locate information about trials not registered in MEDLINE, EMBASE or CENTRAL, either published or unpublished, by searching the reference lists of relevant articles and review articles. We will handsearch the conference proceedings of the International Society for Paediatric Oncology (SIOP) (from 2005 to 2009), the American Society of Clinical Oncology (ASCO) (from 2005 to 2009), and the Multinational Association for Supportive Care in Cancer (MASCC) (from 2005 to 2009). We will scan the ISRCTN Register and the National Institute of Health (NIH) Register for ongoing trials: http://www.controlled-trials.com.

We will not impose language restrictions. We will update the searches every two years.

Data collection and analysis

Selection of studies

After employing the search strategy described above, two authors will independently identify studies meeting the inclusion criteria. We will resolve discrepancies between authors by consensus. If this is impossible, we will achieve final resolution by using a third party arbitrator. We will obtain in full any study which seems to meet the inclusion criteria on the grounds of the title, abstract, or both, for closer inspection. We will clearly state reasons for exclusion of any study considered for the review.

Data extraction and management

Two authors will perform data extraction independently using standardized forms. We will extract data on the following items.

1. Study design.
2. Participants, including:
    a. age;
    b. sex;
    c. number of patients entering the trial;
    d. number of patients randomized;
    e. number of patients excluded (with reasons);
    f. number of patients evaluable (for each outcome);
    g. degree of neutropenia at the moment of presentation with febrile neutropenia.
3. Intervention:
    a. duration of admittance to the hospital in hours/days until discharge from in-hospital treatment.
    b. In-hospital treatment: no, oral, or intramuscular/intravenous antibiotic treatment.
    c. Treatment after discharge: no, oral, or intramuscular/intravenous antibiotic treatment.
4. Outcome measures.
5. Length of follow up.

When data are missing in a published report, we will attempt to contact the authors for the missing information. In cases of disagreement, we will re-examine the abstracts and articles and undertake discussion until consensus is achieved. If this is impossible, we will achieve final resolution using a third party arbitrator.

Assessment of risk of bias in included studies

Two authors will independently asses the risk of bias of the included randomized controlled trials and controlled clinical trials, according to the following criteria:

- sequence generation;
- concealment of allocation;

- blinding of care provider/patients/outcome assessors;
- intention-to-treat analysis; and
- completeness of follow up.

For the quality items we will use the definitions as described in the module of the Childhood Cancer Group, based on the Cochrane Handbook for Systematic Reviews of Interventions.[11,12] We will resolve discrepancies between authors by consensus. If this is impossible, we will achieve final resolution using a third party arbitrator. In the analyses, we will take the quality of studies into account in the interpretation of the review results.

Measures of treatment effect

We will enter data into Review Manager (RevMan)[13] and undertake analyses according to the guidelines of the Cochrane Handbook for Systematic Reviews of Interventions.[12] We will analyze dichotomous variables using risk ratio or relative risk (RR). We will analyze continuous outcomes using the weighted mean difference (WMD). All results will be presented with the corresponding 95% confidence interval (CI).

Dealing with missing data

When information relevant to study selection, data extraction and/or assessment of risk of bias is missing, we will attempt to contact the authors in order to obtain the missing data.

When applicable, we will extract data by allocated intervention, irrespective of compliance with the allocated intervention, in order to allow an intention-to-treat analysis. If this is not possible, this will be stated and we will perform an as treated analysis.

Assessment of heterogeneity

We will assess heterogeneity both by visual inspection of the forest plots and by a formal statistical test for heterogeneity, the I^2 statistic. In the absence of substantial heterogeneity ($I^2 < 50\%$)[12], we will use a fixed-effect model for the estimation of treatment effects. Otherwise, we will explore possible reasons for the occurrence of heterogeneity and take appropriate measures by using the random-effects model.

Assessment of reporting biases

We will construct a funnel plot to ascertain the risk of publication bias graphically.[12]

Data synthesis

If possible we will analyze data for different types of malignancies. We will include outcome measures in this systematic review only if it was the intention of the study to perform the necessary assessments in all patients (i.e. not optional or only performed in some centers). When less than 50% of the patients of a study have an acceptable follow up for a particular outcome measure, due to the associated high risk of attrition bias, we will not report the results of this outcome measure. If pooling is not possible we will summarize the results qualitatively.

Subgroup analysis and investigation of heterogeneity

If possible we will perform subgroup analyses for the following.

1. In-hospital treatment:
   a. in-hospital treatment with intravenous or intramuscular antibiotics versus oral antibiotics;
   b. in-hospital treatment with intravenous or intramuscular antibiotics versus no antibiotics;
   c. in-hospital treatment with oral antibiotics versus no antibiotics.
2. Treatment after discharge:
   a. treatment after discharge with intravenous or intramuscular antibiotics versus oral antibiotics;
   b. treatment after discharge with intravenous or intramuscular antibiotics versus no antibiotics;
   c. treatment after discharge with oral antibiotics versus no antibiotics.
3. Age: 0 to < 4 years versus 4 to < 21 years.
4. Type of malignancy: haematological malignancies versus solid tumours.
5. Degree of neutropenia at the moment of presentation with febrile neutropenia; absolute neutrophil count of 0.1 to 0.5 x 10^9 cells/l versus < 0.1 x 10^9 cells/l.

Sensitivity analysis

We will perform a sensitivity analysis using quality criteria.

Results

Description of studies

See: Characteristics of included studies.

Results of the search

The above search strategy identified one potential study for inclusion (Santolaya 2004).

Included studies

Santolaya 2004 is a study which compared outcome and cost of ambulantory versus hospitalized management among children with fever during neutropenia at low risk for invasive bacterial infection. Children with cancer, eighteen years and younger, were eligible when presenting with febrile neutropenia. They were categorized as high or low risk for invasive bacterial infection. The criteria for high risk were: serum level of C-reactive protein (CRP) ≥90 mg/L, hypotension, identification of relapse of leukemia as the cancer type, platelet count of ≤50,000 platelets/mm^3, and recent (<8 days since) receipt of chemotherapy. Children were considered to be at low risk when they had none of the risk factors or either a platelet count of ≤50,000 platelets/mm^3 or recent receipt of

chemotherapy as a sole risk factor.[15] A total of 390 episodes occurred in 313 children; 222 episodes were classified as high risk at enrolment and 168 as low risk. High risk patients received aggressive intravenous antibiotic treatment. After the second assessment of the low risk patients five patients appeared to be high risk; they were excluded from the study and received high risk treatment. Of the 161 children (41%) at low-risk for invasive bacterial infections 12 could not be randomized due to various reasons. The remaining 149 children were randomly assigned after 24 to 36 hours of hospitalization to receive ambulantory (78) or hospital-based (71) treatment and they were monitored until episode resolution. Antibiotic treatment consisted of intravenous ceftriaxone and teicoplanin. Intravenous antibiotic treatment was switched to oral cefuroxim after 72 hours when the clinical evolution was favorable. Outcome and costs were determined for each episode and compared between both groups using predefined definitions and questionnaires. Outcome was favourable in 74 (95%) of the ambulantory and 67 (94%) of the hospital-based treatment episodes (Figure 1; Figure 2). Mean duration of antimicrobial treatment was 6.1 and 6.4 days for the ambulatory- and inhospital-treated children respectively (Figure 3). Eight patients had an unfavorable outcome; four in each treatment group (Figure 1). After therapy modifications seven children recovered completely and one child, in the inhospital-treatment group, died (Figure 2). The costs for ambulatory treatment were significantly less than for inhospital treatment with a mean of US $638 (; 95% CI, $572 to $703) and US $903 (95% CI, $781 to $1,025; P=.003) respectively (Figure 4).

Excluded studies

None.

Risk of bias in included studies

Santolaya 2004

- blinding of randomization (allocation concealment) – yes;
- blinding of intervention - no;
- completeness of follow-up - yes;
- blinding of outcome measurement - unknown.

Effects of interventions

Due to the availability of only one suitable study, it was not possible to pool results. The data from Santolaya 2004 is suitable for possible meta-analysis and has been entered into RevMan.

Santolaya 2004

Primary outcomes

- The study stated that rehospitalization and/or adjustment of antimicrobial treatment, within the same neutropenic episode, occurred in 4 out of the 78 patients in the early

discharge group (5%) and in 4 out of the 71 patients in the non-early discharge group (6%).

- The study stated that there were no deaths due to complications of (febrile) neutropenia within the same neutropenic episode in the early discharge group. There was one death in the non-early discharge group.

Secondary outcomes

- The predefined outcome measure quality of life was not assessed in this study.
- The study stated that the mean costs for early discharge treatment were significantly lower than the costs for the non-early discharge treatment (US $638; 95% CI, $572 to $703 versus US $903; 95% CI, $781 to $1,025; P = .003).
- The study stated that the mean duration of antimicrobial treatment was 6.1 days (95% CI, 5.4 to 6.8 days) in the early discharge group and 6.4 days (95% CI, 5.9 to 7.0 days) in the non-early discharge group (P = not significant).

Other outcome measures reported in the study that were not pre-specified

- Mean duration of intravenous antimicrobial treatment was 4.3 days (95% CI, 3.7 to 5.0 days) in the early discharge group and 4.8 days (95% CI, 4.4 to 5.3 days) in the non-early discharge group (P = not significant).
- Mean duration of oral antimicrobial treatment was 1.8 days (95% CI, 1.2 to 2.3 days) in the early discharge group and 1.6 days (95% CI, 1.1 to 2.1 days) in the non-early discharge group (P = not significant).

Discussion

There was only one study that met the inclusion criteria for this review. Santolaya 2004 compared an early discharge group with a non-early discharge group. They evaluated safety, duration of treatment and costs of treatment. They did not evaluate quality of life.

Criteria for unfavourable outcome were; hemodynamic instability (not attributed to volume loss), fever for more than 3 days, reappearance of fever after a 48 hour afebrile period persisting for at least 24 hours, an ascending CRP curve or a nondescending curve over normal limits (defined as a value > 40 mg/L and < 30% decrease from a previous recording), and isolation of a bacterial pathogen from a significant sample obtained on day 3. In the study performed by Santolaya et al, unfavourable outcome led to an adjustment of antimicrobial treatment in both treatment groups and rehospitalization in the early discharge group. These criteria are objective parameters, which seem to be adequate. However, in our opinion fever for more than 2 or 3 days is not adequate as a parameter for treatment failure as it can also be caused by a viral infection. Therefore, we did not mention it as an item in our outcome criteria. Eventually we valued rehospitalization and/or adjustment of antimicrobial treatment related to febrile

neutropenia and death as adequate parameters of treatment failure, for both the inhospital and the outpatient treatment group.

Over the last decades there has been a tendency to earlier discharge and/or treatment with oral or no antibiotics in febrile, neutropenic patients considered to be at low risk for serious bacterial infections or infectious complications. Parameters for risk stratification in the heterogeneous group of patients with febrile neutropenia have changed over the years.[14] The prediction model for low risk for invasive bacterial infections provided by Santolaya et al consists of objective items and is reliable in their study cohort.[15] It would be interesting to see results of this prediction model in studies performed in oncology units in other countries, to establish whether or not these results can be replicated in other populations with different genetic and environmental factors. The studies accomplished by Ammann et al and Miedema et al illustrate that there can be a different outcome in a different population.[16,17] The recent study of Ammann et al prospectively evaluated a risk assessment model for adverse events (AEs) during febrile neutropenia, consisting of the items; preceding chemotherapy more intensive than acute lymphoblastic leukemia maintenance (weight 4), hemoglobin ≥90 g/L (weight 5), leukocyte count less than 0.3 G/L (weight 3), and platelet count less than 50 G/L (weight 3). In which a score (sum of weights) ≥9 predicted future AEs.[16] One difference between the two risk assessment models is the moment of assessment; at presentation in the study performed by Santolaya et al and at presentation and within 8-24 hours after admittance to the hospital in the study performed by Ammann et al. Another main difference are the parameters included in the risk assessment; the only corresponding item is the platelet count. Both research groups have objective parameters. The risk assessment model used by Ammann et al accurately predicted adverse events in their population of pediatric cancer patients. In the retrospective study performed by Miedema et al the use of the identical risk assessment model had different sensitivity and specificity levels. This could be due to the retrospective nature of the study, however, the different treatment protocol, the different genetic background of the study population and environmental factors may also play a role. Both authors stressed the necessity of prospective validation of the risk assessment score before broad clinical application and evaluation of the potential of markers of inflammation to increase its predictive performance.[17,18]

Since only one study met the inclusion criteria for this review, there are very limited data on the safety of (very) early discharge in pediatric cancer patients with febrile neutropenia (and a low risk of invasive bacterial infection). There were no publications on very early discharge versus early on non-early discharge in controlled clinical trials or randomized controlled trials.

In the study by Santolaya et al there was no risk of bias concerning randomization or completeness of follow-up. Blinding of the intervention was not possible as it is obvious for patients and personnel whether patients are treated inside or outside the hospital.

This can cause bias as patients can report their symptoms differently because they feel unsafe having fever and not being admitted to the hospital or this could lead to underreporting symptoms as patients or their parents want them to stay at home. Moreover, the attending doctor could adjust antimicrobial treatment faster in outpatients and readmit them to the hospital, whereas he would wait and see if the patient was in the inpatient treatment arm. However, due to the clear parameters for treatment failure this risk seems to be small. It was unclear whether or not there was blinding of the outcome measurement; if there was no blinding of outcome measurement this could inflict bias in favour of both treatment arms depending on the beliefs of the person handling the outcome measures.

Subgroup analyses were not possible because of lack of subgroups or due to not available data.

Author's conclusions

Implications for practice

The current available evidence suggests that early discharge of pediatric cancer patients with febrile neutropenia and low risk of bacterial infection is as safe as non-early discharge in a carefully selected, carefully instructed and well monitored group. It is our opinion that, as there is only one available study, early discharge should only be practised in a trial setting in hospitals/ oncology wards where close monitoring of early discharged patients by well trained employees is garanteed.

Implications for research

Further research is required to confirm or contradict that early discharge or even very early discharge is as safe as non-early discharge. Any further trials containing (very) early discharge should compare this to non-early discharge. To make sure that research results are applicable in other trial settings parameters for assessment for low risk for invasive bacterial infection should be as objective as possible. In our opinion it would also be valuable to have more information on quality of life, costs and duration of treatment.

Acknowledgements

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Contributions of authors

EMP, WJT and ESB performed the searches.
HMB and EMP performed the statistical analysis.
EMP wrote the review.
WJT, ESB and HMB revised the review.

Declaration of interest

None known.

Differences between protocol and review

HMB was added as co-author for statistical expertise and revision of the manuscript. The contact person is changed from Esther M te Poele to Eveline SJM de Bont. In the "Background" the word "results in" in the sentence "In 1966 it was shown that low numbers of granulocytes result in an increased risk of severe infections.[2]" was replaced by "was associated with" as this is more accurate. The word "high" was inserted between "the" and "relative" in the sentence "Due to the relative risk of infections and infectious complications, standard care for pediatric cancer patients with febrile neutropenia (neutropenia with fever) consists of routine hospitalization and parenteral administration of broad spectrum antibiotics." as this appeared to be more accurate. In the sentence "Low risk of bacterial infection was defined as absence of severe comorbidity, good clinical condition, negative blood cultures, no methicillin-resistant Staphylococcus Aureus (MRSA) in cultures in the last 12 weeks, control of local infection, and afebrile for the last 24 hours.[9,10]", "Low risk of bacterial infection was defined as" was replaced by "In this study low risk for bacterial infection was defined as proposed by Paganini et al and Shenep et al[9,10];" and "Pseudomonas aeruginosa or" was inserted between "no"and "methicillin". In the "Objectives" the sentence "And moreover, to evaluate whether very early discharge is not inferior to early discharge from in-hospital treatment." was included. In the "Objectives" the sentence ",however, not in randomized controlled studies" was included in "Various research groups have studied the adjustment of standard care to shorten in-hospital treatment for patients with low-risk febrile neutropenia[5,9,10]".The "Objectives" were changed from ".... with regard to rehospitalization and death." to ".... with regard to rehospitalization and/or adjustment of antimicrobial treatment and death." as rehospitalization is not a good parameter for treatment failure

for patients who receive inhospital treatment. Adjustment of antimicrobial treatment is a more accurate parameter. For the same reason the "Primary outcomes" were changed from ".... rehospitalization due to febrile neutropenia." to ".... rehospitalization and/or adjustment of antimicrobial treatment related to febrile neutropenia." In "Other references" Santolaya 2002 was included in the "additional references as results of the study were used in "Included studies" in the "Results". In "Other references" te Poele 2009, Ammann 2010 and Miedema 2011 were included in the "additional references as results of these studies were used in "Discussion". The following sections were completed: abstract, plain language summary, results, discussion, anthors' conclusions, contributions of authors, differences between protocol and review, characteristics of studies, summary of findings tables, references to studies, data and analyses, and figures.

Tables

Characteristics of studies

Characteristics of included studies

Santolaya 2004	
Methods	Randomized controlled trial.
Participants	Children with cancer 18 years and younger. The study enrolled 149 patients with a low risk for invasive bacterial infections.
Interventions	Patients were randomised to ambulatory or hospital-based treatment of febrile neutropenia after 24 to 36 hours after presentation. After a minimum of three days of intravenous antibiotics the decision to switch to oral antibiotics was made on an individual basis, based on predefined criteria.
Outcomes	Primary outcomes • Unfavourable: possible invasive bacterial infection Secondary outcomes • Duration of antimicrobial treatment • Duration of intravenous antimicrobial treatment • Duration of oral antimicrobial treatment • Costs
Notes	

Santolaya 2004: Risk of bias table		
Bias	Authors' judgement	Support for judgement
Random sequence generation (selection bias)	Low risk	
Allocation concealment (selection bias)	Unclear risk	method not described
Blinding of participants and personnel (performance bias)	High risk	blinding of participants and personnel is not possible
Blinding of outcome assessment (detection bias)	Unclear risk	
Incomplete outcome data (attrition bias)	Low risk	no patients were lost to follow-up
Selective reporting (reporting bias)	Unclear risk	
Other bias	Unclear risk	

Characteristics of ongoing studies

Ammann 2007	
Study name	Low-risk fever and neutropenia in children with cancer: safety and efficacy of oral antibiotics in an outpatient setting.
Methods	Randomized controlled trial.
Participants	Children with cancer and febrile neutropenia, 1 to 18 years of age.
Interventions	Inpatient observation period of 8-22 hours with empirical intravenous antibiotic treatment. Than low risk patients were randomized to either inhospital treatment with intravenous antibiotics or outpatient treatment with oral antibiotics.
Outcomes	Primary outcomes: • Safety: No serious medical complication due to infection (death, treatment in Intensive Care Unit, potentially life-threatening complication) • Efficacy: Response without rehospitalization or changing randomized antibiotics Secondary outcomes (observational study part): • Improved prediction of low-risk episodes of fever and neutropenia • Description of characteristics of low-risk episodes of fever and neutropenia • Description of characteristics of high-risk episodes of fever and neutropenia
Starting date	January 2004
Contact information	Through e-mail contact on 8 July 2011 professor Ammann notified that the analyses have been done and the manuscript is currently being written.
Notes	

References to studies

Included studies

Santolaya 2004

Santolaya ME, Alvarez AM, Avilés CL, Becker A, Cofré J, Cumsille MA, O'Ryan ML, Payá E, Salgado C, Silva P, Tordecilla J, Varas M, Villarroel M, Viviani T, Zubieta M. Early hospital discharge followed by outpatient management versus continued hospitalization of children with cancer, fever, and neutropenia at low risk for invasive bacterial infection. J Clin Oncol 2004;22:3784-9.

Ongoing studies

Ammann 2007

[ClinicalTrials.gov: NCT00107081]

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Data and analyses

1 Treatment failure

Outcome or Subgroup	Studies	Participants	Statistical Method	Effect Estimate
1.1 Rehospitalization and/or adjustment of antimicrobial treatment	1	149	Risk Ratio (M-H, Fixed, 95% CI)	0.91 [0.24, 3.50]
1.2 Death	1	149	Risk Ratio (M-H, Fixed, 95% CI)	0.30 [0.01, 7.34]

2 Treatment costs

Outcome or Subgroup	Studies	Participants	Statistical Method	Effect Estimate
2.1 Costs [US$]	1	149	Mean Difference (IV, Fixed, 95% CI [US$])	-265.00 [-403.14, -126.86]

3 Duration of treatment

Outcome or Subgroup	Studies	Participants	Statistical Method	Effect Estimate
3.1 Duration of antibiotic treatment [days]	1	149	Mean Difference (IV, Fixed, 95% CI [days])	-0.30 [-1.22, 0.62]

Figure 1 (Analysis 1.1): Forest plot of comparison: 1 Treatment failure, outcome: 1.1 Rehospitalization and/or adjustment of antimicrobial treatment.

Study or Subgroup	Early discharge Events	Total	Non-early discharge Events	Total	Weight	Risk Ratio M-H, Fixed, 95% CI
Santolaya 2004	4	78	4	71	100.0%	0.91 [0.24, 3.50]
Total (95% CI)		**78**		**71**	**100.0%**	**0.91 [0.24, 3.50]**
Total events	4		4			

Heterogeneity: Not applicable
Test for overall effect: Z = 0.14 (P = 0.89)

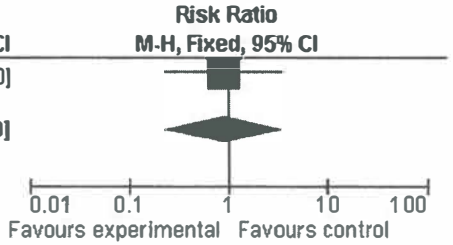


Figure 2 (Analysis 1.2): Forest plot of comparison: 1 Treatment failure, outcome: 1.2 Death.

Study or Subgroup	Early discharge Events	Total	Non-early discharge Events	Total	Weight	Risk Ratio M-H, Fixed, 95% CI
Santolaya 2004	0	78	1	71	100.0%	0.30 [0.01, 7.34]
Total (95% CI)		**78**		**71**	**100.0%**	**0.30 [0.01, 7.34]**
Total events	0		1			

Heterogeneity: Not applicable
Test for overall effect: Z = 0.73 (P = 0.46)

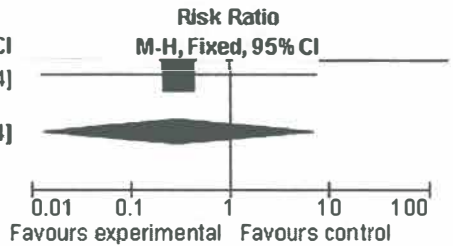


Figure 3 (Analysis 3.1): Forest plot of comparison: 3 Duration of treatment, outcome: 3.1 Duration of antibiotic treatment (days).

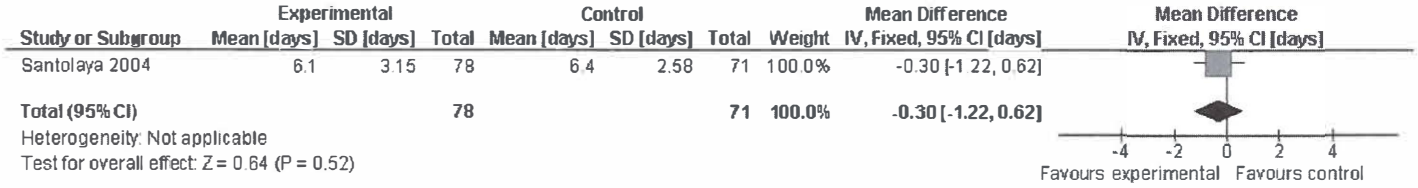
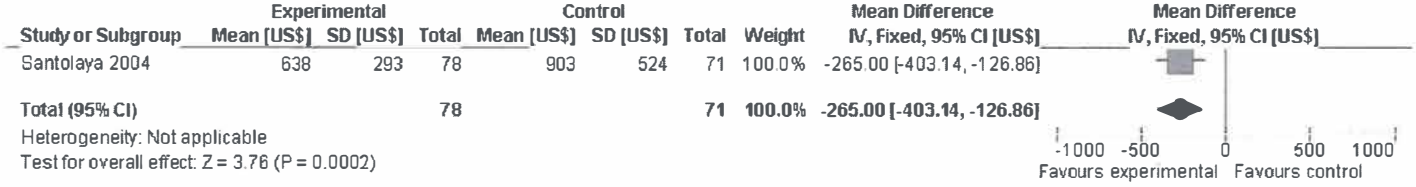


Figure 4 (Analysis 2.1): Forest plot of comparison: 2 Treatment costs, outcome: 2.1 Costs (US$).



Sources of support

Internal sources

No sources of support provided.

External sources

The Foundation of Pediatric Oncology Groningen (SKOG 03-001), Netherlands Stichting Kinderen Kankervrij (KiKa), The Netherlands.

Appendices

1 Search Strategy for MEDLINE/PubMed

1. For **Early discharge** the following MeSH headings and text words will be used:

   discharge* OR patient discharge OR patient discharge* OR ambulatory care OR "outpatient management" OR home treatment OR out-patient OR outpatient OR outpatients OR outpatient care OR outpatient health service* OR early discontinuation OR discontinue

2. For **Neutropenia** the following MeSH headings and text words will be used:

   febrile neutropenia OR fever OR febrile neutropenic OR neutropenia OR fevers OR hyperthermia* OR pyrexia* OR neutropenias OR febrile neutropenias

3. For **Children** the following MeSH headings and text words will be used:

   infant OR infan* OR newborn OR newborn* OR new-born* OR baby OR baby* OR babies OR neonat* OR perinat* OR postnat* OR child OR child* OR schoolchild* OR schoolchild OR school child OR school child* OR kid OR kids OR toddler* OR adolescent OR adoles* OR teen* OR boy* OR girl* OR minors OR minors* OR underag* OR under ag* OR juvenil* OR youth* OR kindergar* OR puberty OR puber* OR pubescen* OR prepubescen* OR prepuberty* OR pediatrics OR pediatric* OR paediatric* OR peadiatric* OR schools OR nursery school* OR preschool* OR pre school* OR primary school* OR secondary school* OR elementary school* OR elementary school OR high school* OR highschool* OR school age OR schoolage OR school age* OR schoolage* OR infancy OR schools, nursery OR infant, newborn

4. For **Childhood cancer** the following MeSH headings and text words will be used:

(((leukemia OR leukemi* OR leukaemi* OR (childhood ALL) OR AML OR lymphoma OR lymphom* OR hodgkin OR hodgkin* OR T-cell OR B-cell OR non-hodgkin OR sarcoma OR sarcom* OR sarcoma, Ewing's OR Ewing* OR osteosarcoma OR osteosarcom* OR wilms tumor OR wilms* OR nephroblastom* OR neuroblastoma OR neuroblastom* OR rhabdomyosarcoma OR rhabdomyosarcom* OR teratoma OR teratom* OR hepatoma OR hepatom* OR hepatoblastoma OR hepatoblastom* OR PNET OR medulloblastoma OR medulloblastom* OR PNET* OR neuroectodermal tumors, primitive OR retinoblastoma OR retinoblastom* OR meningioma OR meningiom* OR glioma OR gliom*) OR (pediatric oncology OR paediatric oncology)) OR (childhood cancer OR childhood tumor OR childhood tumors)) OR (brain tumor* OR brain tumour* OR brain neoplasms OR central nervous system neoplasm OR central nervous system neoplasms OR central nervous system tumor* OR central nervous system tumour* OR brain cancer* OR brain neoplasm* OR intracranial neoplasm*) OR (leukemia lymphocytic acute) OR (leukemia, lymphocytic, acute[mh])

5. For **RCTs** and **CCTs** the following MeSH headings and text words will be used:

(randomized controlled trial[pt] OR controlled clinical trial[pt] OR randomized[tiab] OR placebo[tiab] OR drug therapy[sh] OR randomly[tiab] OR trial[tiab] OR groups[tiab]) AND humans[mh]

6. 1 AND 2 AND 3 AND 4 AND 5

[pt = publication type; tiab = title, abstract; sh = subject heading; mh = MeSH term; RCT = randomized controlled trial; CCT = controlled clinical trial]

2 Search Strategy for EMBASE/Ovid

1. For **Early discharge** the following Emtree terms and text words will be used:

1. (discharge$ or patient discharge$).mp.
2. patient discharge.mp. or exp Hospital Discharge/
3. ambulatory care.mp. or exp Ambulatory Care/
4. outpatient management.mp.
5. home treatment.mp. or exp Home Care/
6. out-patient.mp.
7. exp OUTPATIENT CARE/ or outpatient.mp. or exp OUTPATIENT/
8. outpatients.mp.
9. outpatient health service$.mp.
10. (early discontinuation or discontinue).mp.
11. or/1-10

2. For **Neutropenia** the following Emtree terms and text words will be used:

1. febrile neutropenia.mp.
2. febrile neutropenias.mp.
3. febrile neutropenic.mp.
4. exp Febrile Neutropenia/
5. exp NEUTROPENIA/ or neutropenia.mp.
6. fever.mp. or exp FEVER/
7. fevers.mp.
8. Hyperthermia/
9. hyperthermia$.mp.
10. hyperthermia.mp.
11. pyrexia.mp.
12. pyrexia$.mp.
13. (neutropenias or neutropaenia or neutropaenias).mp.
14. or/1-13

3. For **Childhood cancer** the following Emtree terms and text words will be used:

1. (leukemia or leukemi$ or leukaemi$ or (childhood adj ALL) or acute lymphocytic leukemia).mp.
2. (AML or lymphoma or lymphom$ or hodgkin or hodgkin$ or T-cell or B-cell or non-hodgkin).mp.
3. (sarcoma or sarcom$ or Ewing$ or osteosarcoma or osteosarcom$ or wilms tumor or wilms$).mp.
4. (nephroblastom$ or neuroblastoma or neuroblastom$ or rhabdomyosarcoma or rhabdomyosarcom$ or teratoma or teratom$ or hepatoma or hepatom$ or hepatoblastoma or hepatoblastom$).mp.
5. (PNET or medulloblastoma or medulloblastom$ or PNET$ or neuroectodermal tumors or primitive neuroectodermal tumor$ or retinoblastoma or retinoblastom$ or meningioma or meningiom$ or glioma or gliom$).mp.
6. (pediatric oncology or paediatric oncology).mp.
7. ((childhood adj cancer) or (childhood adj tumor) or (childhood adj tumors) or childhood malignancy or (childhood adj malignancies) or childhood neoplasm$).mp.
8. ((pediatric adj malignancy) or (pediatric adj malignancies) or (paediatric adj malignancy) or (paediatric adj malignancies)).mp.
9. ((brain adj tumor$) or (brain adj tumour$) or (brain adj neoplasms) or (brain adj cancer$) or brain neoplasm$).mp.
10. (central nervous system tumor$ or central nervous system neoplasm or central nervous system neoplasms or central nervous system tumour$).mp.
11. intracranial neoplasm$.mp.

12. LEUKEMIA/ or LYMPHOMA/ or brain tumor/ or central nervous system tumor/ or teratoma/ or sarcoma/ or osteosarcoma/
13. nephroblastoma/ or neuroblastoma/ or rhabdomyosarcoma/ or hepatoblastoma/ or medulloblastoma/ or neuroectodermal tumor/ or retinoblastoma/ or meningioma/ or glioma/ or childhood cancer/
14. or/1-13

4. For **Children** the following Emtree terms and text words will be used:

1. infant/ or infancy/ or newborn/ or baby/ or child/ or preschool child/ or school child/
2. adolescent/ or juvenile/ or boy/ or girl/ or puberty/ or prepuberty/ or pediatrics/
3. primary school/ or high school/ or kindergarten/ or nursery school/ or school/
4. or/1-3
5. (infant$ or newborn$ or (new adj born$) or baby or baby$ or babies or neonate$ or perinat$ or postnat$).mp.
6. (child$ or (school adj child$) or schoolchild$ or (school adj age$) or schoolage$ or (pre adj school$) or preschool$).mp.
7. (kid or kids or toddler$ or adoles$ or teen$ or boy$ or girl$).mp.
8. (minors$ or (under adj ag$) or underage$ or juvenil$ or youth$).mp.
9. (puber$ or pubescen$ or prepubescen$ or prepubert$).mp.
10. (pediatric$ or paediatric$ or peadiatric$).mp.
11. (school or schools or (high adj school$) or highschool$ or (primary adj school$) or (nursery adj school$) or (elementary adj school) or (secondary adj school$) or kindergar$).mp.
12. or/5-11
13. 4 or 12

5. For **RCTs** and **CCTs** the following Emtree terms and text words will be used:

1. (randomized controlled trial or controlled clinical trial).mp.
2. (randomized or placebo or randomly or trial or groups).ti,ab.
3. drug therapy.sh.
4. 1 or 2 or 3
5. limit 4 to human

6. 1 and 2 and 3 and 4 and 5

[mp = title, abstract, subject headings, heading word, drug trade name, original title, device manufacturer, drug manufacturer name; sh = subject heading; ti,ab = title, abstract; / = Emtree term; RCT = randomized controlled trial; CCT = controlled clinical trial]

3 Search strategy for Cochrane Central Register of Controlled Trials (CENTRAL)

1. For **Early discharge** the following text words will be used:

discharge* OR patient discharge OR patient discharge* OR ambulatory care OR "outpatient management" OR home treatment OR out-patient OR outpatient OR outpatients OR outpatient care OR outpatient health service* OR early discontinuation OR discontinue

2. For **Neutropenia** the following text words will be used:

febrile neutropenia OR fever OR febrile neutropenic OR neutropenia OR fevers OR hyperthermia* OR pyrexia* OR neutropenias OR febrile neutropenias

3. For **Children** the following text words will be used:

infant OR infan* OR newborn OR newborn* OR new-born* OR baby OR baby* OR babies OR neonat* OR perinat* OR postnat* OR child OR child* OR schoolchild* OR schoolchild OR school child OR school child* OR kid OR kids OR toddler* OR adolescent OR adoles* OR teen* OR boy* OR girl* OR minors OR minors* OR underag* OR under ag* OR juvenil* OR youth* OR kindergar* OR puberty OR puber* OR pubescen* OR prepubescen* OR prepuberty* OR pediatrics OR pediatric* OR paediatric* OR peadiatric* OR schools OR nursery school* OR preschool* OR pre school* OR primary school* OR secondary school* OR elementary school* OR elementary school OR high school* OR highschool* OR school age OR schoolage OR school age* OR schoolage* OR infancy

4. For **Childhood cancer** the following text words will be used:

leukemia OR leukemi* OR leukaemi* OR childhood ALL OR AML OR lymphoma OR lymphom* OR hodgkin OR hodgkin* OR T-cell OR B-cell OR non-hodgkin OR sarcoma OR sarcom* OR Ewing* OR osteosarcoma OR osteosarcom* OR wilms tumor OR wilms* OR nephroblastom* OR neuroblastoma OR neuroblastom* OR rhabdomyosarcoma OR rhabdomyosarcom* OR teratoma OR teratom* OR hepatoma OR hepatom* OR hepatoblastoma OR hepatoblastom* OR PNET OR medulloblastoma OR medulloblastom* OR PNET* OR primitive neuroectodermal tumors OR retinoblastoma OR retinoblastom* OR meningioma OR meningiom* OR glioma OR gliom* OR pediatric oncology OR paediatric oncology OR childhood cancer OR childhood tumor OR childhood tumors OR brain tumor* OR brain tumour* OR brain neoplasms OR central nervous system neoplasm OR central nervous system neoplasms OR central nervous system tumor* OR central nervous system tumour* OR brain cancer* OR brain neoplasm* OR intracranial neoplasm* OR acute lymphocytic leukemia

5. 1 AND 2 AND 3 AND 4

The search will be performed in title, abstract or keywords.

Chapter 4

Dexamethasone in the maintenance phase of acute lymphoblastic leukaemia treatment: Is the risk of lethal infections too high?

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Abstract

We report an increased incidence of infectious deaths during maintenance treatment of the ninth protocol for acute lymphoblastic leukaemia of the Dutch Childhood Oncology Group (DCOG-ALL-9). The main difference in maintenance treatment between DCOG-ALL-9 and the DCOG-ALL-7 and DCOG-ALL-8 protocols is the interruption of methotrexate and 6-mercaptopurine by vincristine (2 mg/m^2 weekly) and dexamethasone (6 mg/m^2 daily) for 14 days every 7 weeks in the DCOG-ALL-9 protocol. The 1107 children treated with the DCOG-ALL-7, DCOG-ALL-8 or DCOG-ALL-9 protocol were included and screened for infectious death during maintenance treatment (July 1988-July 2002). Seven of the 510 children died of severe infections during the maintenance phase of DCOG-ALL-9, compared to none of the 597 patients during the DCOG-ALL-7 and DCOG-ALL-8 protocols (1.37% versus 0.0%; p = 0.013). Results from the current study suggest that repeated, prolonged exposure to dexamethasone results in an increase of lethal infections from 0% to 1.37%. In the dosing-schedule used, the advantage of dexamethasone may not outweigh the higher risk of infectious death.

Keywords: Acute lymphoblastic leukaemia, ALL child, Dexamethasone, Glucocorticoids, Corticosteroids, Infection

1. Introduction

A number of children with leukaemia die during treatment due to causes other than progressive leukaemia.[1] Since the start of the ninth protocol for acute lymphoblastic leukaemia treatment of the Dutch Childhood Oncology Group (DCOG-ALL-9), in January 1997, we have noticed an increase in lethal infections in children with acute lymphoblastic leukaemia (ALL). These patients presented with severe infections, mostly in the second half of the maintenance treatment. The main difference between the maintenance treatment of the DCOG-ALL-9 and the previous DCOG-ALL-7 and DCOG-ALL-8 protocols is the interruption of methotrexate (MTX) and 6-mercaptopurine (6MP) by vincristine (VCR) (2 mg/m^2 i.v. weekly) combined with dexamethasone (6 mg/m^2 p.o. daily) for 14 days every 7 weeks in the DCOG-ALL-9 protocol.

Dexamethasone has an important role in the treatment of ALL. The advantages of dexamethasone over prednisone in ALL treatment are its 5.5 to 16 times stronger antileukaemic activity[2,3] and its greater penetration into and the longer half-life in cerebrospinal fluid,[4] resulting in a decreased number of central nervous system relapses.[5,6] Randomised studies have shown that dexamethasone is appreciably more effective than prednisone in treatment of childhood ALL with significantly higher event-free survivals as a result.[7,8] However, dexamethasone also appears to have an anti-inflammatory action about nine times as strong as the anti-inflammatory action of prednisolone.[9]

In ALL induction therapy, an increased incidence in infectious deaths from 1% to 11% was reported when dexamethasone (6 mg/m^2/d) was substituted for prednisone (40 mg/m^2/d).[10] Others however, did not find an increase in infectious deaths, when dexamethasone (6 mg/m^2/d) was used instead of prednisone (40-60 mg/m^2/d) during ALL induction therapy.[7,11] During maintenance treatment in (standard risk) ALL, no difference was found in the incidence of infectious deaths between short courses (5 days every 4 weeks) of dexamethasone (6 mg/m^2/d) and prednisone (40 mg/m^2/d).[7] In contrast, the DCOG-ALL-9 protocol administered dexamethasone for 2 weeks every 7 weeks (in addition to two weekly VCR dosages), resulting in a higher dexamethasone dose-intensity and prolonged episodes of dexamethasone treatment. When scanning the literature, no reports of infectious deaths related to VCR use were found.

Patients treated for ALL often suffer from chemotherapy induced neutropenia, resulting in a diminished inflammatory response and an increased risk for serious infections. Therefore, standard treatment of febrile neutropenia consists of hospitalisation and empirical broad-spectrum intravenous antibiotic therapy.

In this article the patient and episode characteristics as well as the incidence of infectious deaths during the maintenance phase of the DCOG-ALL-9 treatment are described and compared with the incidence of infectious deaths during the maintenance treatment of the preceding DCOG-ALL-7 and DCOG-ALL-8 protocols.

2. Patients and methods

2.1. *Patients*

Of the 1289 children diagnosed in the Netherlands between July 1988 and July 2002 with ALL and treated with one of the consecutive DCOG-ALL-7, DCOG-ALL-8 or DCOG-ALL-9 protocol, 1139 (88%) entered maintenance treatment. The 1107 (97%) patients from whom we received complete data, concerning the start and stop dates of maintenance treatment, treatment outcome, and date of death when applicable, were included in this retrospective study. Data were complete in all DCOG-ALL-7 and DCOG-ALL-8 patients; 32 DCOG-ALL-9 patients were excluded due to missing data. Patient characteristics were obtained from the Dutch Childhood Oncology Group data office. Infectious death was defined as death with clinical and/or microbiologic evidence of a localised or generalised infection during complete remission. Death during maintenance was defined as death in the period from the day maintenance therapy started until the end of maintenance treatment. Neutropenia was defined as an absolute neutrophil count (ANC) $<0.5 \times 10^9$/l, or a leucocyte count $<1.0 \times 10^9$/l when ANC was not available.

2.2. *Treatment*

From July 1988 until January 1997, children with ALL were enrolled in DCOG-ALL-7[12] and DCOG-ALL-8 studies.[13] Briefly, maintenance treatment consisted of daily 6MP 50 mg/m^2 and weekly MTX 20 mg/m^2 orally. From January 1997 until January 2005, children with ALL were treated according to the DCOG-ALL-9 protocol. The induction therapy, central nervous system treatment and two intensification blocks (for high risk patients only) were followed by a maintenance phase up to 2 years after diagnosis. In contrast to the maintenance treatment of the two previous protocols, DCOG-ALL-9 consists of intrathecal triple medication every 7 weeks for 1 year, weekly doses of VCR 2 mg/m^2 intravenously two times and daily oral dexamethasone 6 mg/m^2 for 14 days, alternating with 5 weeks of weekly MTX 30 mg/m^2 (intravenously in high risk patients, orally in non-high risk patients) and daily oral 6MP 50 mg/m^2.

2.3. *Statistical methods*

The χ^2-test was used to compare infectious deaths during the maintenance treatment of the different ALL-protocols. The Cox proportional hazards model was used to test the influence of the duration of maintenance treatment on risk of infectious death. The

Mann-Whitney U test was used to test whether there were age differences between the DCOG-ALL-7/8 and the DCOG-ALL-9 groups.

3. Results

During the DCOG-ALL-9 protocol, there were increasing reports of patients dying of infectious complications during their maintenance treatment. In the first 5.5 years, seven infectious deaths occurred during the maintenance phase. Patient- and episode-characteristics are shown in Table 1. The patients (4 boys, 3 girls) had a median age of 5 years (range: 3-18 years), 3 were on high risk and 4 on non-high risk treatment. All but one patient had used dexamethasone recently, defined as within 2 days of presentation with infection. Five patients died in the second half of their maintenance phase. All but two patients had fever at admission, four in combination with neutropenia and/or leucocytopenia. At presentation most patients appeared in a relatively good clinical condition: five patients were alert; hypotension was reported in one patient (two unknown). Despite the prompt introduction of i.v. broad-spectrum antibiotic treatment, six patients deteriorated within 12 h after admission (one within 18 h). Causative pathogens were found in six patients. The incidence of infectious deaths during the maintenance therapy of DCOG-ALL-9 differed from the incidence of infectious deaths during the DCOG-ALL-7 and DCOG-ALL-8 protocols. DCOG-ALL-9 maintenance treatment was given to 510 evaluable patients. Seven of the 510 patients died of infectious complications. During the DCOG-ALL-7 and DCOG-ALL-8 protocols, 597 children started maintenance treatment and none of these children died of infectious complications or due to uncertain causes. There were significantly more infectious deaths during DCOG-ALL-9 than during the DCOG-ALL-7 and DCOG-ALL-8 maintenance treatments (7/510 versus 0/597; respectively, 1.37% versus 0.0%; $p = 0.013$), even more so when the uncertain causes of death ($n = 1$) were taken into account (8/510 versus 0/597; respectively, 1.57% versus 0.0%; $p = 0.007$). Additional adjustment for the duration of the maintenance treatment in the different protocols did not change these results. Therefore, the higher incidence of infectious deaths during the DCOG-ALL-9 protocol is not due to longer duration of the maintenance treatment of this protocol, compared to the previous protocols.

4. Discussion

Seven infectious deaths were seen during the first 5.5 years of DCOG-ALL-9 maintenance treatment, compared to none during DCOG-ALL-7 and DCOG-ALL-8 (1.37% versus 0.0%, respectively). These patients had hardly any clinical complaints and they presented relatively late with a severe infection. Five of the seven patients were still alert on

admission, however, all but one patient's condition deteriorated quickly and despite intensive care treatment all seven patients died.

In this study 32 of the 542 patients who started DCOG-ALL-9 maintenance treatment had been excluded from analysis due to incomplete data. If these patients were included and analysed as having had no infectious deaths during maintenance treatment, they would decrease the incidence of infectious deaths from 1.37% to 1.29%. The difference in infectious deaths during DCOG-ALL-7 and DCOG-ALL-8 compared to DCOG-ALL-9 would still be significant (p = 0.016).

Table 1: Characteristics of seven patients with a lethal infection during the maintenance phase of their DCOG-ALL-9 treatment

	Patient						
	A	B	C	D	E	F	G
Age (years)	18	3	16	5	5	15	3
Sex	Female	Male	Male	Female	Male	Female	Male
Risk group	HR	NHR	HR	NHR	HR	NHR	NHR
Day – since start of last dexamethasone administration[a]	15	33	13	8	15	14	16
Period of dexamethasone use (HR 11, NHR 14 periods in total)	9th	12th	6th	12th	7th	7th	2nd
ANC at presentation (10^9/l)	NA	3.7	NA	0	0.1	2.9	0.03
ALC at presentation (10^9/l)	0.1	5.8	2.4	1.5	2.3	4.2	1.6
Mental state at presentation	Alert	Apathic and groggy	Alert	Somnolent	Alert	Alert	Alert
Hypotension at presentation	No	NA	NA	Yes	No	No	No
Febrile at presentation	Yes	No	No	Yes	Yes	Yes	Yes
CRP at presentation (mg/l)	238	5	1	83	74	469	111
Start of deterioration (ours after admission)	<2	<12	<18	3	~4	~10	~10
Cultured microorganisms in blood (or otherwise mentioned)	Escherichia coli	Shigella flexneri 4a (faeces), Toxoplasma myocarditis	Herpes simplex virus	Escherichia coli	None	Pseudomonas aeruginosa, Streptococcus mitis	Salmonella (faeces)

HR: high risk; NHR: non-high risk; ANC: absolute neutrophil count; NA: not available; ALC: absolute leucocyte count; CRP: C-reactive protein; <: less than; ~: about; ICU: intensive care unit.

[a] The first day of dexamethasone administration being day 1; day 14 being the last day of dexamethasone administration during that treatment cycle (7 weeks).

During the DCOG-ALL-6 maintenance treatment (1984-1988), which was identical to DCOG-ALL-9 maintenance treatment, the incidence of infectious deaths was even higher: 1.63% (3/184) (3-year event-free survival: 80%). However, the total number of patients was

smaller.[14] In the UK ALL study, event-free survival was significantly improved among the 1603 randomised patients when dexamethasone was given instead of prednisone (84.2% versus 75.6% for the prednisone arm). Steroids were given 5 days out of every 4 weeks, total death in remission was, with 4.1% versus 3.0%, not statistically significantly different.[8] The study of the Children's Cancer Group of the United States[7] also used dexamethasone at a dose of 6 mg/m^2/d and showed a superior event-free survival than the prednisone arm of this randomised study; they reported one fatal complication among approximately 500 patients during maintenance. The more prolonged exposure to dexamethasone per treatment cycle, 14 consecutive days every 7 weeks (DCOG-ALL-9) versus 5 subsequent days every 4 weeks,[7] could explain the higher incidence of infectious deaths seen in the DCOG-ALL-9 group compared to Bostrom and colleagues (7/510 versus 1/about 500 patients, respectively).

A possible explanation for the increased incidence of infectious deaths under the DCOG-ALL-9 may lie in daily dexamethasone for 2 weeks in the maintenance treatment of the DCOG-ALL-9 protocol. In the literature, *in vitro* experiments are described in which effector cells of the innate immunity were (pre)incubated with dexamethasone, stimulated with several pro-inflammatory agents, and the pro-inflammatory cytokine production was measured. The inhibited production of inflammatory cytokines results in a slower rise of cytokine levels *in vitro*.[15-18] A longer preincubation with dexamethasone gave an even stronger inhibition of cytokine production *in vitro*.[17] *In vivo*, this attenuated cytokine response could have contributed to a diminished and later appearance of clinical symptoms of infection. This blunted inflammatory response will attenuate the clinical symptoms of a serious and potentially lethal infection. It can be hypothesised that the host response of these infectious deaths was diminished due to the use of dexamethasone.

Another explanation for the increased incidence of infectious deaths during the maintenance treatment of DCOG-ALL-9 may be an inadequate stress response. Stressors like inflammation stimulate the hypothalamic-pituitary-adrenal axis (HPA axis); this results in the increased synthesis and secretion of cortisol. Increased cortisol levels cause, e.g. anti-inflammatory effects and maintenance of the vascular tone. The HPA axis is of vital importance to build this stress response to an infection. Exposure to synthetic glucocorticosteroids can cause HPA axis suppression,[19-22] resulting in de creased cortisol levels in response to inflammatory stimuli. Several studies have shown great variation in the incidence and duration of HPA axis suppression.[23-25] A recent study in ALL patients demonstrated an attenuated cortisol response upon adrenocorticotropic hormone (ACTH) (low dose) in 46% of the patients 2 weeks after the discontinuation of 28 days prednisone.[26] Even after 1 week of prednisone treatment the ACTH-induced cortisol response is suppressed for at least 2 days after discontinuation.[27] We think that at the later phases of DCOG-ALL-9 maintenance treatment, that is after consecutive blocks of dexamethasone (14 days every 7 weeks), the HPA axis recovery might have become even

worse, resulting in periods of (more severe) HPA axis suppression towards the end of the treatment. We hypothesise that this inquate stress response results in a rapid deterioration of the patients' condition following a slow initiation of the inflammatory response.

5. Conclusion

Seven children died of infectious complications during the maintenance phase of the DCOG-ALL-9 protocol compared to none during the DCOG-ALL-7 and DCOG-ALL-8 protocols. The recurrent, prolonged use of dexamethasone could have been the crucial difference between DCOG-ALL-9 and DCOG-ALL-7/8. Dexamethasone treatment may result in a blunted inflammatory response with a slower and delayed rise of pro-inflammatory cytokine levels. This results in a delayed and masked presentation of infections in these patients. The effect of the recurrent and extended periods of dexamethasone usage may be accumulative and could lead to a hampered inflammatory response on the one hand and to an inadequate stress response on the other hand, resulting in an increased risk for lethal infections. Although dexamethasone has shown to have a stronger antileukaemic effect than prednisone[7,8] and could therefore be useful in a prolonged usage to maintain remission in ALL, the current findings suggest that this can lead to unacceptably high risks of lethal infections. It is questionable whether the advantage of dexamethasone administration in this repetitive and prolonged dosing-schedule during maintenance treatment, especially in the favourable prognostic groups, outweighs the disadvantage.

Conflict of interest statement

None declared.

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Chapter 5

Encouraging outcome in pediatric oncology patients admitted to the intensive care unit

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Abstract

Background

Survival rates for pediatric oncology patients have improved substantially due to more intensive chemotherapy protocols. However, current treatments are associated with life-threatening events requiring pediatric intensive care unit (PICU) admission. Over the last 20 years, mortality rates of children with malignancies admitted to the PICU have decreased, with significant differences between ventilated and not-ventilated patients.

Purpose

To assess survival until PICU discharge and determinants of survival in pediatric oncology patients treated in our tertiary university hospital pediatric oncology referral center.

Methods

Between January 2001 and December 2005, all non-elective PICU-admissions of cancer patients were included. Data collected included: demographic variables, interval between diagnosis and PICU admission, PICU-admission indication, Pediatric Risk of Mortality (PRISM) score, inotropic support, sepsis, transfusion of blood products, days on mechanical ventilation, and survival to PICU discharge.

Results

107 acute admissions were included. Mechanical ventilation was necessary in 58 admissions (54%), combined with inotropic support in 21 admissions (20%); another 8 admissions (7%) required inotropic support only. Overall PICU mortality was 14 of 107 (13%), the PRISM-predicted mortality 12%. There was a significantly higher mortality in sepsis- (9 of 41; 22%) than in non-sepsis admissions (5 of 66; 8%)(p= 0.032).

Conclusions

Overall PICU mortality was 13%; mortality was higher in sepsis than in non-sepsis patients. Mechanical ventilation, inotropic support and transfusion of blood products were independently associated with increased mortality. However, even the sickest patients have encouraging survival rates, and thus should receive intensive care support.

Introduction

Survival rates for pediatric oncology patients have improved substantially over the last decades. This has been attributed to better understanding of the disease, improved treatment protocols and increased levels of supportive care. Disease related or treatment-induced complications, however, may result in life-threatening events that require admission to the pediatric intensive care unit (PICU).[1] In the eighties, mortality rates in pediatric oncology patients admitted to the PICU who required respiratory or circulatory support were reported to be more than 84%.[2,3] More recently mortality rates in pediatric oncology patients admitted to the PICU have shown improvement. Keengwe *et al.* have shown that the mortality rates in non-elective PICU admissions of oncology patients decreased to 34% during the period 1990-1997.[4] In ventilated patients, decreased mortality rates have also been reported.[4,5]

The PICU mortality predictors, such as the acute physiology and chronic health evaluation score (APACHE-II) and the Pediatric Risk of Mortality score (PRISM), were shown to be higher in non-survivors compared to survivors to PICU discharge.[1,3,4,6-9] Moreover, variables like use of inotropic support, severe leucopenia, neutropenia, septic shock, sepsis, and fungal sepsis have been associated with mortality in pediatric cancer patients.[2-4,6-11]

In pediatric oncology patients other than bone marrow recipients few studies have prospectively evaluated PRISM scores[12] and no studies have shown whether Pediatric Index of Mortality (PIM)[13] can accurately predict PICU mortality. We studied survival until PICU discharge in a cohort of pediatric oncology patients. Also we evaluated PIM and PRISM scores in relation to PICU mortality and we tried to identify independent variables associated with PICU mortality.

Methods

Study Population

All patients included in the present study met the following criteria: 1) age: 0-18 years; 2) non-elective admission to the PICU between January 1, 2001, and December 31, 2005 due to imminent or manifest life-threatening disorders; 3) an underlying diagnosis of cancer. On our tertiary-care pediatric oncology ward cord cell transplantations and allogeneic bone marrow transplantations are not performed. No patients with stem cell transplantations elsewhere were admitted. Eligible patients were identified by cross-matching the PICU database and the oncology database.

Patients are admitted to the PICU of The Beatrix Children's Hospital if they require respiratory or circulatory support for life-threatening disorders. Patients are managed by the attending intensivist in cooperation with the attending oncologist. Mechanical

ventilation is according to a lung protective strategy i.e. with tidal volumes of approximately 6-7 ml/kg body weight. Fluid resuscitation comprises administration of fluids to correct hypoperfusion and restore blood pressure and/or central venous pressure. The first line of inotropic support usually consists of dopamine infusion, often in combination with noradrenalin. Patients are treated with broad-spectrum intravenous antibiotics, antifungals, and antiviral agents at the discretion of the intensivist, oncologist, and infectious disease consultant. Enteral nutritional support is provided when possible, and parenteral nutrition is used otherwise. Patients receive packed red blood cell and platelet transfusions if necessary. Patients are treated with hematopoietic colony-stimulating factors (granulocyte colony-stimulating factors) at the discretion of the attending oncologist. Coagulopathy is managed with transfusions of fresh frozen plasma, cryoprecipitate, and vitamin K.

Data Collection

The following information was retrieved from our PICU and oncology databases: demographic variables, oncology diagnosis and remission date, time between oncology diagnosis and PICU admission, indication for PICU admission, absolute neutrophil count on admission, sepsis, use of inotropic support, use of mechanical ventilation, development of hypertension, blood culture results, ventilator days, PICU length of stay, transfusions of blood products (erythrocytes, platelets and fresh frozen) and survival to PICU discharge.

PIM[13] and PRISM[12] scores (version I and version II respectively) have been prospectively recorded in our PICU since 2001. Standardized mortality ratios (SMRs) were then calculated by dividing the observed number of deaths by the predicted number of deaths. A SMR of 1 indicates the number of observed deaths equals the predicted deaths. If the SMR is >1 the prediction model underestimates mortality. If the SMR is <1 the prediction model overestimates mortality.

Sepsis coding was performed at PICU discharge and included a proven or suspected infection in the presence of systemic inflammatory response syndrome.[14-16] Neutropenia was defined as an absolute neutrophil count (ANC) < 0.5 x 10^9/l, or a leukocyte count < 1.0 x 10^9/l when ANC was not available. Patients with Langerhans cell histiocytosis, hemophagocytic lymphohistiocytosis or myelodysplastic syndrome were included as "other hematological disorders". Complete remission (CR) in hematological malignancy was defined according to current international protocol criteria.

Statistical Analysis

ROC curves were used to determine the sensitivity and specificity of mortality predictors PRISM and PIM, by plotting sensitivity against specificity, with 'death' as dependent variable. Univariate statistical analysis was performed to compare characteristics of

survivors and non-survivors. The chi-square test and Fisher's exact test were used to analyze categorical variables. Student's *t*-test (parametric data) and Wilcoxon rank sum test (nonparametric data) were used to analyze continuous variables and the mortality predictors. In univariate analysis, a *p* value <.05 was considered significant (tested 2-sided). To identify variables associated with survival a multivariate logistic regression was done. All variables with a *p* value of <.10 in univariate analysis were entered step forward into the model, and those with a *p* value of <.05 after logistic regression analysis were considered significant and remained in the model. Several patients were admitted more than once to the PICU. Therefore, we also analyzed the data in a univariate analysis only using the first admission per patient to the PICU.

Results

Demographics

A total of 82 pediatric oncology patients were non-electively admitted to PICU 107 times (107 of 2323 PICU admissions: 4.6%). The median age of these patients was 5.9 years (range 0.1 – 18 years) and 57% were male. Forty-eight percent of patients had a solid malignancy and 52% a hematological malignancy (table 1). The most common PICU admission indications were sepsis (38%) and primary respiratory failure (22%) (table 2). Mechanical ventilation was used in 34%, inotropic support in 7% and both mechanical ventilation and inotropic support in 20% of admissions.

Table 1: Oncology diagnoses. Oncology diagnoses of pediatric oncology patients with non-elective pediatric intensive care unit admissions.

Oncology diagnosis	Number (%)
Acute non-lymphatic leukemia	16 (19%)
Acute lymphatic leukemia	15 (18%)
Brain tumor	10 (12%)
Wilms / other kidney tumor	9 (11%)
Lymphoma	8 (10%)
Neuroblastoma	8 (10%)
Sarcoma	7 (9%)
Other solid tumor	5 (6%)
Other hematological tumor	4 (5%)
Total	**82 (100%)**

Mortality

Fourteen children died on the PICU (i.e. 13.1% of 107 PICU admissions). When only first admissions were analyzed, mortality was 14.6% (12 of 82 admissions).

Mechanical ventilation was associated with a significantly higher mortality rate: 22% (13 of 58) versus 2% (1 of 49) when mechanical ventilation was not required (p=0.002). Inotropic support was associated with a higher mortality rate: 38% (11 of 29) versus 4% (3 of 78) no inotropic support (p<0.001). When both mechanical ventilation and inotropic support were required, the mortality rate was 48% (10 of 21). The mortality rate in sepsis patients (9 of 41 admissions: 22%) was significantly higher compared to patients without sepsis (5 of 66 admissions: 8%; p=0.032). If cultures yielded Gram negative bacteria or fungi, mortality rates where high (respectively 4/10: 40% and 5/7: 71%), particularly when compared to Gram positive bacteria or negative cultures (respectively 0/17 and 0/7: 0%). The incidence of mechanical ventilation in sepsis (61%) was not significantly different compared to mechanical ventilation in non-sepsis (50%; p=0.27). The incidence of inotropic support was higher in sepsis (63%) compared to non-sepsis (4.5%; p<0.001).

Table 2: Pediatric intensive care unit admission indications. Indications of non-elective pediatric intensive care unit admissions in pediatric oncology patients.

PICU admission indication	Number (%)	Mortality (%)
Sepsis	41 (38%)	9 (22%)
Respiratory failure (no pneumonia)	23 (22%)	2 (9%)
Neurological (convulsions, coma)	11 (10%)	3 (27%)
Hypertension	8 (7%)	0 (0%)
Pneumonia	5 (5%)	0 (0%)
Shock (non-septic)	5 (5%)	0 (0%)
Electrolyte disorders	2 (2%)	0 (0%)
Acute abdomen	2 (2%)	0 (0%)
Other	10 (9%)	0 (0%)
Total	**107 (100%)**	**14 (13%)**

Variables associated with mortality

Table 3 compares survival and non-survival (univariate analysis) in the 107 admissions included in the study. Significant differences were found for sepsis, fungal sepsis, mechanical ventilation, use of inotropic support, number of blood product transfusions per day and PIM and PRISM score. However, age, gender, type of malignancy (hematological versus solid), time since oncology diagnosis and admission, neutropenia on admission and hypertension were not significantly different. Survival rates in patients with hematological malignancies were comparable between patients who were considered to be in CR and patients not in CR (mortality in CR 4/25 (16%) versus not CR 5/27 (19%); p>0.05).

Table 3: Univariate analysis of variables associated with intensive care unit mortality

Variable	Survivors (%) N = 93	Non-survivors (%) N = 14	*p*-value
Age (years)*	5.8 [0.1-16.5]	10.2 [0.2-18.5]	0.64
Male sex	54/93 (58%)	7/14 (50%)	0.57
Hematological malignancy	34/93 (37%)	9/14 (64%)	0.21
Interval oncology diagnosis – PICU admission* (months)	3.0 [0-158]	3.5 [0-87]	0.89
Sepsis	32/93 (34%)	9/14 (64%)	**0.03**
Neutropenia on admission	37/93 (40%)	10/14 (71%)	0.06
Fungal sepsis	2/93 (2%)	5/14 (36%)	**<0.001**
Mechanical ventilation	45/93 (48%)	13/14 (93%)	**0.002**
Use of inotropic support	18/93 (19%)	11/14 (79%)	**<0.001**
Hypertension	19/93 (20%)	0/14 (0%)	0.07
Number of blood products/day*	0.3 [0-3.5]	1.7 [0-8.3]	**<0.001**
PIM-score*	-3.9 [-6.4- -0.1]	-2.7 [-4.9- -0.3]	**0.03**
PRISM-score*	9.0 [0-37]	16.5 [5-31]	**0.001**

Abbreviations: PICU, pediatric intensive care unit; PIM-score, Pediatric Index of Mortality-score; PRISM-score, Pediatric Risk of Mortality-score. Significant p-values are bold. * median [range].

Multivariate analysis of variables associated with mortality

Multivariate analysis identified three variables independently associated with mortality: use of inotropic support (p=0.008; OR 8.2, 95%CI 1.7-38.7), number of blood products per day (p=0.008; OR 3.5, 95%CI 1.4-8.8), and mechanical ventilation (p=0.026; OR 14.6, 95%CI 1.4-156.3).

Predicted and observed mortality

The overall mortality was 13.1%. The predicted PRISM mortality was 12.3% and the predicted PIM mortality was 6.2%. Both PRISM and PIM scores were predictive of death (area under the curve PRISM=0.77 (p=0.001), respectively area under the curve PIM=0.68 (p=0.034), under a nonparametric assumption) (figure 1A and figure 1B).

SMR-PRISM and SMR-PIM in pediatric oncology patients were 1.06 (SMR-PRISM all PICU admissions: 0.85) and 2.09 (SMR-PIM all PICU admissions: 1.17), respectively.

First admission per patient to the PICU

Seventy patients (85%) were admitted once to the PICU and 12 patients (15%) were admitted more than once (range 1-6). When only the first PICU admission per patient (82 admissions in 82 patients) was included in the univariate analysis, significant differences between survivors and non-survivors were found for mechanical ventilation, use of inotropic support, fungal sepsis, number of blood products per day and PRISM scores. Multivariate analysis was not done due to of the small number of patients.

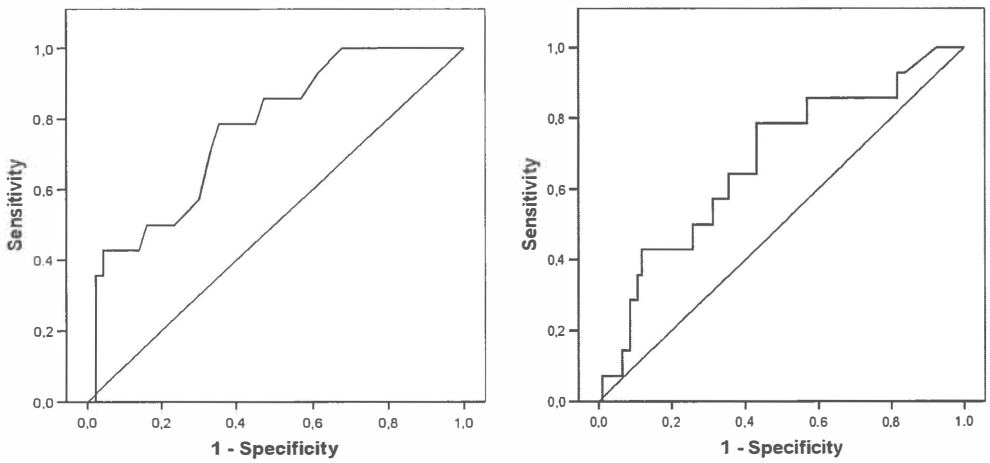

Figure 1: ROC curves of mortality predictor scores.

A. ROC curve of Pediatric Risk of Mortality-score predicting death in oncology patients admitted to the pediatric intensive care unit. AUC: 0.77, p= 0.001. CI: 0.644-0.89. Diagonal segments are produced by ties.

B. ROC curve of Pediatric Index of Mortality-score predicting death in oncology patients admitted to the pediatric intensive care unit. AUC: 0.68, p= 0.034. CI: 0.53-0.83. Diagonal segments are produced by ties.

Discussion

In this study, oncology patients (non-bone marrow recipients) admitted to the PICU for life-threatening events had an overall PICU survival rate of 87%. This result is in keeping with the trend of increasing survival of pediatric oncology patients on the PICU over the last decades (figure 2). The predicted PRISM mortality was 12.3% and the predicted PIM mortality was 6.2% corresponding to SMRs of 1.06 and 2.09, respectively. High scores obtained using the mortality predictors PRISM and PIM were associated with high mortality rates. The association of the PRISM score with mortality appeared to be stronger than the association of the PIM score with mortality (table 3, figure 1A and figure 1B). Variables associated with mortality were the use of inotropic support, a high number of blood product transfusions per day and mechanical ventilation.

Mortality

Even children admitted with of a sepsis that required respiratory and/or circulatory support had a survival rate of 78%. Comparison of our mortality rate with other published series must be guarded. Our series did not include post-operative elective patients and focused on acute life-threatening admissions to the PICU. Including elective patients to our series would result in a much lower mortality whereas clinicians are mostly interested in the specific outcome of severely ill pediatric oncology patients. Our mortality rate,

concerning patients in the time frame 2001-2005, is lower than previously reported rates and supports earlier observations that the outcome of pediatric oncology patients (excluding bone marrow recipients) admitted to the PICU has been improving over the last decades (figure 2).[2-4,7-10]

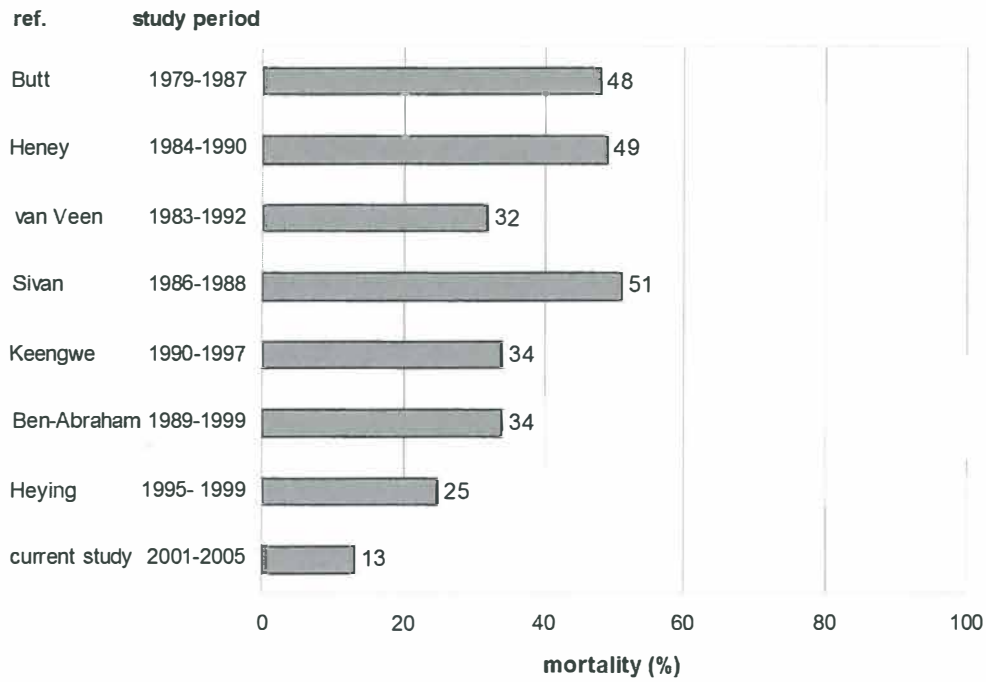

Figure 2: Historical overview of mortality of pediatric oncology patients admitted to the pediatric intensive care unit. Mortality of pediatric oncology patients (age < 21 yrs) admitted to the pediatric intensive care unit (non-bone marrow recipients, non-electively admitted) [2-4,7-10]. ref.; reference.

Variables associated with mortality & multivariate analysis

In our study, independent variables associated with mortality in pediatric oncology patients admitted to the PICU were the use of inotropic support, a high number of blood product transfusions per day and mechanical ventilation. One should realize that these variables are aspects of treatment and as such are not *predictors* of mortality. In the literature several variables are related to outcome in pediatric oncology patients admitted to the intensive care. In many studies, mechanical ventilation, inotropic support, and operative status have been used as measures of severity of illness, especially as relevant to outcome.[1,4,5] In our study, the need for mechanical ventilation was significantly higher in non-survivors compared to survivors. Fiser et al. have, retrospectively, identified bone marrow transplantation, fungal sepsis, use of multiple inotropes and PRISM-III as independent variables for mortality in septic oncology patients using both mechanical ventilation and inotropic support.[6] In our institution, no allogenic bone marrow

transplantations are performed, but we confirmed the association of both inotropic support and PRISM with mortality. Possibly due to the small number of episodes, fungal sepsis was not independently associated with mortality. However, particularly patients with gram-negative and fungal sepsis had high mortality rates.

The association between a higher number of blood product transfusions and an increased mortality has not been reported previously in pediatric oncology patients, possibly because it is not included in most of the analyses.[1,6,7] The high requirements of transfusions may be related to the severity of bone marrow suppression due to either the underlying disease, or the effect of therapy for the disease, or to "cutoff" levels for transfusion commonly used in severely ill children with cancer. The benefits of transfusion in severely ill children are still matter of debate. One multi-center study in pediatric patients showed that a restrictive transfusion strategy was as safe as a liberal transfusion strategy and decreased the rate of exposure to red cells.[17] We did not study the level of hemoglobin at which transfusions were given in our patients. To our knowledge no data exists on the influence of blood product transfusions on mortality in pediatric oncology patients admitted to the PICU.

Predicted and observed mortality

In cohorts of critically ill children, the severity of illness and the risk of mortality at PICU admission can be accurately assessed by easily acquired scores, such as PRISM and PIM.[12,13] These scores should, however, not be used to make decisions about individual patients. This implies that PRISM and PIM scores can be used to compare performance between PICUs or to look at trends within a PICU over time. In this way they are useful as quality instruments, provided that the admission criteria and the patient cohorts are comparable. Our study shows that PRISM and PIM, when applied in regular PICU patient care over several years, helps to predict mortality rates in cohorts of pediatric oncology patients.

In our study, PRISM was a better mortality predictor than PIM; PIM performed poorly in both the SMR and the AUC (ROC analysis). In studies performed by Heying et al and Ben Abraham et al PRISM scores were significantly higher in the patients who did not survive PICU discharge.[7,9] Obviously PRISM scores are significantly higher in non-survivors than in survivors. However, they should not be used for mortality prediction on an individual basis, as low scores appear in the non-survivor group and high scores in the survivor group. Compared to the overall performance of PRISM and PIM during the study period, the pediatric oncology patients' SMRs were higher by a factor 1.3 to 1.8 respectively. In centers with a high volume of pediatric oncology patients the overall PRISM and PIM may therefore underestimate overall mortality. In addition, one must consider that PRISM was originally developed in an acute setting. In patients with ongoing oncology treatment, some of the score variables, such as serum electrolytes, may be significantly affected by

the pre-PICU treatment. This may result in lower PRISM scores in pretreated compared to untreated patients.[7]

The lack of disease specific variables in the mortality prediction scores may also influence the performance of the PIM and PRISM. Because of their underlying condition, previous therapy, and existing organ dysfunction, accurate assessment of the overall condition and prognosis in pediatric oncology patients admitted to the PICU is difficult. However, the risk of mortality in pediatric oncology patients is influenced by these variables which are usually not included in Pediatric Mortality Risk scores. In pediatric oncology patients certain variables are most probably associated with decreased (PICU-) survival such as bone marrow involvement, treatment schedule, and treatment induced neutropenia and thrombocytopenia. An ideal scoring system would incorporate all pertinent clinical data, reflect the nuances of children with cancer, and be simple to use. Not incorporating these data in Pediatric Mortality Risk scores may either over- or underestimate mortality in pediatric oncology patients.

These considerations have led to the introduction of the oncologic PRISM (O-PRISM) score, which has been designed specifically for the post-bone marrow transplant patient.[18] Addition of variables such as macroscopic bleeding, graft-versus-host disease and an increased C-reactive protein to the PRISM score may improve its performance in pediatric bone marrow recipients admitted to the PICU.[19] However, this score has not been validated in pediatric oncology patients outside the bone marrow transplantation scenario. Then again, this would be of questionable value as graft-versus-host disease is not observed outside in this population.

This study has some limitations. The retrospective nature of the study renders it susceptible to potential bias, however, PIM and PRISM scores were recorded prospectively. We did not use the more recent PIM 2[20] and PRISM III[21] since performance of these scores has not been extensively tested in the Dutch population. Moreover, this study reflects the experience of a single center without a bone marrow transplantation service. Additionally, we might have a low threshold for PICU admission of oncology patients when compared to other hospitals. However, more than half of the patients required mechanical ventilation. Moreover, our study population reflects the clinical practice of our institution and indeed of many other institutions.

Conclusions

In the past, support of a septic oncology patient requiring ventilation and inotropic support has been argued to be a futile use of costly and limited PICU resources.[11] The survival rate of 87% found in our study further supports the current literature that reveals an increasing PICU-survival rate in pediatric oncology patients (non-bone marrow recipients). Even the sickest patients have encouraging survival rates. Thus, admission to

the PICU for a child with cancer should not be denied based solely on the diagnosis or severity of illness, as most children are now expected to survive until PICU discharge. Utilization of PICU resources should be addressed on a case-by-case basis to match the objectives of therapy. PRISM and PIM are useful mortality predictors in cohorts of pediatric oncology patients but should not be used for individual patients.

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Chapter 6

Pegfilgrastim in pediatric cancer patients

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Abstract

Chemotherapy-induced neutropenia is a major dose-limiting side effect of intensive chemotherapy in cancer patients. Recently, pegfilgrastim (a product with a long half-life, resulting in once-per-cycle dosage) was introduced to prevent neutropenia in adults. The authors report 32 episodes of pegfilgrastim use in seven pediatric cancer patients to diminish chemotherapy-induced neutropenia. Feasibility was assessed by adherence to treatment protocol and safety was assessed by adverse effects. There were only two treatment delays (6%) due to neutropenia. No short-term adverse effects were recorded. The use of pegfilgrastim is feasible in pediatric cancer patients, without short-term adverse effects or major treatment delay due to neutropenia.

Key words: Chemotherapy-induced neutropenia, Neutropenia, Pegfilgrastim, Children, Cancer

Introduction

Chemotherapy-induced neutropenia is a major dose-limiting side effect of intensive chemotherapy in cancer patients. Prolonged neutropenia results in delay of chemotherapy and an increased risk of infections. Granulocyte colony-stimulating factors (filgrastim and lenograstim) stimulate the proliferation and maturation of neutrophil precursors in the bone marrow and activate neutrophils. After administration of granulocyte colony-stimulating factor, the absolute neutrophil count (ANC) increases within 24 hours; this rise indicates an early release of stored, mature granulocytes and accelerated neutrophil maturation.[1] In cancer patients filgrastim treatment after chemotherapy produced a reduced depth of ANC nadirs and a reduced duration and incidence of chemotherapy-induced neutropenia, resulting in tighter adherence to treatment schedules.[2-7] However, whether the incidence of febrile (neutropenic) episodes decreases with filgrastim remains controversial.[3-6,8] Filgrastim has a half-life of 3.5 hours, which is mainly due to renal excretion; therefore, doses of subcutaneous filgrastim are given daily until the ANC has recovered. Mild to moderate musculoskeletal pain, the most common short-term adverse effect, can be treated with simple analgesics.[2-4,7]

Recently, pegfilgrastim was introduced on the market for adults. Due to the addition of a polyethylene glycol (PEG) molecule to filgrastim, the molecular size has increased. Instead of renal excretion, clearance through binding to neutrophils becomes the primary elimination mechanism. This saturable neutrophil-mediated clearance results in a "self-regulating" elimination mechanism where the serum concentration pegfilgrastim remains elevated until the ANC rises.[9,10]

Due to the different route of clearance, the half-life of pegfilgrastim is extended to about 42 hours, which makes once-per-cycle administration sufficient.[10] In adults, a single dose of 100 μg/kg as well as a fixed dose of 6 mg pegfilgrastim per chemotherapy cycle is at least as effective as one dose of filgrastim 5 μg/kg per day during an average of 11 days. There was no significant difference in terms of reducing the duration and incidence of neutropenia, febrile neutropenia, and chemotherapy dose intensity when pegfilgrastim and filgrastim were compared.[9,11,12] Pegfilgrastim appears as safe as filgrastim, with musculoskeletal pain as the most common adverse event.[9-12]

Recently the first results of pegfilgrastim in pediatric cancer patients suggested that pegfilgrastim is as effective and safe as filgrastim in pediatric sarcoma patients.[13] The purpose of this study was to evaluate whether a single subcutaneous injection of pegfilgrastim 100 μg/kg per chemotherapy cycle was feasible and safe in children with various malignancies.

Methods

The opportunity to receive pegfilgrastim instead of filgrastim was offered to consecutive patients of the Department of Pediatric Oncology of the University Medical Center Groningen, when patients treated for solid tumors were expected to have prolonged neutropenia (expected duration >7 days) due to chemotherapy schedules. Informed consent was obtained from parents and children when appropriate, following institutional medical ethical board procedures. The inclusion criterion for this study was use of pegfilgrastim to diminish chemotherapy-induced neutropenia. Pegfilgrastim was administered in a dose of 6 mg in patients over 45 kg and 100 μg/kg in children less than 45 kg, 24 hours after the last chemotherapy was given. When pegfilgrastim administration was not followed by chemotherapy, these episodes were excluded from the study. Feasibility was assessed by adherence to the treatment protocol. Treatment delay was defined as a delay of 7 days or more after the episode of pegfilgrastim use. WHO criteria for thrombocytopenia (grade 1, 75-150 x 10^9/L; grade 2, 50-75 x 10^9/L; grade 3, 10-50 x 10^9/L; grade 4, <10 x 10^9/L) and neutropenia grade 4 (<0.5 x 10^9/L) were used. Safety was assessed by recording adverse events such as musculoskeletal pain (WHO criteria).

Results

Thirty-two episodes in seven patients (four boys, three girls; median age 9 years [range 4-16 years]) were evaluated. The group was severely pretreated; four of the seven children were on relapse therapy (Table 1). Feasibility was accessed by protocol adherence and evaluated in the 32 episodes.

Adherence to the treatment protocol was possible in 21 of the 32 episodes. In the 11 episodes of treatment delay, the mean delay was 1.5 weeks (range 1-3 weeks). Four of the five chemotherapy dose reductions followed a treatment delay (according to protocol). Interestingly, only two treatment delays (6%) were due to neutropenia (one combined with thrombocytopenia and fever); the remaining nine treatment delays were caused by holiday (n = 1), thrombocytopenia (n = 7), and thrombocytopenia with fever (n = 1). Eight of the 11 treatment delays appeared in two patients; 5 of these 8 delays followed administration of a chemotherapy course containing carboplatin and etoposide. Febrile neutropenia occurred in 7 of the 32 episodes (22%); all patients fully recovered.

No short-term adverse effects, such as musculoskeletal pain, were found.

Table 1. Patient and episode characteristics

Patient	Diagnosis	Weight (kg)	Episode	Neutropenic Chemotherapeutics (total dosage)	Count Recovery Criteria	Delay (weeks)	Cause of Delay
1	Medulloblastoma	16	1	Cyclo (3 g/m2)	A	0	
			2	Cyclo (3 g/m2)	A	1	N
2	Wilms tumor; relapse	25	3	Cyclo (73,5 mg/kg)/VP-16 (16,5 mg/kg)	A	0	
			4	Carbo (83,5 mg/kg)/VP-16 (16,5 mg/kg)	B	0	
			5	Cyclo (73,5 mg/kg)/VP-16 (16,5 mg/kg)	A	2	F & T3
			6	Carbo (83,5 mg/kg)/VP-16 (16,5 mg/kg)	B	3	FN & T4
			7	Cyclo (37 mg/kg)/VP-16 (8,25 mg/kg)	A	1	H
			8	Cyclo (73,5 mg/kg)/VP-16 (16,5 mg/kg)	B	2	T3
3	Neuroblastoma	20	9	Cyclo (4,2 g/m2)/Adria (75 mg/m2)	B	0	
			10	CDDP (200 mg/m2)/VP-16 (600 mg/m2)	B	0	
			11	Cyclo (4,2 g/m2)/Adria (75 mg/m2)	B	0	
4	Embryonal rhabdomyo-sarcoma	36	12	ACT-D (0,045 mg/kg)/Cyclo (2,2 mg/m2)	A	0	
			13	ACT-D(0,045 mg/kg)/Cyclo (2,2 mg/m2)	A	0	
			14	ACT-D (0,045 mg/kg)/Cyclo (2,2 mg/m2)	A	0	
			15	ACT-D (0,045 mg/kg)/Cyclo (2,2 mg/m2)	A	2	T3
			16	ACT-D (0,045 mg/kg)/Cyclo (1,9 mg/m2)	A	1	T3
5	M. Hodgkin; relapse	32	17	CDDP (100 mg/m2)/Ara-C (4 g/m2)	B	0	
			18	VP-16 (270 mg/m2)/MTX (60 mg/m2)/IFO (6 g/m2)	B	0	
			19	VP-16 (270 mg/m2)/MTX (60 mg/m2)/IFO (6 g/m2)	B	0	
6	M. Hodgkin; relapse	54	20	CDDP (100 mg/m2)/Ara-C (4 g/m2)	A	0	
			21	MX (12 mg/m2)/PCB (1500 mg/m2)	A	0	
			22	MX (12 mg/m2)/PCB (1500 mg/m2)	A	0	
			23	Adria (50 mg/m2)/BLM (20 mg/m2)/VDS (12 mg/m2)/DTIC (750 mg/m2)	A	0	
			24	MX (12 mg/m2)/PCB (1500 mg/m2	A	0	
			25	Adria (50 mg/m2)/BLM (20 mg/m2)/VDS (12 mg/m2)/DTIC (750 mg/m^2)	A	0	
			26	MX (12 mg/ m^2)/PCB (1500 mg/ m^2)	A	0	
			27	Adria (50 mg/ m^2)/BLM (20 mg/ m^2)/VDS (12 mg/ m^2)/DTIC (750 mg/ m^2)	A	0	
7	Osteosarcoma; relapse	56	28	Carbo (600 mg/ m^2)/VP-16 (600 mg/ m^2)	C	0	
			29	Carbo (600 mg/ m^2)/VP-16 (600 mg/ m^2)	C	1	T3
			30	Carbo (600 mg/ m^2)/VP-16 (600 mg/ m^2)	C	1	T1
			31	Carbo (600 mg/ m^2)/VP-16 (600 mg/ m^2)	C	1	T3
			32	Carbo (600 mg/ m^2)/VP-16 (600 mg/ m^2)	C	1	T3

Count recovery criteria, A, neutrophilic granulocyte count >1.0 x 10^9/L and thrombocyte count >100 x 10^9/L; B, neutrophilic granulocyte count >0.75 x 10^9/L and thrombocyte count >75 x 10^9/L; C, neutrophilic granulocyte count >0.75 x 10^9/L and thrombocyte count >100 x 10^9/L; Delay, delay of next chemotherapy cycle; Cyclo, cyclophosphamide; N, neutropenia; NA, not applicable; VP-16, etoposide; Carbo, carboplatin; F, fever; T, thromocytopenia; numbers, grade of thrombocytopenia according to WHO criteria (T1, grade 1: 75-150 x 10^9/L; T2, grade 2: 50-75 x 10^9/L; T3, grade 3: 10-50 x 10^9/L; T4, grade 4: <10 x 10^9/L); H, holiday; Adria, adriamycin; CDDP, cisplatin; ACT-D, actinomycin-D; Ara-C, cytosine arabinoside; MTX, methotrexate; IFO, ifosfamide; MX, mitoxantrone; PCB, procarbazine; BLM, bleomycin; VDS, vindesine; DTIC, dacarbazine.

Discussion

In this patient group, pegfilgrastim administration appeared to be feasible and safe. Adherence to the treatment protocol was possible in 21 of the 32 episodes, with neutropenia causing a treatment delay in only 6% of the episodes. Thrombocytopenia, but not neutropenia, appeared to be the major limiting factor in adherence to the treatment schedule in 25% of the episodes. Thrombocytopenia up to grade 4 (WHO criteria) has been described in less than 5% of the treatment cycles where pegfilgrastim or filgrastim is given.[10,12] No association between pegfilgrastim and thrombocytopenia was found in the literature. However, thrombocytopenia might be an unwanted side effect of pegfilgrastim in patients with an already compromised hematopoiesis. Nonetheless, it remains likely that the patients in our study had prolonged thrombocytopenias as a result of bone marrow exhaustion, due to heavy pretreatment with myelosuppressive agents. Five of the 11 treatment delays occurred in two relapse patients after receiving carboplatin and etoposide combination chemotherapy. However, due to the small sample size, more conclusive remarks cannot be made.

Febrile neutropenia occurred in 22% of the episodes, with full recovery of all patients. A general statement about the frequency of febrile neutropenia in this population would be improper, as the patient group was small and the patients were selected to receive pegfilgrastim on the basis of expected prolonged neutropenia due to their heavy and long-lasting chemotherapy schedules.

There is some pediatric experience with pegfilgrastim, and recently the results of a study by Wendelin *et al* suggested that pegfilgrastim is effective and safe in pediatric sarcoma patients.[13] A large clinical trial to determine the efficacy of pegfilgrastim in pediatric sarcoma patients started in May 2003.[14] In this study we showed its feasibility in patients with various kinds of cancer.

In conclusion, in this small number of patients with various kinds of cancer, we found that with the use of pegfilgrastim, prolonged neutropenia caused treatment delay in only 6% of the treatment courses, and no adverse events were recorded. However, in terms of general implementation, the above-mentioned trial will give more accurate insights as to its feasibility and safety in children.[14]

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Part 2

Genetic aspects

Chapter 7

Association of polymorphisms in the *TLR4* gene with the risk of developing neutropenia in children with leukemia

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Abstract

Infections are a major cause of morbidity and mortality in children with acute lymphoblastic leukemia (ALL). Susceptibility to infections increases as the neutrophil count decreases. Despite identical treatment patients vary considerably in the number of neutropenic episodes. Toll-like receptor 4 (TLR4) has been shown to have a role in inhibiting apoptosis of neutrophils. Therefore, we hypothesized that polymorphisms in the *TLR4* gene may influence the number of chemotherapy-induced neutropenic episodes. Eight single-nucleotide polymorphisms (SNPs) of the *TLR4* gene were determined in 194 children aged 0-17 years, who were diagnosed with ALL. We compared the genotype distributions of the SNPs with the frequency of neutropenic episodes during treatment with chemotherapeutic regimens. The number of neutropenic episodes varied from 0 to 17, with a median of four neutropenic episodes. Four SNPs in the *TLR4* gene (rs10759931, rs11536889, rs1927911 and rs6478317) were associated with an increased risk of developing chemotherapy-induced neutropenia, each sustaining correction for multiple testing. Further studies are required to elucidate whether pediatric patients with ALL with the particular SNPs in the *TLR4* gene also experience more infections and would benefit from prophylactic antibiotic treatment, by a reduction of morbidity and mortality due to infections.

Keywords: Neutropenia, TLR4, Polymorphisms, Children

Introduction

Acute lymphoblastic leukemia (ALL) is the most common form of cancer in children. The long-term, event-free survival of children with ALL has increased considerably during the past decades, due largely to more aggressive chemotherapeutic regimens. Unfortunately, this type of chemotherapy has secondary effects detrimental to a variety of normal, rapidly growing cells causing side effects like neutropenia, which is defined as an absolute neutrophil count (ANC) of $<0.5 \times 10^9$/l.[1] Neutropenia increases the risk of severe bacterial infections, still a major cause of morbidity and mortality in pediatric patients with ALL.[2] Moreover, neutropenia calls for chemotherapeutic treatment delay and/or dose reduction until the ANC has recovered, which may compromise overall survival in cancer patients.[3]

Despite identical chemotherapeutic treatment patients vary considerably in the number, depth and duration of neutropenic episodes.[4–7] This variance is only partially explained by patient factors including comorbidity, age and individual chemotherapeutical pharmacokinetics and pharmacodynamics.[8] Genetic variance may also contribute to the risk of developing neutropenia during chemotherapy, but to date this possibility has not been investigated. A candidate gene that might be associated with the risk of developing chemotherapy-induced neutropenia is *Toll-like receptor 4* (*TLR4*), as TLR4 has been shown to have a role in inhibition of neutrophil apoptosis, and extension of the functional lifespan of neutrophils during stimulation with lipopolysaccharide.[9–13]

TLR4 is 1 of 11 known mammalian Toll-like membrane receptors that have a key role in innate immunity by detecting and eliminating invading pathogens. Expression of TLR4 has been detected on neutrophils.[12,14,15] Stimulation of TLR4 by ligands such as lipopolysaccharide,[16,17] heat shock proteins 60,[18] high mobility group box-1 protein, fibrinogen peptides[19] or other endogenous ligands leads to activation of the nuclear factor κB signaling pathway. Nuclear factor κB is an essential transcription factor for proinflammatory signaling, which regulates the production of various cytokines, adhesion molecules and anti-apoptotic factors.[20–22] Nuclear factor κB is suggested to have a major role in TLR4-mediated neutrophil survival.[13,23,24] Other TLRs were shown to have no or hardly any effect on neutrophil apoptosis, for example, TLR2.[12]

Certain polymorphisms of *TLR4* are associated with hyporesponsiveness to TLR4 ligands *in vivo* and *in vitro*, and have been associated with the pathogenesis of multiple diseases.[25–28] Therefore, we hypothesized that polymorphisms located in the *TLR4* gene may influence apoptosis and the lifespan of neutrophils during chemotherapeutic treatment, and in this way influence the occurrence of chemotherapy-induced neutropenia.

Materials and methods

Study population

The study population consisted of children who were diagnosed with ALL between 1984 and 2001 in the Beatrix Children's Hospital of University Medical Center of Groningen or in the Wilhelmina Children's Hospital of University Medical Center Utrecht, The Netherlands. The patients were treated according to one of the consecutive protocols of the Dutch childhood oncology group (DCOG) ALL6, DCOG-ALL7, DCOG-ALL8 or DCOG-ALL9.[29-32] We obtained patient data from the hospital records on sex, age, ALL subtype (T-ALL or precursor B-ALL) and ALL risk group, ANC and the number of neutropenic episodes during the maintenance phase of the chemotherapeutic treatment regimen. The maintenance treatment of these protocols is identical, except for the use of daily oral Dexamethasone and weekly intravenous Vincristin during 2 weeks every 7 weeks in DCOG-ALL6 and DCOG-ALL9. During this phase of the protocol, the blood cell count is measured at least once a week. We defined neutropenia as an ANC of $<0.5 \times 10^9$/l or a leukocyte count of $<1.0 \times 10^9$/l if ANC was not available. DNA was successfully obtained from 221 patients. Eighteen patients of non-Caucasian origin were excluded from analyses. Nine children were lost to follow up or had died before complete remission. The local medical ethics committee approved the study. All parents, as well as from the children aged 12 years and older, gave their written informed consent.

Selecting SNP and genotyping

We extracted genomic DNA from buccal swabs, blood or bone marrow samples. For extracting DNA from blood or bone marrow samples we used the Nucleospin Blood L kit (Macherey-Nagel, Düren, Germany) and for frozen samples we used the NucleoSpin Tissue kit (Macherey-Nagel); both according to manufacturer protocols. For isolating DNA from buccal swabs we used the protocol described by Meulenbelt *et al.*[33] We amplified DNA by using REPLI-g UltraFast technology (Qiagen, Venlo, The Netherlands). We selected seven haplotype-tagging single-nucleotide polymorphisms (SNPs) and one functional SNP (Asp299Gly) of the *TLR4* gene from the HapMap database (Table 1).

Genotyping was performed by competitive allele-specific PCR using KASPar (Hoddesdon, UK) genotyping chemistry, performed under contract by K-Biosciences (Herts, UK), with extensive quality control measures.

Statistical methods

We tested the genotype and allele frequencies for deviations from the Hardy-Weinberg equilibrium with 1df χ^2 tests at a $P>0.05$. Pair wise linkage disequilibrium (LD) was assessed between single SNPs by using the r^2 statistic as implemented in Haploview version 4.1 (Broad Institute, Cambridge, UK). We tested the number of neutropenic

episodes as a continuous variable by SNP genotypes using the Kruskall-Wallis test. Multivariate linear regression analysis was performed to estimate the explained proportion of the variation of neutropenic episodes. Subsequently, we divided the cohort into two groups based on the median value of neutropenic episodes, whereby 0-4 was considered a 'low number of neutropenic episodes' and ≥5 a 'high number of neutropenic episodes'. We used a 2df χ^2-test to compare genotype frequencies of the eight SNPs between the two groups. To account for multiple testing, we calculated the false discovery rate as described by Benjamini and Hochberg using a *P*-value of 0.05 as a cutoff value.[34] For SNPs showing a significant association with neutropenia, we used logistic regression analyses to estimate odds ratios (ORs) with 95% confidence intervals (CIs) for the best-fitting model, that is, additive, dominant or recessive. To analyze the effect of the combination of SNPs, we performed logistic regression analysis using the backward selection model. The two SNPs that remained in the final model at a *P*-value of <0.1 were included in logistic regression analysis to estimate OR and 95% CI for either one or combined two risk genotypes. In this model, subjects with low-risk genotype in both SNPs were coded as 0; subjects with one risk genotype in either of the two SNPs as 1; and carriers of risk genotypes in both of the SNPs as 2, that is, the highest TLR4-associated risk group. Analyses were performed using the statistical software package SPSS version 16.0 (SPSS, Chicago, IL, USA).

Results

Study population

The study population consisted of 194 children, 117 males (60%) and 77 females (40%), with ALL. Their ages ranged from 0 to 17 years (median 4 years). The subtypes of ALL were T-ALL (*n*=23; 12%) and precursor B-ALL (*n*=142; 73%), the subtype being unknown in 29 patients (15%). Patients were treated according to DCOG-ALL6 (*n*=9; 5%), DCOG-ALL7 (*n*=26; 13%), DCOG-ALL8 (*n*=55; 28%) or DCOG-ALL9 (*n*= 104; 54%). During the study period, we found a total of 877 neutropenic episodes. The number of neutropenic episodes per patient varied from 0 to 17, with a median of four neutropenic episodes (Figure 1). One hundred six patients (54.6%) had 0-4 neutropenic episodes, that is, a low number of neutropenic episodes; and 88 patients (45.4%) had 5-17 neutropenic episodes, that is, a high number of neutropenic episodes. The characteristics of these two groups of patients are compared in Table 2. Age, type of ALL and risk group were significantly different among the groups. The T-ALL and high-risk groups were over-represented among patients with a high number of neutropenic episodes as a result of receiving more aggressive treatment. The frequencies of SNPs genotypes, however, were evenly distributed among the two ALL subtypes and the risk group (data not shown).

Table 1: SNPs in the *TLR4* gene

SNP	SNP type	Location	MA	MAF	HWE *P*-value	Call rate (%)
rs2770150	Promoter	-3612	C	0.27	0.53	95
rs10759931	Promoter	-2604	A	0.39	0.94	95
rs6478317	Promoter	-2570	G	0.34	0.38	97
rs10759932	Promoter	-1607	C	0.12	0.24	97
rs1927911	Intron	3304	T	0.24	0.08	97
rs11536878	Intron	4803	A	0.42	0.12	99
rs4986790	Exon, Asp299Gly, non-synonymous	8552	G	0.06	0.56	98
rs11536889	3' UTR	11381	C	0.40	0.23	99

Abbreviations: HWE, Hardy-Weinberg equilibrium; MA, minor allele; MAF, minor allele frequency; SNP, single-nucleotide polymorphism; UTR, untranslated region.

Genotyping and LD analysis

Table 1 shows the function and location of the eight SNPs of the *TLR4* gene. None of the SNPs deviated from Hardy-Weinberg equilibrium ($P<0.05$). On average the call rate was 97%. We constructed an LD plot based on our own data (Figure 2). The associated SNPs did not show LD with $r^2>0.75$ with other SNPs. The strongest linkage was found between rs6478317 and rs1927911 ($r^2=0.67$). Moderate linkage was found between rs10759932 and rs1927911 ($r^2=0.40$) and between rs1927911 and rs11536878 ($r^2=0.42$).

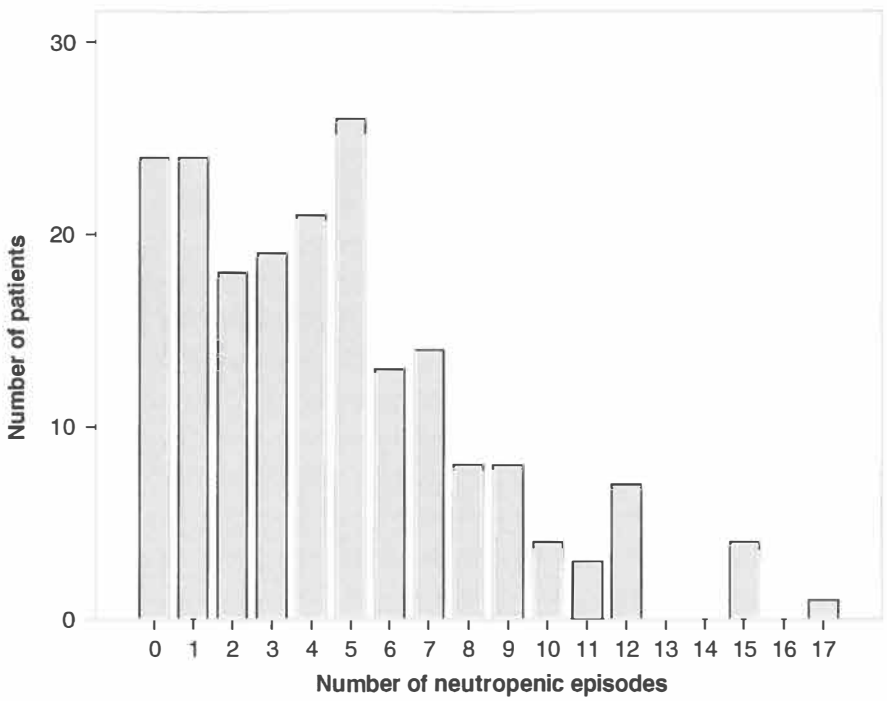

Figure 1: Total number of neutropenic episodes. The vertical black line represents the median.

Table 2: Patient characteristics of the group with a low number of neutropenic episodes and the group with a high number of neutropenic episodes

	0–4 Neutropenic episodes	5–17 Neutropenic episodes	*P*-value
Number of patients (%)	106 (54.6%)	88 (45.4%)	
Age-median years (range)	5.2 (0–17)	3.7 (0–17)	**<0.002**
Sex			0.54
Male (%)	66 (62%)	51 (60%)	
Female (%)	40 (38%)	37 (42%)	
Type of ALL			**0.01**
T-ALL	5	18	
Precursor B-ALL	81	61	
Unknown	20	9	
Protocol			0.78
DCOG-ALL6	4	5	
DCOG-ALL7	13	14	
DCOG-ALL8	29	26	
DCOG-ALL9	60	44	
Risk group			**0.0005**
ALL6 NHR	4	5	
ALL7 SRG	8	3	
ALL7 RG	5	9	
ALL7 EG	0	1	
ALL8 SRG	14	6	
ALL8 MRG	10	14	
ALL8 HRG	3	6	
ALL9 NHR	48	17	
ALL9 HR	12	26	

Abbreviations: ALL, acute lymphoblastic leukemia; DCOG, Dutch Child Oncology Group; EG, experimental group; HR, high risk; HRG, high-risk group; MRG, medium risk group; NHR, non-high risk; RG, risk group; SRG, standard risk group. Bold values are statistically significant.

Single SNP association analysis

When analyzing the neutropenic episodes as a continuous variable, four SNPs in the *TLR4* gene, rs10759931 (*P*=0.047), rs11536889 (*P*=0.048), rs1927911 (*P*=0.017) and rs6478317 (*P*=0.007), showed a significant association with the risk of developing neutropenia, see Table 3. Multivariate linear regression analysis to the four associated SNPs showed that 8.1% of the variation in the number of neutropenic episodes was explained by these four SNPs. The four SNPs in combination with age, risk group and type of ALL explained 27.2% of the variation in the number of neutropenic episodes.

Comparing patients with a low number of neutropenic episodes (0-4) with patients with a high number of neutropenic episodes (5-17), the same four SNPs, rs10759931, rs11536889, rs1927911 and rs6478317, showed a significant difference in genotype frequencies between patients with low and high number of neutropenic episodes while adjusting for age. All

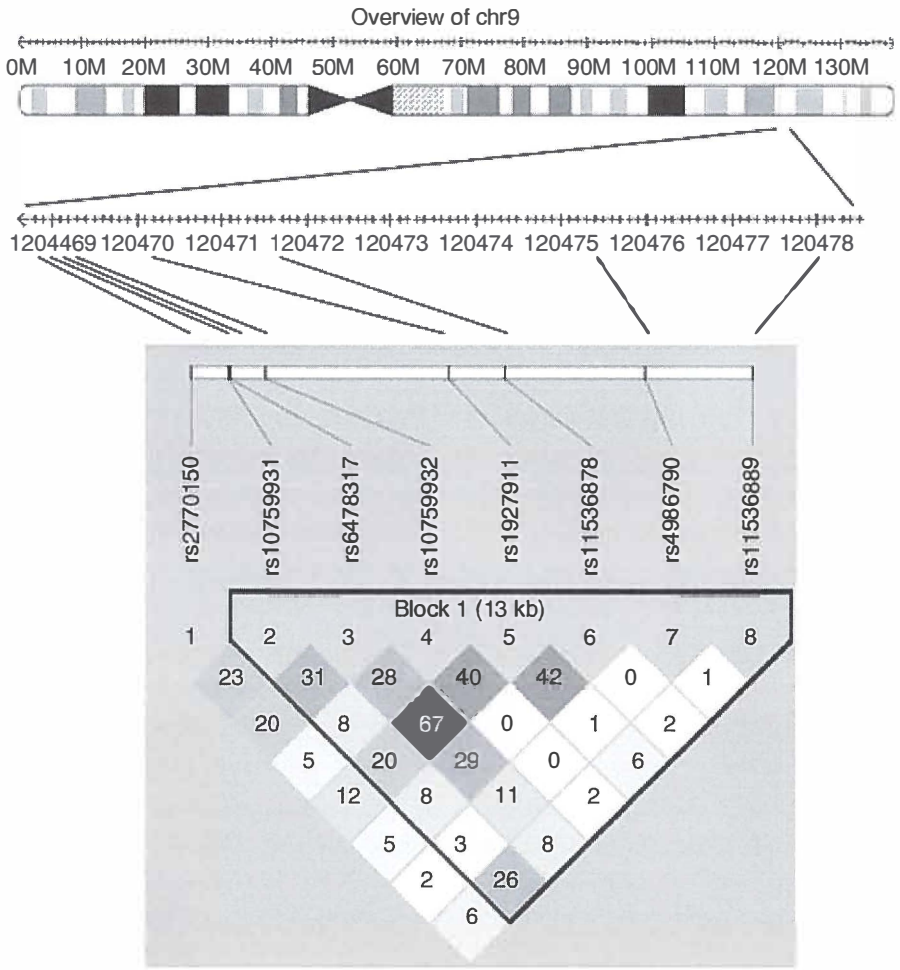

Figure 2 Genetic layout of inheritance of the eight SNPs of the *TLR4* gene with LD plot based on studied SNPs.

four significant SNPs sustained correction for multiple testing (Table 3). The SNPs rs10759931 and rs11536889 fitted best in a dominant model. The presence of one or two minor alleles in both SNPs resulted in an increased risk of a high number of neutropenic episodes (OR 2.23, 95% CI 1.20-4.14, *P*=0.011 and OR 2.86, 95% CI 1.48-5.53, *P*=0.002, respectively). The SNPs rs1927911 and rs6478317 fitted best in a recessive model. For these two SNPs, the presence of two minor alleles resulted in a decreased risk of a high number of neutropenic episodes (OR 0.25, 95% CI 0.07-0.91, *P*=0.035 and OR 0.27, 95% CI 0.10-0.77, *P*=0.014, respectively) (Table 4). Figure 3 shows the genotype frequencies of the four significant SNPs in their best-fitting model.

The backward selection approach showed that two of the eight SNPs remained in the final model, namely rs6478317 (*P*=0.08) and rs11536889 (*P*=0.005), while adjusting for age.

Despite low power, combination analysis showed that carriers of one TLRs-associated risk genotype had an increased risk of neutropenia, compared with carriers of no combined TLRs-associated risk genotype group (OR 2.78, 95% CI 0.97-7.98, *P*=0.06); this risk was significantly increased to more than double for carriers of two risk genotypes, that is, with the highest TLR4-associated risk (OR 7.38, 95% CI 2.35–23.20, *P*=0.001).

Table 3: Genotype frequencies of SNPs in the *TLR4* gene associated with frequency of neutropenic episodes

SNP	Genotype	Median (range)	*P*-value	0–4 Neutropenic episode (n=106) N (%)	≥5 Neutropenic episode (n=88) N (%)	*P*-value[a]	Correction for multiple testing
rs2770150	TT	4 (0–17)	0.253	51 (49)	49 (61)	0.030	NS
	TC	4 (0–15)		42 (41)	27 (33)		
	CC	2.5 (1–8)		10 (10)	5 (6)		
rs10759931	GG	3 (0–15)	**0.047**	47 (46)	23 (28)	**0.019**	**Significant**
	GA	5 (0–17)		40 (39)	48 (58)		
	AA	4 (0–15)		15 (15)	12 (14)		
rs6478317	AA	4 (0–15)	**0.007**	50 (49)	36 (42)	**0.005**	**Significant**
	AG	5 (0–17)		34 (33)	45 (52)		
	GG	2 (0–12)		19 (18)	5 (6)		
rs10759932	TT	4 (0–15)	0.708	80 (78)	64 (74)	0.899	NS
	TC	4.5 (0–17)		21 (21)	22 (26)		
	CC	4 (0–4)		1 (1)	0 (0)		
rs1927911	CC	4 (0–15)	**0.017**	67 (64)	48 (55)	**0.011**	**Significant**
	CT	5 (0–17)		25 (24)	37 (42)		
	TT	2 (0–8)		13 (12)	3 (3)		
rs11536878	CC	4 (0–17)	0.368	81 (78)	70 (80)	0.639	NS
	CA	4 (0–12)		19 (18)	17 (19)		
	AA	1.5 (0–8)		4 (4)	1 (1)		
rs4986790 (Asp299Gly)	AA	4 (0–17)	0.774	91 (89)	79 (90)	0.468	NS
	AG	3.5 (0–15)		10 (10)	9 (10)		
	GG	2 (2–2)		1 (1)	0 (0)		
rs11536889	GG	3.5 (0–15)	**0.048**	83 (81)	52 (60)	**0.006**	**Significant**
	GC	5 (0–17)		17 (17)	29 (34)		
	CC	5 (1–11)		2 (2)	5 (6)		

Abbreviations: NS, not significant; SNP, single-nucleotide polymorphism.
[a] Adjusted for age.

Table 4: Associations between minor alleles of SNPs in the TLR4 gene with neutropenia

SNP	Alleles[a]	Risk of developing neutropenia OR[b] (95% CI)	*P*-value
rs10759931	G/A	2.229 (1.201–4.138) D	0.011
rs6478317	A/G	0.273 (0.097–0.765) R	0.014
rs1927911	C/T	0.250 (0.069–0.907) R	0.035
rs11536889	G/C	2.856 (1.477–5.525) D	0.002

Abbreviations: CI, confidence interval; OR, odds ratio; SNP, single-nucleotide polymorphism.
[a] Wild type is mentioned first; ORs are calculated for minor alleles using wild type as a reference group.
[b] OR is calculated by logistic regression (95% CI) in their best-fitting model (D=minor allele is dominant and R=minor allele is recessive).

Discussion

We showed that four SNPs in the *TLR4* gene (rs6478317, rs1927911, rs10759931 and rs11536889) are significantly associated with the risk of developing chemotherapy-induced neutropenia in children with ALL. To our knowledge, this is the first study to examine the association between genetic polymorphisms and the risk of developing neutropenia during chemotherapeutic treatment.

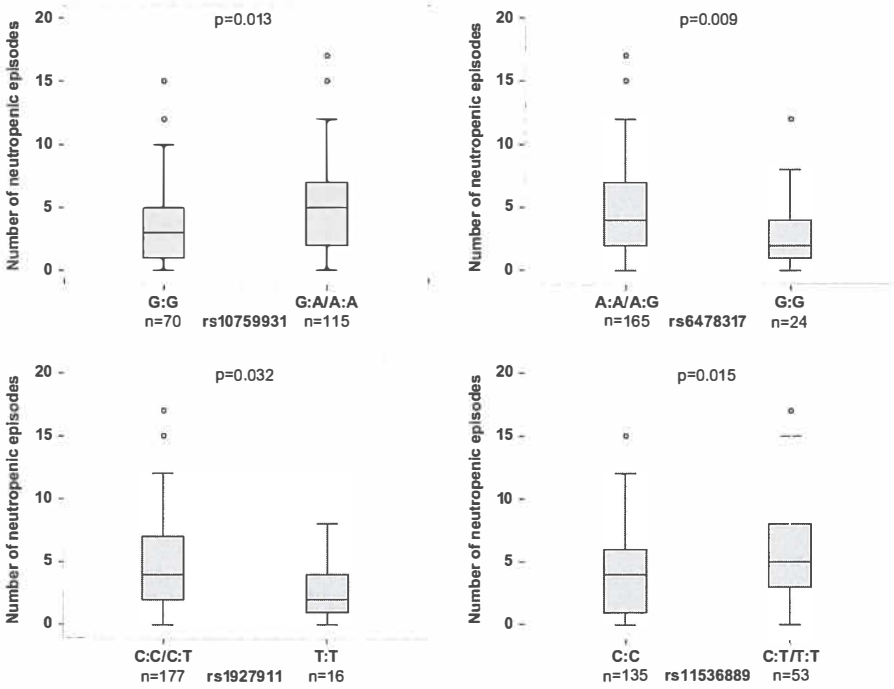

Figure 3: Frequencies of neutropenic episodes per genotype or combination of genotypes (depending on the best-fitting model: dominant or recessive). The median of the number of neutropenic episodes is marked by the horizontal line in the central box. The boxes are limited by the 25th and 75th percentile. The whiskers represent minimum and maximum number of neutropenic episodes. Outliers are depicted separately by white circles.

Neutropenia is a frequent side effect in children receiving chemotherapy and requires serious attention. The lower the blood neutrophil number and the longer patients remain neutropenic, the more patients are susceptible to developing serious bacterial or fungal infections. When the ANC falls below 1.0, 0.5 and 0.1 × 10^9/l, the frequency of life-threatening infections rises steeply from 10 to 18 and 28%, respectively.[1] Moreover, when a patient is neutropenic, the chemotherapeutic treatment course is usually delayed and/or the dose reduced. This may lead to a reduction of the patients' chances of survival.[3]

In this study, we found genetic variation in the *TLR4* gene to be significantly associated with the number of neutropenic episodes in pediatric patients with ALL. Several studies

previously demonstrated an association of the TLR4 gene with enhanced neutrophil survival by inhibiting neutrophil apoptosis.[9,11-13] Dick *et al.*[10] showed that neutrophil survival is inhibited when neutrophils are transduced with a lentivirus encoding a dominant-negative TLR4 protein, and that neutrophils can be genetically manipulated to enhance or inhibit survival.

The question arises whether the SNPs that we found to be associated with neutropenia are relevant with respect to their function. This clearly requires further study. We did study one known functional SNP, rs4986790, with low minor allele frequency, that has previously been shown to influence the responsiveness of TLR4 for its ligands.[25-28] In our study, however, this particular SNP showed no association with the risk of neutropenia. It is not known whether the other SNPs are functional. Nevertheless, the other seven SNPs were haplotype-tagging SNPs, implying that one or more of these SNPs may cover a functional region of the gene.

Our findings demonstrate that only a small proportion, a mere 8%, of the variation in neutropenic episodes is explained by the four SNPs in the *TLR4* gene. Other factors that contribute to the risk of developing chemotherapy-induced neutropenia are patient-specific factors like comorbidity, age, and individual chemotherapeutical pharmacokinetics and pharmacodynamics, probably in combination with other genetic polymorphisms besides the four we found in the TLR4 gene. It would be interesting to know whether the four *TLR4* SNPs are also associated with sepsis or other infectious complications. Given the fact that a lower neutrophil count leads to higher chances of infection,[1] this seems likely. In fact, a study at the intensive care unit showed that variation in the *TLR4* gene was associated with a decreased risk of complicated sepsis as well.[35] Future studies should focus on infectious complications as outcome measure, as severe infections constitute the most important threat during neutropenia.

There are some limitations to this study. Although we showed the influence of SNPs in the *TLR4* gene on the risk of developing neutropenia in a reasonable large, homogeneous group of patients, one might argue that these results were coincidental. The fact that we performed corrections for multiple testing makes this less likely even though this still does not completely rule out the common problem in candidate gene studies of finding false positive results. For this reason our study needs to be replicated in an equally large or, preferably, an even larger group of patients.

The strengths of this study lay in the number and homogeneity of the included patients, the wide variety of number of neutropenic episodes, and the standard procedure we used for all patients to determine neutropenia. Neutropenia was defined by the number of neutropenic episodes during the maintenance phase of the ALL protocol, instead of taking the nadir and/or the duration of the neutropenia. During the maintenance phase of the protocol, blood was drawn from the patients once a week, at least. The neutrophil count during the rest of the week was unknown. Logically, when a patient was shown to

be neutropenic at the time of withdrawal and out of neutropenia at the next time of withdrawal, it was unclear whether the measured neutrophil count was the nadir, and the same accounts for the duration of the neutropenic episode. Therefore, we believe that the number of neutropenic episodes in a certain phase of the protocol is a good tool to define susceptibility to neutropenia.

In conclusion, this study showed for the first time that four SNPs in the *TLR4* gene were associated with an increased risk of developing chemotherapy-induced neutropenia in children with leukemia. Given its clinical implication this is an important finding, as neutropenia is often seen in pediatric oncology and related to an increased risk of developing serious infections. Future studies have to elucidate whether pediatric patients with ALL, who possess the particular SNPs in the *TLR4* gene, also experience more infections and/or sepses and whether they would benefit from prophylactic antibiotic treatment, by a reduction of morbidity and mortality as a result of infections.

Conflict of interest

The authors declare no conflict of interest.

Author contributions

KGEM: designed and performed the research, analyzed and interpreted data, performed statistical analysis and wrote the manuscript; EMP: designed and performed the research and revised the manuscript; WJET: designed research, performed research, analyzed and interpreted data and wrote the manuscript; DSP: designed the research, interpreted data and revised the manuscript; GHK: designed the research, interpreted data and revised the manuscript; AP: performed research and collected data; WAK: interpreted data and revised the manuscript; BZA: analyzed and interpreted data, performed statistical analysis and wrote the manuscript; MHB: designed research, analyzed and interpreted data, performed statistical analysis and revised the manuscript; ESJMB: designed research, analyzed and interpreted data and wrote the manuscript.

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Chapter 8

MBL2 and fever during neutropenia in children with acute lymphoblastic leukaemia

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Keywords: Paediatric cancer, Fever during neutropenia, Mannan-binding lectin, Innate immunity, Acute lymphoblastic leukaemia

Innate immunity is the first line of defence against infections. Mannan-binding lectin (MBL) plays a central role in the innate immune defence and its deficiency predisposes children to infectious illness. Single nucleotide polymorphisms (SNPs) in *MBL2* influence serum MBL levels.[1,2] The various SNPs, genotype combinations and haplotypes are associated with (very) low, intermediate and high MBL levels. Fever during neutropenia (FN) in cancer patients can indicate an emerging bacterial sepsis. Several research groups have investigated and rejected the relationship between FN and *MBL2* in children with cancer.[3-5] However, these negative results could result from a lack of power due to the relatively small sample sizes, a too brief observation period and the heterogeneity of the population with respect to the types of cancer and treatments. Larger groups of subjects with longer observation periods are therefore needed to firmly establish whether *MBL2* SNPs are associated with FN.

Table I: Patient characteristics of children with acute lymphoblastic leukaemia

		Number	Percentage
All patients		203	
Maintenance treatment		194	
Male		117	60
Age		0-17 years	Median 4 years
ALL subtype	T-ALL	23	11.9
	Pre B-ALL	142	73.2
	Unknown	29	14.9
DCOG-ALL	6	9	4.6
	7	26	13.4
	8	55	28.4
	9	104	53.6
N	0 times	26	13.4
	1-17 times	168	86.6
FN	0 times	82	48.8
	1-8 times	86	51.2

ALL, acute lymphoblastic leukaemia; DCOG-ALL, Dutch Childhood Oncology Group ALL protocol; N, neutropenic episode; FN: fever during neutropenia.

We hypothesized that *MBL2* SNPs that contribute to low levels of MBL increase the risk of FN in children with acute lymphoblastic leukaemia (ALL) during maintenance treatment.

The study population consisted of 203 Caucasian children with ALL, diagnosed between 1984 and 2001 in the Beatrix Children's Hospital of the University Medical Center Groningen or the Wilhelmina Children's Hospital of the University Medical Center Utrecht, the Netherlands. The patients were treated according to one of the consecutive Dutch Childhood Oncology Group protocols. The maintenance phase of these protocols is the longest part of the treatment and is relatively similar. During this phase, the blood cell count is measured at least once a week. Patient data were obtained from hospital records

on, e.g. absolute neutrophil count (ANC) (Table I). Neutropenia was defined as an ANC <0.5 x 109/l or a leucocyte count <1.0 x 109/l when ANC was not available. FN was defined as fever (temperature >38.4 °C, or two readings of 38.0-38.4 °C within 6 h) during a neutropenic episode (N). Patients with FN were compared with patients without FN. DNA was successfully obtained from 194 patients entering maintenance treatment. Patients with at least one N were studied for FN. The local medical ethics committee approved the study. Written informed consent was obtained from parents and children aged 12 and older.

Genomic DNA was derived from buccal swabs, blood or bone marrow samples using the NucleoSpin® Blood L, the NucleoSpinTissue kit (http://www.bioke.com MACHEREY-NAGEL, Germany), or the protocol described in Meulenbelt et al.[6] DNA was amplified using the REPLI-g Ultrafast technology (Qiagen, Venlo, The Netherlands). Twelve *MBL2* haplotype tagging SNPs or SNPs with known functionality were selected and genotyping was performed by KBiosciences (http:// www.kbiosciences.co.uk), as described by Ruskamp et al[7] (Table IIA). We investigated the distribution of these polymorphisms, genotype combinations (YA/YA and YA/XA 'high producers'; XA/XA and YA/O 'intermediate producers'; XA/O and O/O 'low producers'[4,5]), and haplotypes for the research question (Table II).

Data were analysed for deviations from Hardy-Weinberg equilibrium (Haploview version 4.1, MIT/Harvard Broad Institute, Boston, MA, USA). To analyse the association between the *MBL2* polymorphisms and the occurrence of FN, differences in SNP, genotype combination and haplotype distributions between the group with and the group without FN were tested using a Chi-squared test (2 df) using Haploview (version 4.1) and SPSS 16.0 (SPSS, Chicago, IL, USA).

There were 877 N in 194 patients. Twenty-six children did not experience at least one N and were thus not analysed in the group of patients without FN. In the group with 1-17 N (n = 168), 86 children suffered from FN 1 to 8 times (Table I).

There was no significant difference between the group with and the group without FN in the distribution of the various genotype combinations of 'high', 'intermediate', and 'low' producers (data not shown). There was a significant difference in the distribution of the homozygotes of both alleles of SNPs rs11003125 (P = 0.040), rs1838065 (P = 0.018) and rs1838066 (P = 0.023); these were less common in the group with FN (Table IIB) than in the group without FN. The haplotype LXPA (intermediate producing) was more common in the FN group than in the group without FN (25% versus 15.7%, P = 0.034) and was thus associated with higher FN numbers (Table IIC). We also found no consistent associations between causes of FN, such as particular pathogens (e.g., E. coli bloodstream episodes) and the genetic variants under study.

Table II: *MBL2* single nucleotide polymorphisms, genotype combinations and haplotypes

(A) Investigated *MBL2* single nucleotide polymorphisms, modified from Ruskamp et al.[7] Three polymorphisms in exon 1 of the *MBL2* gene at codons 52, 54, and 57, encode the mutant alleles D, B and C respectively (collectively known as the O allele); the wild-type allele is A. Three SNPs in the *MBL2* promoter area, located at positions -619, -290 and -66 encode for the alleles H/L, Y/X and P/Q respectively; the SNPs influence MBL serum levels.[7,8] **(B)** Significant differences for single nucleotide polymorphism distribution for fever during neutropenia episodes. **(C)** Haplotype distribution for fever during neutropenic episodes. The structural exon 1 alleles are in linkage disequilibrium with the promoter SNPs and every individual carries two of the seven haplotypes: HYPA, LYQA, LYPA, LXPA, LYPB, LYQC and HYPD.[9] Functional analyses of these variants showed that specific haplotypes produced high, intermediate and (very) low levels of MBL.[8]

A

dbSNP identifier	SNP	Allele name	SNP type	MAF (*n*=194)	Minor allele
rs11003125	-619G/C	H/L	Promoter	0.419	C
rs7096206	-290G/C	Y/X	Promoter	0.203	G
rs7095891	-66A/G	P/Q	5' UTR	0.031	A
rs5030737	154C/T Arg52Cys	D	Exon 1, codon 52	0.065	T
rs1800450	161G/A Gly54Asp	B	Exon 1, codon 54, Ht	0.121	A
rs1800451	170G/A Gly57Glu	C	Exon 1, codon 57	0.030	A
rs4647964	297A/G		Ht	0.154	A
rs1838066	2071A/G		Ht	0.418	G
rs1838065	2139A/G		Ht	0.419	G
rs930507	3130C/G		Exon 4, Ht	0.168	G
rs10824792	5190C/T		Exon 4, Ht	0.427	C
rs2120132	5356C/T		Ht	0.265	C

B

rs number	Genotype	FN=0 (*n*=82) %	FN=1-8 (*n*=86) % (*P*-value, 2 df)	
rs11003125	G:G	44.3	31.7	
	G:C	30.4	50.0	
	C:C	25.3	18.3	(0.040)
rs1838065	A:A	44.3	29.8	
	A:G	31.6	53.6	
	G:G	24.1	16.7	(0.018)
rs1838066	A:A	45.5	29.8	
	A:G	31.2	52.4	
	G:G	23.4	17.9	(0.023)

C

Haplotype	Predicted MBL serum level	HF (*n*=203)	FN=0 (*n*=82)	FN=1-8 (*n*=86)	*P*-value
HYPA	Intermediate	0.359	0.346	0.371	0.6342
LXPA	Intermediate	0.211	0.157	0.250	**0.034**
LYQA	Intermediate	0.129	0.147	0.111	0.3221
LYPB	Low	0.130	0.145	0.096	0.1661
HYPD	Low	0.066	0.063	0.066	0.9043
LYPA	High	0.075	0.110	0.071	0.2129
LYQC	Very low	0.030	0.032	0.035	0.8914

ALL, acute lymphoblastic leukaemia; Ht, haplotype tagging; MAF, minor allele frequency; SNP, single nucleotide polymorphism; UTR, untranslated region; N, neutropenic episode; FN, fever during neutropenia; HF, haplotype frequency. The significant P-value is shown in bold.

This study explored *MBL2* polymorphisms in relation to the risk of FN in children with ALL during maintenance treatment. Our cohort is a relatively large and homogenous group with a long observation period, while patients all had the same type of cancer and hence comparable chemotherapy regimens. Patients without N were excluded from the

analysis, which avoids possible noise. This enabled us to analyse the role of *MBL2* polymorphisms in the susceptibility to FN. The main result of this study is that the genotypes and/or the haplotypes resulting in low MBL levels did not confer an increased risk of FN.

This is in agreement with findings of other studies. In a cohort of 106 children with ALL, Lausen et al found no difference in the frequency of infectious events during induction treatment (50 d) between the *MBL2* genotypes encoding for low and normal levels of MBL.[5] However, their observation period could have been too short to find significant differences. Neth et al studied 100 children with various kinds of malignancies and did not report a significant difference in the FN incidence between MBL- sufficient and MBL-insufficient children.[3] Likewise, in the study by Frakking et al the median FN number was similar between the various *MBL2* genotypes.[4] Our study underscores these results. A possible explanation for the limited influence of the *MBL2* polymorphisms, genotype combinations and haplotypes on the frequency of FN might be that MBL needs some phagocytic function to be functionally effective against bacteria. This is supported by Mullighan et al, who found that MBL coding mutations in stem cell donors only influenced infections following phagocytic recovery.[10]

In conclusion, *MBL2* low-producing genotypes and haplotypes do not influence the occurrence of FN during maintenance treatment of ALL in children.

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Chapter 9

Polymorphisms in the *TLR6* gene associated with the inverse association between childhood acute lymphoblastic leukemia and atopic disease

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Abstract

Little is known about the etiology of childhood acute lymphoblastic leukemia (ALL). The presence of atopic disease has been shown to protect against developing childhood ALL. The aim of this study was to examine whether single nucleotide polymorphisms (SNPs) in innate immunity genes previously associated with atopic disease, can elucidate the inverse association between childhood ALL and atopic disease. We studied 525 children, including 192 with childhood ALL, 149 with atopic disease and 184 healthy control subjects. We compared genotype distributions of 29 SNPs in genes of *TLR2, TLR4, TLR6, TLR9, TLR10*, and *CD14* between the three groups and corrected for multiple testing. The genotype distributions of two SNPs in the *TLR6* gene, rs5743798 and rs6531666, differed significantly between children with ALL, children with atopic disease and control subjects. Particularly in children with atopic eczema, risk alleles for atopic disease were observed more often than in control subjects, and less often in children with ALL than in control subjects. These findings support the immune surveillance hypothesis as an explanation for the protective association of atopic disease on childhood ALL. Further investigation is warranted to examine in more detail the role of innate immunity in the development of childhood ALL.

Keywords: Childhood acute lymphoblastic leukemia, Atopic disease, TLR6, Polymorphisms, Innate immunity

Introduction

Acute lymphoblastic leukemia (ALL) is the most common form of cancer among children, which accounts for about 30% of all cancer cases in children under the age of 15 years. There is a specific age peak at 2-5 years, mainly due to an excess of pre-B ALL cases in this age range. Little is known about the etiology of childhood ALL. Interestingly, there is growing evidence of an inverse association between childhood ALL and atopic disease.[1-3] Many epidemiological studies have shown a protective association of atopic disease on the risk of childhood ALL. The presence of an atopic condition is thought to increase the vigilance of the immune system in monitoring for, identifying, and eliminating malignant cells.[4] Therefore, atopy may lead to early elimination of malignant cells and thus prevent the development of malignancies like childhood ALL.

A multifactorial background has been suggested for atopic disease with genetic as well as environmental factors contributing to disease susceptibility. Various studies examined the role of innate immunity genes on the risk of atopic disease. Particularly SNPs in *toll-like receptor 2 (TLR2), TLR4, TLR6, TLR9, TLR10,* and *cluster of differentiation 14 (CD14)*[5-9] have been associated with atopic disease. TLRs are membrane receptors that act as the gatekeepers of the innate immunity by recognizing microbial components and initiating activation of an adequate immune response. CD14 acts as a co-receptor, along with TLR4, for the detection of bacterial lipopolysaccharide. Variations in genes encoding *TLRs* and *CD14* are suggested to alter the capability to recognize microbes or alter the amount of gene product, leading to inadequate immune responses and increasing the susceptibility for atopic disease.[5,9] In the case of childhood ALL much less is known about the role of genes involved in innate immunity.[10] Examining the genetic basis of the inverse association between childhood ALL and atopic disease might help towards elucidating the etiology of childhood ALL.

The aim of this study was to examine whether genetic variations in the genes of *TLR2, TLR4, TLR6, TLR9, TLR10,* and *CD14* are associated with the inverse association between childhood ALL and atopic disease. We hypothesized that risk alleles for atopic disease are observed more often in atopic children than in healthy controls, and are observed less often in children with ALL than in healthy controls.

Material and methods

Study population

This study was conducted in three cohorts. The first cohort included 210 children, aged 0-15 years, with ALL who were diagnosed between 1984 and 2001 in Beatrix Children's Hospital of University Medical Center Groningen in Groningen, the Netherlands, or between 1997 and 2001 in Wilhelmina Children's Hospital of University Medical Center Utrecht in Utrecht, the Netherlands. Eighteen subjects were excluded because of their non-Caucasian background.

The second cohort included 149 atopic children, aged 0-8 years old, derived from the PIAMA study,[11] see Table S1. The PIAMA study is a prospective birth cohort study on *P*revention and *I*ncidence of *A*sthma and *M*ite *A*llergy. The participating children were born between May 1996 and December 1997 in three regions of the Netherlands. They were selected using a validated screening questionnaire with questions on asthma and respiratory allergies administered to the mother at her first visit to the midwife. The midwives were requested to ask every new pregnant woman to fill in the screening questionnaire. A total of 10232 pregnant women completed the questionnaire and 2949 (29%) of them reported asthma or respiratory allergy and were defined as 'allergic'. Based on this screening, 7862 women were invited to participate, and 4146 agreed and gave informed consent. After birth the baseline study population consisted of 3963 children. All children from allergic mothers (n=1327) and a random sample of children from non-allergic mothers (n=663) were selected for more extensive investigation, including DNA collection at 4 years of age. DNA was obtained from 1046 of these children and genotypes were obtained from 1037 children. Children with a non-Caucasian ethnicity were excluded from the analyses (n=51). Atopic disease was determined by elevated specific IgE-levels (≥0.70 IU/ml) and/or positive allergy skin test, in combination with asthma and/or eczema, since these more stringent definitions of atopy were used in epidemiological studies observing a reverse association between childhood ALL and atopy. Specific IgE to mite (*Dermatophagoides pteronyssinus*), cat (Fel d1), dog (Can f1), grass (*Dactylis glomerata*), birch (*Betula verrucosa*), and mould (*Alternaria alternata*) was measured by radioallergosorbent test. In PIAMA, allergy skin tests were performed with: *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, *Alternaria alternata*, mixed grass pollen, mixed tree pollen, cat, and dog. A positive skin test was defined as a mean wheal diameter of ≥3 mm in response to one or more allergens provided the control was negative (<3 mm) and the positive control was positive (≥3 mm). Asthma was defined as an episode of wheezing or dyspnea, or use of inhalation steroids in the past twelve months at the age of eight years old. Eczema was defined as having reported an itching rash at least twice in the past twelve months, intermittently present at the usual eczema locations (e.g. elbows, back of the knees, front of the ankles, around the eyes or ears, in the neck), for at least two years in a row in the age 0-8 years. In all, 82 children (55%)

were diagnosed with atopic asthma, and 115 (77.2%) with atopic eczema. Forty-eight of the children (32%) were diagnosed both with asthma and eczema.

The third cohort consisted of 184 healthy control children, aged 0-8 years, also derived from the PIAMA cohort. These children did not have positive skin tests, their specific serum IgE-levels were not increased (<0.70 IU/ml), and they were not diagnosed with either asthma or eczema.

The local medical ethics committees of participating institutes approved all studies. Written informed consent was obtained from all participants and/or their parents.

Genotyping

Genomic DNA was extracted from buccal swabs, blood, or bone-marrow samples. DNA extraction from blood or bone-marrow samples was performed by using the NucleoSpin® Blood L kit (Macherey-Nagel, Germany). For frozen samples the NucleoSpin® Tissue kit was used. Both kits were used according to the manufacturer's protocol. For DNA isolation from buccal swabs, the protocol described in Meulenbelt et al. (1995) was used.[12] DNA was amplified using REPLI-g UltraFast technology (Qiagen, Venlo, The Netherlands). We selected (potential) functional SNPs complemented with known haplotype-tagging SNPs in the genes for *TLR2* (rs3804099, rs3804100, rs4969480), *TLR4* (rs4986790, rs6478317, rs10759932, rs11536878, rs11536889, rs1927911, rs10759931, rs2770150), *TLR6* (rs1039559, rs5743788, rs5743810, rs6531666, rs5743798), *TLR9* (rs352140, rs187084, rs5743836), *TLR10* (rs4274855, rs10856839, rs11096956, rs11096957, rs11466652, rs4129009), and *CD14* genes (rs2563298, rs2569190, rs256191, rs5744455). Genotyping was performed by Competitive Allele-Specific PCR using KASParTM genotyping chemistry, performed under contract by K-Biosciences (Herts, UK). Quality of genotype data was guaranteed by standards of K-Biosciences with extensive quality control as described before. [13]

The genotype data of the healthy control subjects were analyzed for deviations from Hardy-Weinberg equilibrium using 1 df chi-square tests. None of the SNPs deviated from HWE (p<0.01). On average, the call rate was 97%, but one SNP (rs6531666) had a call rate of 86%. We therefore performed an extensive quality control on this SNP: the allele frequencies were similar between the cohorts and compared to near-lying SNPs (p>0.05), and genotypes were in Hardy-Weinberg equilibrium (p≥0.01). Thus, as the quality of this SNP was good, we included it in the analyses. The 29 SNPs selected for *TLR2*, *TLR4*, *TLR6*, *TLR9*, *TLR10*, and *CD14*, data source and allele frequencies are shown in Table 1. Pair wise linkage disequilibrium (LD) was assessed between single SNPs by using the r^2 statistic as implemented in Haploview version 4.1 (Broad Institute, Cambridge, UK).

Data analyses

Table 1: SNPs genotyped in *TLR2, TLR4, TLR6, TLR9, TLR10,* and *CD14*, and their location, minor allele frequency, call rate, and deviation from Hardy-Weinberg equilibrium in controls (*n*=184)

Gene	Location	SNP	Allele	SNP type	MAF	Call rate	HWE *P* value
TLR2	4q32	rs3804099	T/C	Ht, exon, synonymous, 199Asn	0.47	96	0.03
		rs3804100	T/C	Ht, exon, synonymous, 450Ser	0.06	98	0.48
		rs4696480	T/A	Ht, promoter	0.46	98	0.35
TLR4	9q32-q33	rs4986790	A/G	Exon, nonsynonymous, Asp299Gly	0.07	97	0.26
		rs6478317	A/G	Ht, promoter	0.32	98	0.35
		rs10759932	T/C	Ht, promoter	0.11	97	0.94
		rs11536878	C/A	Ht, intron	0.12	98	0.32
		rs11536889	G/C	Ht, 3'UTR	0.15	98	0.11
		rs1927911	C/T	Ht, intron	0.24	99	0.10
		rs10759931	G/A	Ht, promoter	0.40	95	0.11
		rs2770150	T/C	Ht, promoter	0.28	96	0.36
TLR6	4p14	rs1039559	T/C	Ht, intron	0.47	95	0.72
		rs5743788	C/G	Ht, intron	0.48	97	0.37
		rs5743810	C/T	Ht, exon, nonsynonymous, Ser249Pro	0.41	96	0.19
		rs6531666	T/C	Ht, intron	0.28	86	0.23
		rs5743798	C/T	Ht, intron	0.25	96	0.72
TLR9	3p21.3	rs352140	T/C	Ht, exon, synonymous, G2848A	0.43	97	0.85
		rs187084	T/C	Ht, intron	0.45	96	0.15
		rs5743836	T/C	Ht, intron	0.14	97	0.59
TLR10	4p14	rs4274855	G/A	Ht, 5'UTR	0.20	98	0.18
		rs10856839	A/C	Ht, 5'UTR	0.17	97	0.21
		rs11096956	G/T	Ht, exon, synonymous	0.24	95	0.79
		rs11096957	A/C	Ht, exon, non-synonymous, Asn241His	0.39	95	0.71
		rs11466652	A/G	Ht, synonymous	0.14	97	0.76
		rs4129009	A/G	Ht, exon, Ile2322Val	0.20	98	0.31
CD14	5q31.1	rs2563298	C/A	Ht, 3'UTR	0.26	98	0.09
		rs2569190	G/A	Ht, 5'UTR	0.45	97	0.05
		rs2569191	T/C	Ht, promoter	0.46	98	0.08
		rs5744455	C/T	Ht, promoter	0.26	98	0.12

Abbreviations: Ht – haplotype-tagging; HWE – Hardy-Weinberg equilibrium; MAF – minor allele frequency; SNP – single nucleotide polymorphism; UTR – untranslated region

Differences in distribution of genotype and allele frequencies were tested using 4 df chi-square test. To account for multiple testing we calculated the false discovery rate (FDR) as described by Benjamini and Hochberg, using a *p*-value of 0.05 as a cut-off value.[14] Further, we performed 10,000 permutation tests to correct for false-positives, where we compared the genotype distributions between ALL versus atopic patients for studied SNPs using permute module, implemented in PLINK software. The two subgroups in the atopic cohort, i.e. asthmatic children and eczematous children, were analyzed separately in an additive model by using chi-square statistics. Subsequently, we compared children with childhood ALL and children with atopic disease separately with healthy controls by using logistic regression, to estimate the odds ratios (ORs) and 95% confidence intervals (CIs)

for the best-fitting model (i.e. dominant, recessive, or additive). *P*-values <0.05 were considered statistically significant. The statistical software package used was SPSS 16.0 (SPSS, Chicago, IL).

Results

Study population

Table 2 shows the patient characteristics of the three cohorts. A total of 525 children were included in the study: 192 children with childhood ALL (61% male), 149 children with atopic disease (55% male), and 184 healthy control children (48% male). There was a significant difference in sex distribution among the three cohorts (p=0.041).

Table 2. Patient characteristics of the three cohorts

	ALL *N* (%)	Controls *N* (%)	Atopic disease *N* (%)	*P* value
Total number of patients	192	184	149	
Male (%)	115 (61%)	88 (48%)	82 (55%)	0.041
Age, years – median (range)	4.4 (0-15)	8*	8*	
ALL subtype:				
- B-lineage ALL	163 (84.9%)			
- T-lineage ALL	20 (10.4%)			
- Infant ALL	2 (1.0%)			
- Undifferentiated	2 (1.0%)			
- Unknown	5 (2.6%)			
Cytogenetic aberrations:				
- Hyperdiploid (>50 chromosomes)	50 (26.0%)			
- T(12;21)	20 (10.4%)			
Atopic symptom:				
- Asthma			82 (55%)	
- Eczema			115 (77%)	
- Asthma and eczema			48 (32%)	
- Elevated IgE-levels			145 (97%)	
- Positive Skin Tests			115 (77%)	

Abbreviations: ALL, acute lymphoblastic leukemia. * The control and atopic children were examined annually until the age of 8 years.

Association analysis

Table 3 shows the genotype and allele frequencies of the 29 SNPs among the three cohorts. The genotype distribution of two SNPs in the *TLR6* gene, rs6531666 (p=0.0025) and rs5743798 (p=0.0005), was significantly different among children with ALL, control subjects, and children with atopic disease. Both SNPs sustained correction for multiple testing. When correcting for sex, both rs6531666 and rs5743798 remained significantly different among the three cohorts (p=0.0035 and p=0.0006, respectively). The largest differences in genotype distributions were observed between children with ALL and

Table 3: Comparison of genotype and allele frequencies of SNPs in *TLR2*, *TLR4*, *TLR6*, *TLR9*, *TLR10*, and *CD14* among children with ALL, healthy control subjects, and children with atopic disease.

Gene	SNP	Genotype	ALL	Control	Atopic disease	*P* value	FDR
			N (%)	*N* (%)	*N* (%)		
TLR2	rs3804099	TT	51 (28)	48 (27)	35 (24)		
		TC	94 (52)	102 (57)	77 (53)		
		CC	37 (20)	28 (16)	34 (23)	0.472	NS
		C allele	46%	44%	50%	0.401	NS
	rs3804100	TT	170 (90)	165 (90)	120 (84)		
		TC	18 (10)	18 (10)	22 (15)		
		CC	1 (1)	0 (0)	1 (1)	0.331	NS
		C allele	5%	5%	8%	0.136	NS
	rs4696480	TT	42 (23)	60 (33)	52 (36)		
		TA	99 (54)	83 (46)	65 (45)		
		AA	44 (24)	38 (21)	29 (20)	0.102	NS
		A allele	51%	44%	42%	0.065	NS
TLR4	rs4986790	AA	168 (89)	151 (85)	126 (88)		
		AG	20 (11)	28 (16)	17 (12)		
		GG	0 (0)	0 (0)	1 (1)	0.314	NS
		G allele	5%	8%	7%	0.392	NS
	rs6478317	AA	83 (44)	85 (47)	73 (50)		
		AG	82 (44)	74 (41)	59 (41)		
		GG	22 (12)	22 (12)	13 (9)	0.785	NS
		G allele	34%	33%	29%	0.470	NS
	rs10759932	TT	140 (75)	144 (80)	120 (83)		
		TC	45 (24)	33 (18)	25 (17)		
		CC	1 (1)	3 (2)	0 (0)	0.192	NS
		C allele	13%	115	9%	0.398	NS
	rs11536878	CC	150 (79)	139 (78)	114 (77)		
		CA	35 (18)	39 (22)	29 (20)		
		AA	5 (3)	1 (1)	5 (3)	0.411	NS
		A allele	12%	11%	13%	0.784	NS
	rs11536889	GG	134 (72)	129 (72)	104 (70)		
		GC	44 (24)	49 (27)	42 (28)		
		CC	7 (4)	1 (1)	2 (1)	0.195	NS
		C allele	16%	14%	16%	0.843	NS
	rs1927911	CC	111 (58)	109 (61)	92 (63)		
		CT	65 (34)	57 (32)	46 (31)		
		TT	15 (8)	14 (8)	9 (6)	0.919	NS
		T allele	25%	24%	22%	0.641	NS
	rs10759931	GG	70 (38)	62 (35)	48 (34)		
		GA	87 (48)	76 (43)	71 (51)		
		AA	26 (14)	38 (22)	21 (15)	0.318	NS
		A allele	38%	43%	40%	0.364	NS
	rs2770150	TT	98 (54)	92 (53)	67 (46)		
		TC	69 (38)	73 (42)	63 (43)		
		CC	15 (8)	10 (6)	15 (10)	0.429	NS
		C allele	27%	27%	32%	0.252	NS
TLR6	rs1039559	TT	47 (26)	53 (30)	44 (31)		
		TC	87 (48)	85 (48)	69 (49)		
		CC	47 (26)	38 (22)	29 (20)	0.716	NS
		C allele	50%	46%	45%	0.345	NS
	rs5743788	CC	45 (25)	49 (27)	43 (30)		
		CG	92 (50)	96 (53)	69 (47)		
		GG	47 (26)	36 (20)	34 (23)	0.628	NS
		G allele	51%	46%	47%	0.483	NS
	rs5743810	CC	63 (35)	61 (34)	54 (38)		
		CT	82 (45)	94 (53)	66 (46)		
		TT	36 (20)	24 (13)	23 (16)	0.437	NS
		T allele	43%	40%	39%	0.625	NS

Table 3 (cont.): Comparison of genotype and allele frequencies of SNPs in *TLR2*, *TLR4*, *TLR6*, *TLR9*, *TLR10*, and *CD14* among children with ALL, healthy control subjects, and children with atopic disease.

Gene	SNP	Genotype	ALL	Control	Atopic disease	*P* value	FDR
			N (%)	*N* (%)	*N* (%)		
	rs6531666	TT	104 (56)	72 (49)	49 (41)		
		TC	76 (41)	66 (45)	54 (45)		
		CC	5 (3)	9 (6)	15 (13)	**0.0025**	**Significant**
		C allele	23%	29%	33%	**0.0283**	NS
	rs5743798	CC	118 (65)	100 (56)	66 (46)		
		CT	60 (33)	68 (38)	59 (41)		
		TT	5 (3)	10 (6)	19 (13)	**0.00047**	**Significant**
		T allele	19%	25%	34%	**0.00011**	**Significant**
TLR9	rs352140	TT	67 (37)	57 (32)	45 (31)		
		TC	85 (46)	88 (49)	74 (51)		
		CC	31 (17)	36 (20)	26 (18)	0.772	NS
		C allele	40%	44%	43%	0.509	NS
	rs187084	TT	57 (31)	50 (28)	44 (30)		
		TC	88 (48)	98 (55)	71 (49)		
		CC	37 (20)	31 (17)	30 (21)	0.767	NS
		C allele	45%	45%	45%	0.985	NS
	rs5743836	TT	133 (72)	138 (76)	107 (75)		
		TC	45 (24)	40 (22)	34 (24)		
		CC	7 (4)	4 (2)	2 (1)	0.667	NS
		C allele	16%	13%	13%	0.490	NS
TLR10	rs4274855	GG	123 (64)	112 (62)	100 (69)		
		GA	54 (28)	56 (31)	42 (29)		
		AA	14 (7)	12 (7)	3 (2)	0.245	NS
		A allele	21%	22%	17%	0.161	NS
	rs10856839	AA	131 (69)	124 (69)	97 (69)		
		AC	55 (29)	47 (26)	39 (28)		
		CC	3 (2)	8 (5)	5 (4)	0.592	NS
		C allele	17%	18%	17%	0.930	NS
	rs11096956	GG	110 (59)	98 (56)	86 (63)		
		GT	65 (35)	65 (37)	47 (34)		
		TT	13 (7)	12 (7)	4 (3)	0.472	NS
		T allele	24%	25%	20%	0.270	NS
	rs11096957	AA	66 (36)	57 (33)	55 (39)		
		AC	91 (50)	88 (51)	72 (51)		
		CC	27 (15)	29 (17)	15 (11)	0.571	NS
		C allele	39%	42%	36%	0.302	NS
	rs11466652	AA	136 (74)	130 (73)	111 (76)		
		AG	46 (25)	45 (25)	31 (21)		
		GG	2 (1)	4 (2)	5 (3)	0.588	NS
		G allele	14%	15%	14%	0.890	NS
	rs4129009	AA	125 (66)	112 (62)	103 (71)		
		AG	52 (27)	57 (32)	39 (27)		
		GG	13 (7)	11 (6)	3 (2)	0.219	NS
		G allele	21%	22%	16%	0.104	NS
CD14	rs2563298	CC	95 (51)	85 (47)	86 (59)		
		CA	86 (46)	85 (47)	54 (37)		
		AA	5 (3)	11 (6)	6 (4)	0.139	NS
		A allele	26%	30%	23%	0.129	NS
	rs2569190	GG	58 (32)	53 (29)	36 (25)		
		GA	80 (44)	101 (56)	82 (56)		
		AA	42 (23)	26 (14)	29 (20)	0.078	NS
		A allele	46%	43%	48%	0.413	NS
	rs2569191	TT	59 (32)	53 (29)	36 (25)		
		TC	81 (44)	101 (56)	82 (56)		
		CC	46 (25)	28 (15)	28 (15)	0.061	NS
		C allele	47%	43%	47%	0.512	NS
	rs5744455	CC	108 (57)	96 (53)	72 (49)		
		CT	66 (35)	77 (43)	66 (45)		
		TT	14 (7)	8 (4)	9 (6)	0.312	NS
		T allele	25%	26%	29%	0.554	NS

Abbreviations: SNP – single nucleotide polymorphism; ALL – acute lymphoblastic leukemia; FDR – false discovery rate; NS – not significant. Bold values emphasize significance.

children with atopic disease. The minor alleles of both rs6531666 and rs5743798 were associated with an increased risk of atopic disease when comparing with the controls, and at the same time associated with a decreased risk of ALL when comparing with the controls. After 10,000 permutation test, both rs6531666 (p=0.013) and rs5743798 (p=0.0004) sustained their significant differences in their genotype frequencies between ALL and atopic patients (see Table S2). SNPs in the other *TLR* and/or *CD14* genes were not associated with childhood ALL or atopy.

The frequencies of the minor alleles in rs6531666 and rs5743798 were significantly different among the three groups. For rs6531666 the frequency of the minor allele was 23% in children with ALL, 29% in the control subjects, and 33% in the atopic children (p=0.028). For rs5743798 the frequency of the minor allele was 19% in children with ALL, 25% in the healthy controls, and 34% in the atopic children, (p=0.0001, which sustained correction for multiple testing).

Figure 1 presents the genotype effects (ORs and 95% CIs) of the heterozygous and homozygous minor allele genotype of *TLR6* SNPs rs6531666 and rs5743798 compared to the homozygous major allele genotype on the risk of ALL and atopic disease, using the healthy control subjects as reference group. Subjects homozygous for the minor allele (C) of rs6531666 showed a borderline significant decreased risk of childhood ALL (OR 0.39, 95% CI, 0.12-1.20, p=0.099), whereas they had significantly increased risk of atopic disease (OR 2.61, 95% CI, 1.07-6.39, p=0.035). The same trend was observed for subjects homozygous for the minor allele (T) of rs5743798, with a borderline significant decreased risk of childhood ALL (OR 0.42, 95% CI, 0.14-1.28, p=0.13), and a significantly increased risk of atopic disease (OR 2.88, 95% CI, 1.26-6.58, p=0.012).

As the atopic children and the control subjects were examined annually until the age of 8, we also performed the analysis including only the children with ALL who were 0-8 years (n=157). Although numbers were lower, this analysis still showed that the genotype distributions of the same two *TLR6* SNPs, i.e. rs6531666 (p=0.008) and rs5743798 (p=0.0009), differed significantly among the three groups. Table S3 shows the genotype distributions of the children with ALL who were 0-8 years, compared to the controls and the atopic children.

It has been suggested that different subtypes of ALL, in particular B-lineage ALL, may have different etiologies. Therefore, we performed separate analyses for the groups of children with B-lineage ALL and the children with T-lineage ALL. The results for the group with B-lineage ALL (n=163) were similar to the entire ALL group: the *TLR6* SNPs rs6531666 and rs5743798 were significantly different among the three groups (p=0.005 and p=0.001, respectively). Only SNP rs5743798 sustained correction for multiple testing. ORs did not reach significant levels. When comparing the group with T-lineage ALL (n=20) to the controls and the atopic children, none of the SNPs sustained correction for multiple testing, which may be due to the small number of T-ALL patients.

Among the 149 children with atopic disease, 82 (55%) were diagnosed with asthma. When comparing the genotype frequencies of asthmatic children and children with ALL with the controls, the genotype distribution of both *TLR6* SNP rs6531666 and rs5743798 were significantly different among the three cohorts (p=0.01 and p=0.02, respectively). Both

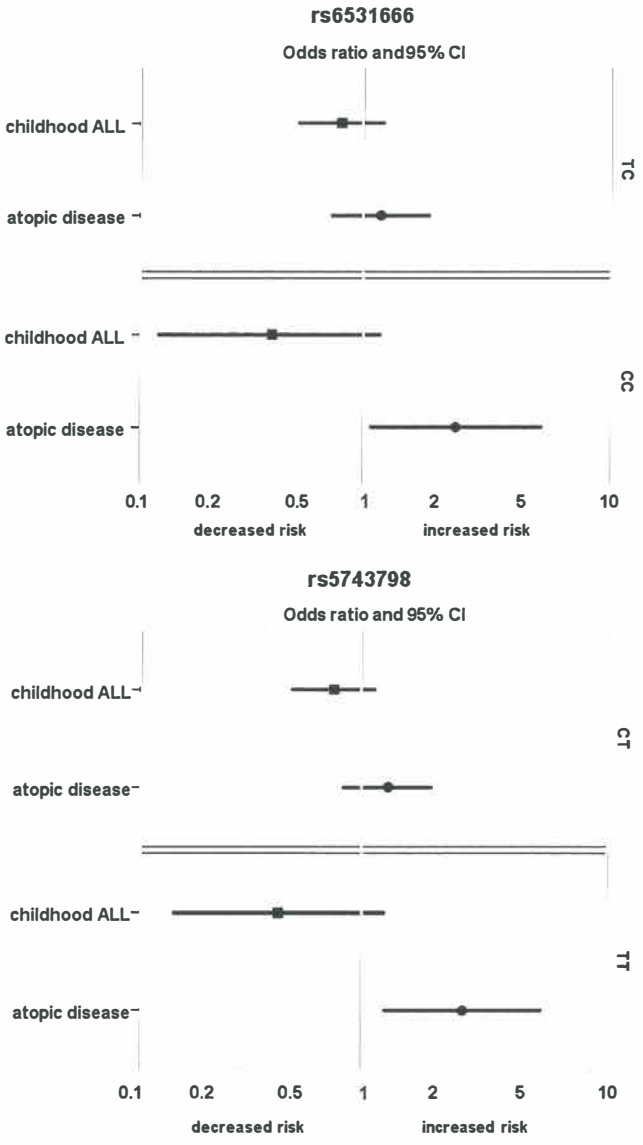

Figure 1: Genotype effects (OR and 95% CI) of the heterozygous and homozygous minor allele genotype of *TLR6* SNPs rs6531666 and rs5743798 compared with the homozygous major allele genotype on the risk of ALL and atopic disease, using the healthy control subjects as a reference group.

SNPs did not sustain correction for multiple testing. Of the 149 atopic children, 115 (77%) were diagnosed with eczema. When comparing the children with eczema and the children with ALL with the control subjects, again both *TLR6* SNP rs6531666 and rs5743798 were significantly different among the three groups (p=0.001 and p<0.001, respectively). Both SNPs sustained correction for multiple testing.

Finally, we constructed an LD plot of the *TLR6* gene, including the SNPs that were associated with the inverse association between childhood ALL and atopic disease. As can be seen in the LD plot of *TLR6*, rs5743798 and rs6531666 show moderately high LD with an r2 of 0.77 (Figure 2). Minor alleles of these SNPs were both associated with an increased risk of atopy and a decreased risk of childhood ALL, as would be expected with this LD.

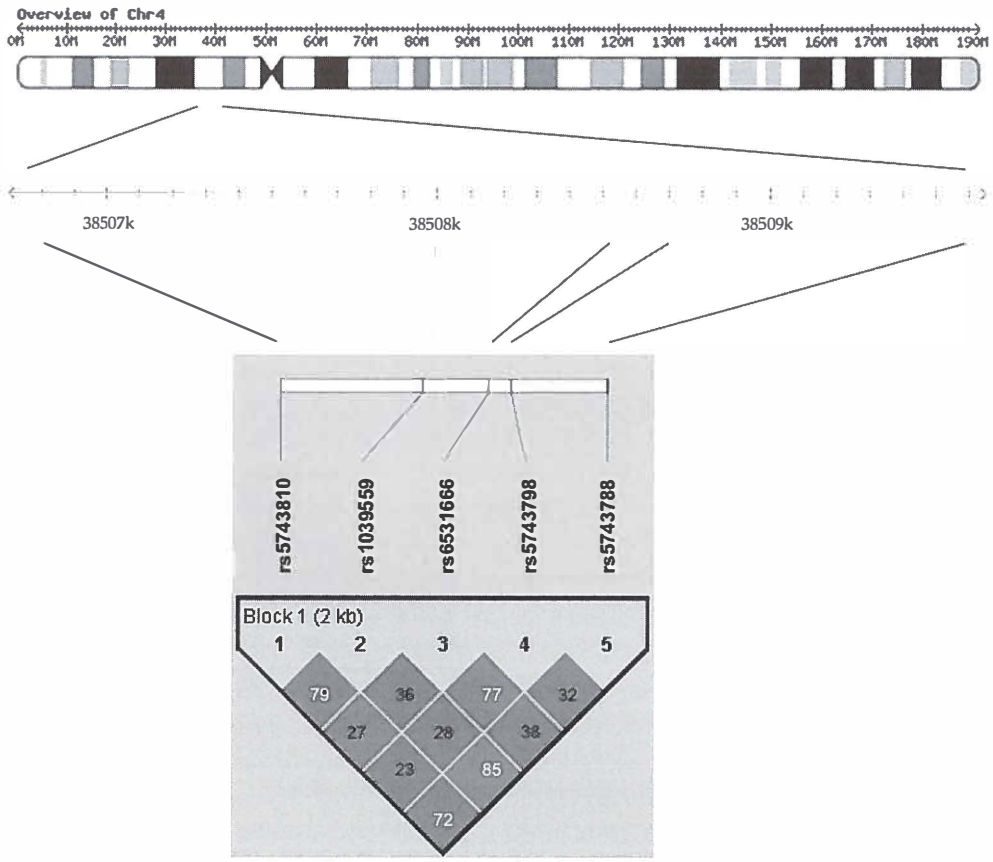

Figure 2. Genetic layout of *TLR6* with LD plot (r^2).

Discussion

In the present study we show that two polymorphisms in the *TLR6* gene are associated with the inverse association between childhood ALL and atopic disease. At the same time, compared to healthy controls, the minor alleles of these two *TLR6* SNPs are associated with an increased risk of atopic disease, as they are also associated with a decreased risk of childhood ALL. Ours was the first study to provide a possible genetic explanation for the protective association of atopic disease on the occurrence of childhood ALL.

Different hypotheses have been proposed to explain the inverse relationship between childhood ALL and atopic disease. The principal factor linking childhood ALL and atopic disease seems to be the rate at which the immune system matures. Hypotheses proposed by Greaves and Kinlen suggest an etiological role for the immune system in the development of childhood leukemia via delayed exposure and abnormal response to childhood infections.[15,16] A hypothesis that has been proposed concerning the inverse association between atopic disease and childhood ALL is the so-called immune surveillance hypothesis. This hypothesis implies that the innate immune system recognizes antigens of malignant cells as foreign and mounts a response to them by triggering adaptive immune responses which prevent a majority of potential cancers from developing.[17] As the vigilance of the immune system may be increased in the presence of atopic disease,[4] this could eliminate malignant cells, and prevent the development of malignancies, such as childhood ALL.

Atopic disease is associated with a predominance of T helper 2 (Th2) cells, essential for the production of IgE, versus T helper 1 (Th1) cells. All infants are born with a Th2-dominated immune profile, characterized by interleukin 4 (IL-4), IL-5, IL-9, IL-10, and IL-13 production. By the age of two years, nonatopic infants have gradually migrated to a Th1-dominant profile, which is characterized by: IL-12, IL-18, interferon-gamma (IFNγ), and tumour necrosis factor α (TNF-α), while infants who develop atopy fail to make this Th2-to-Th1 transition. It has been suggested that one of the driving forces for this immune shift is microbial exposure, which induces innate immunity cells, such as dendritic cells, to produce cytokines important for the development of Th1 responses.[18] Dendritic cells express TLRs and are susceptible to TLR ligands, including microbial stimuli, as well as endogenous ligands. Genetic variation in *TLR*s may influence the activation of T-regulatory cells (responsible for suppressing Th2 responses), and/or skewing of the Th1-Th2 balance. We propose that the two SNPs in the *TLR6* gene as found in our study are related to an altered shift in the Th1-Th2 balance, causing an increased risk of developing with atopic disease, which by means of increased vigilance protects against childhood ALL.

Ligands for TLR6 include diacyl lipopeptides from *Mycoplasma*, yeast zymosan from *Saccharomyces cerevisiae*, and lipoteichoic acid from *group B streptococci* and *staphylococci* which are often found in the upper respiratory tract.[19] Earlier studies found that the production of TNF-α elicited by zymosan and gram-positive bacteria[20] is inhibited in *TLR6* knock-out mice, as is the production of TNF-α in response to mycoplasmal macrophage-activating lipopeptide-2 (MALP-2) from *Mycoplasma fermentans*.[21] These data suggest that the *TLR6* gene controls Th1 differentiation, whereas the absence of TLR6-mediated signals generates Th2 responses. Moreover, Kormann et al. showed another SNP located in the *TLR6* gene, i.e. rs5743789, to be associated with increased mRNA expression. Carriers of the minor allele of this SNP showed increased Th1 cytokine expression, and reduced Th2 cytokine production after stimulation with its ligand.[5] Unfortunately, at the beginning of our study, there was no information available on the possible importance of *TLR6* SNP rs5743789, and it was therefore not included in our study. Alternatively, the *TLR6* SNPs may be associated with a decreased risk of childhood ALL more directly via expression of TLR6 on leukemic cells, as has previously been shown for B chronic lymphoblastic leukemia cells.[22] Future research is warranted to define the association between TLR6 and childhood ALL cells.

Recently, various genome-wide association studies (GWAS) have been performed in order to find risk genes for atopic disease.[23] Although several candidate gene studies did find *TLR6* polymorphisms to be associated with atopy, [5,8,24] this has to date not yet been confirmed in GWAS. However, a GWAS of atopic disease in combination with positive skin tests has not been performed to date. Based on previous epidemiological studies, we specifically defined atopic disease relatively strictly by elevated IgE-levels and/or positive skin tests in combination with asthma and/or eczema.[5,6,24-26] This strengthens our results. Unfortunately, of the children with ALL no information was available on atopy, since IgE-levels and skin tests are not performed regularly in children with leukemia. Since IgE-levels may be influenced by immunosuppressive treatment regimens, however, measuring IgE-levels for the purpose of this study did not seem useful.

As a wide range of atopic conditions exists, it is conceivable that the analysis of allergic subtypes is more accurate than the overall allergy estimate in describing the association, even though numbers are smaller. When comparing the genotype frequencies of the subgroups of children with asthma and eczema to childhood ALL, the strongest inverse association was found between eczematous children and children with childhood ALL. This finding is supported by the literature.[1-3,27]

This study has some limitations. Although the minor allele frequencies of the two *TLR6* SNPs (0.25 and 0.28) are considered common, observations were made in a limited number of subjects and therefore need to be interpreted with caution. Even though we showed a robust association between two SNPs in the *TLR6* gene and the inverse association between childhood ALL and atopic disease, one could argue that these results

were chance findings. This is, however, not very likely since we corrected for multiple testing and performed permutation analysis. Nevertheless, the common problem in candidate gene studies of false positive findings could not be ruled out altogether. Therefore, our study needs to be replicated in study populations of the same or, preferably, larger sample sizes. The control population was derived from the study on Prevention and Incidence of Asthma and Mite Allergy. However, the control population was selected after careful screening for absence of atopic disease, by specific IgE-levels, skin-prick-tests, asthma and eczema, to make sure our control cohort was not biased.

Moreover, the question arises whether the SNPs in the *TLR6* gene that we found are relevant with respect to their function. This clearly requires further study. They are both haplotype-tagging SNPs, implying the effect can be direct or indirect. A direct effect may come from a direct biological influence of (one of) the SNPs, even though they are located in introns. It is now well established that introns may be related to the regulation of gene expression. An indirect effect can by achieved by tagging of a region of the gene that is functional.

Conclusion

We examined the etiology of childhood ALL from a new genetic perspective, and found two *TLR6* polymorphisms to be associated with the inverse association between childhood ALL and atopic disease. Our findings support the immune surveillance hypothesis as an explanation for the protective effect of atopic disease on childhood ALL and provide new insight into the mechanism of the inverse association between childhood ALL and atopic disease. Further investigation is warranted to replicate our findings and to examine in more detail the role of innate immunity in the development of childhood ALL.

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Authorship contribution

Karin G.E. Miedema: designed and performed the research, analyzed and interpreted data, performed statistical analysis, and wrote the manuscript.

Wim J.E. Tissing: designed research, performed research, analyzed and interpreted data, and wrote the manuscript.
Esther M. te Poele: designed and performed the research, collected data, and revised the manuscript.
Willem A. Kamps: interpreted data, and revised the manuscript.
Behrooz Z. Alizadeh: analyzed and interpreted data, performed statistical analysis, and revised the manuscript.
Marjan Kerkhof: analyzed and interpreted data, performed statistical analysis, and revised the manuscript.
Johan H.C. de Jongste: performed research, collected data, and revised the manuscript.
Henriëtte A. Smit: performed research, collected data, and revised the manuscript.
Anne P. de Pagter: performed research, collected data, and revised the manuscript.
Marc Bierings: performed research, collected data, and revised the manuscript.
H. Marike Boezen: designed research, and revised the manuscript.
Dirkje S. Postma: designed the research, interpreted data, and revised the manuscript.
Eveline S.J.M. de Bont: designed research, analyzed and interpreted data, and revised the manuscript.
Gerard H. Koppelman: designed the research, interpreted data, and wrote the manuscript.

Disclosure of conflicts of interest

All authors have no declared conflict of interest.

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Supplementary information

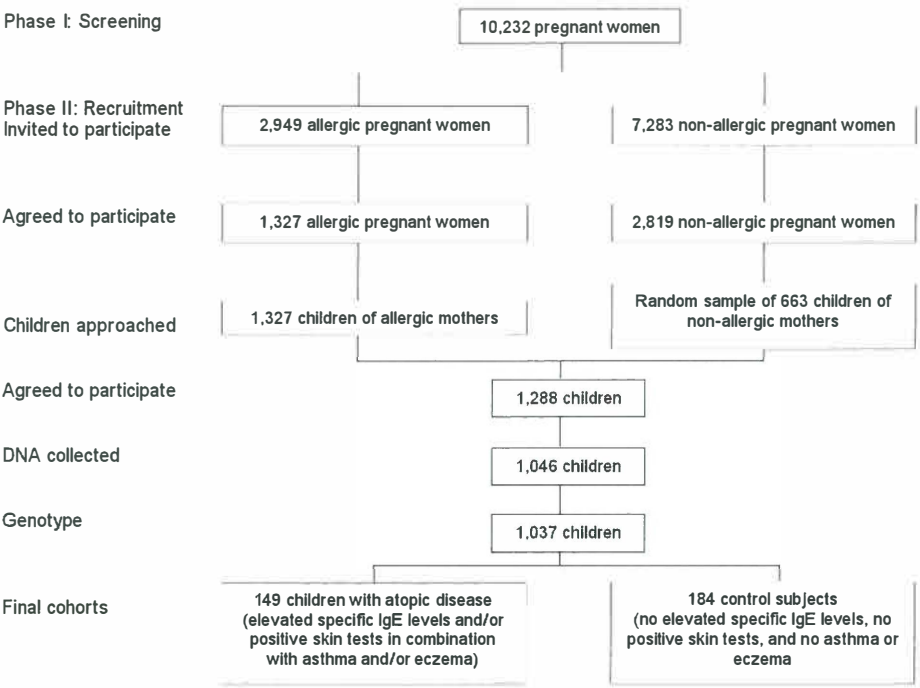

Table S1: The Prevention and Incidence of Asthma and Mite Allergy (PIAMA) recruitment scheme.

Table S2: 10,000 permutation analysis of the 29 SNPs.

SNP	*P* value	Permutation *p* value	Corrected permutation *p* value
rs3804099	0.65	0.34	0.99
rs3804100	0.26	0.12	0.91
rs4696480	0.04	0.031	0.48
rs4986790	0.49	0.48	1
rs6478317	0.49	0.24	0.99
rs10759932	0.20	0.083	0.87
rs11536878	0.88	0.65	1
rs11536889	0.28	0.97	1
rs1927911	0.67	0.37	0.99
rs10759931	0.76	0.54	1
rs2770150	0.38	0.19	0.97
rs1039559	0.42	0.20	0.98
rs5743788	0.59	0.39	0.99
rs5743810	0.66	0.41	0.99
rs6531666	0.00040	0.00020	0.013
rs5743798	0.000097	0.0001	0.00040
rs352140	0.57	0.40	1
rs187084	0.98	0.86	1
rs5743836	0.41	0.37	0.99
rs4274855	0.09	0.13	0.90
rs10856839	0.51	0.64	1
rs11096956	0.27	0.23	0.99
rs11096957	0.53	0.36	0.99
rs11466652	0.27	0.91	1
rs4129009	0.12	0.11	0.87
rs2563298	0.22	0.32	0.99
rs2569190	0.12	0.59	1
rs2569191	0.07	0.83	1
rs5744455	0.19	0.31	0.99

The observed p value for a differential genotype frequencies in patients with atopy compared to ALL patients and the corresponding permutation SNP-wise *p* values and *p* values that are corrected for multiple testing.

Table S3: Comparison of genotype and allele frequencies of SNPs in *TLR2*, *TLR4*, *TLR6*, *TLR9*, *TLR10*, and *CD14* among children with ALL in the age 0-8 years, healthy control subjects, and children with atopic disease

Gene	SNP	Genotype	ALL	Control	Atopic disease	*P* value	FDR
			N (%)	*N* (%)	*N* (%)		
TLR2	rs3804099	TT	41 (27)	48 (27)	35 (24)		
		TC	75 (50)	102 (57)	77 (53)		
		CC	34 (23)	28 (16)	34 (23)	0.390	NS
		C allele	48%	44%	50%	0.395	NS
	rs3804100	TT	143 (92)	165 (90)	120 (84)		
		TC	12 (8)	18 (10)	22 (15)		
		CC	1 (1)	0 (0)	1 (1)	0.189	NS
		C allele	5%	5%	8%	0.082	NS
	rs4696480	TT	36 (24)	60 (33)	52 (36)		
		TA	80 (52)	83 (46)	65 (45)		
		AA	36 (24)	38 (21)	29 (20)	0.226	NS
		A allele	50%	44%	42%	0.124	NS
TLR4	rs4986790	AA	137 (88)	151 (85)	126 (88)		
		AG	18 (12)	28 (16)	17 (12)		
		GG	0 (0)	0 (0)	1 (1)	0.432	NS
		G allele	6%	8%	7%	0.580	NS
	rs6478317	AA	72 (47)	85 (47)	73 (50)		
		AG	64 (41)	74 (41)	59 (41)		
		GG	19 (12)	22 (12)	13 (9)	0.872	NS
		G allele	33%	33%	29%	0.575	NS
	rs10759932	TT	117 (76)	144 (80)	120 (83)		
		TC	38 (25)	33 (18)	25 (17)		
		CC	0 (0)	3 (2)	0 (0)	0.095	NS
		C allele	12%	11%	9%	0.348	NS
	rs11536878	CC	124 (80)	139 (78)	114 (77)		
		CA	27 (17)	39 (22)	29 (20)		
		AA	5 (3)	1 (1)	5 (3)	0.329	NS
		A allele	12%	11%	13%	0.787	NS
	rs11536889	GG	112 (74)	129 (72)	104 (70)		
		GC	35 (23)	49 (27)	42 (28)		
		CC	5 (3)	1 (1)	2 (1)	0.302	NS
		C allele	15%	14%	16%	0.841	NS
	rs1927911	CC	92 (59)	109 (61)	93 (63)		
		CT	52 (33)	57 (32)	46 (31)		
		TT	12 (8)	14 (8)	9 (6)	0.950	NS
		T allele	24%	24%	22%	0.711	NS
	rs10759931	GG	58 (38)	62 (35)	48 (34)		
		GA	70 (46)	76 (43)	71 (51)		
		AA	23 (15)	38 (22)	21 (15)	0.412	NS
		A allele	38%	43%	40%	0.107	NS
	rs2770150	TT	81 (54)	92 (53)	67 (46)		
		TC	56 (37)	73 (42)	63 (43)		
		CC	13 (9)	10 (6)	15 (10)	0.417	NS
		C allele	27%	27%	32%	0.266	NS
TLR6	rs1039559	TT	36 (24)	53 (30)	44 (31)		
		TC	77 (52)	85 (48)	69 (49)		
		CC	36 (24)	38 (22)	29 (20)	0.706	NS
		C allele	50%	46%	45%	0.391	NS
	rs5743788	CC	34 (23)	49 (27)	43 (30)		
		CG	80 (53)	96 (53)	69 (47)		
		GG	36 (24)	36 (20)	34 (23)	0.599	NS
		G allele	51%	46%	47%	0.508	NS
	rs5743810	CC	51 (35)	61 (34)	54 (38)		
		CT	71 (48)	94 (53)	66 (46)		
		TT	26 (18)	24 (13)	23 (16)	0.734	NS
		T allele	42%	40%	39%	0.821	NS
	rs6531666	TT	82 (54)	72 (49)	49 (41)		
		TC	67 (44)	66 (45)	54 (45)		
		CC	4 (3)	9 (6)	15 (13)	**0.008**	NS
		C allele	25%	29%	33%	**0.018**	NS

Table S3 (cont.): Comparison of genotype and allele frequencies of SNPs in *TLR2*, *TLR4*, *TLR6*, *TLR9*, *TLR10*, and *CD14* among children with ALL in the age 0-8 years, healthy control subjects, and children with atopic disease

Gene	SNP	Genotype	ALL	Control	Atopic disease	*P* value	FDR
			N (%)	*N* (%)	*N* (%)		
	rs5743798	CC	95 (63)	100 (56)	66 (46)		
		CT	54 (36)	68 (38)	59 (41)		
		TT	3 (2)	10 (6)	19 (13)	**0.0009**	**Significant**
		T allele	20%	25%	34%	**0.0005**	**Significant**
TLR9	rs352140	TT	57 (38)	57 (32)	45 (31)		
		TC	69 (46)	88 (49)	74 (51)		
		CC	25 (17)	36 (20)	26 (18)	0.690	NS
		C allele	39%	44%	43%	0.423	NS
	rs187084	TT	46 (31)	50 (28)	44 (30)		
		TC	73 (49)	98 (55)	71 (49)		
		CC	31 (21)	31 (17)	30 (21)	0.796	NS
		C allele	45%	45%	45%	0.992	NS
	rs5743836	TT	109 (71)	138 (76)	107 (75)		
		TC	39 (26)	40 (22)	34 (24)		
		CC	5 (3)	4 (2)	2 (1)	0.768	NS
		C allele	16%	13%	13%	0.439	NS
TLR10	rs4274855	GG	100 (64)	112 (62)	101 (69)		
		GA	46 (30)	56 (31)	42 (29)		
		AA	10 (6)	12 (7)	3 (2)	0.313	NS
		A allele	21%	22%	16%	0.160	NS
	rs10856839	AA	111 (72)	124 (69)	97 (69)		
		AC	41 (27)	47 (26)	39 (28)		
		CC	3 (2)	8 (5)	5 (4)	0.779	NS
		C allele	15%	18%	17%	0.662	NS
	rs11096956	GG	89 (58)	98 (56)	86 (63)		
		GT	55 (36)	65 (37)	47 (34)		
		TT	10 (7)	12 (7)	4 (3)	0.512	NS
		T allele	24%	25%	20%	0.267	NS
	rs11096957	AA	54 (36)	57 (33)	55 (39)		
		AC	76 (50)	88 (51)	72 (51)		
		CC	21 (14)	29 (17)	15 (11)	0.578	NS
		C allele	39%	42%	36%	0.302	NS
	rs11466652	AA	115 (75)	130 (73)	111 (76)		
		AG	36 (24)	45 (25)	31 (21)		
		GG	2 (1)	4 (2)	5 (3)	0.714	NS
		G allele	13%	15%	14%	0.814	NS
	rs4129009	AA	101 (65)	112 (62)	104 (71)		
		AG	44 (28)	57 (32)	39 (27)		
		GG	10 (7)	11 (6)	3 (2)	0.251	NS
		G allele	21%	22%	15%	0.094	NS
CD14	rs2563298	CC	80 (53)	85 (47)	86 (59)		
		CA	69 (45)	85 (47)	55 (37)		
		AA	3 (2)	11 (6)	6 (4)	0.121	NS
		A allele	25%	30%	23%	0.120	NS
	rs2569190	GG	46 (31)	53 (29)	36 (25)		
		GA	67 (45)	101 (56)	82 (56)		
		AA	36 (24)	26 (14)	29 (20)	0.107	NS
		A allele	47%	43%	48%	0.369	NS
	rs2569191	TT	46 (30)	53 (29)	36 (25)		
		TC	68 (44)	101 (56)	82 (56)		
		CC	40 (26)	28 (15)	28 (15)	0.080	NS
		C allele	48%	43%	47%	0.401	NS
	rs5744455	CC	87 (57)	96 (53)	72 (49)		
		CT	57 (37)	77 (43)	66 (45)		
		TT	10 (7)	8 (4)	9 (6)	0.601	NS
		T allele	25%	26%	29%	0.607	NS

NB: SNP – single nucleotide polymorphism; ALL – acute lymphoblastic leukemia; FDR – false discovery rate; NS – not significant.

Chapter 10

Summary, general discussion, and future perspectives

Acute lymphoblastic leukaemia (ALL) is the most common form of cancer in children. To cure ALL in children, and cancer in general, treatment protocols are being revised regularly according to the latest insights to increase survival, and to minimize relapse and unwanted side effects. Due to these revisions the outcome of children with ALL improved substantially over the years.[1-5] Nevertheless, the side effects of these intensive cancer treatment protocols sometimes can be life-threatening.

In this thesis several clinical and genetic aspects of infectious side effects of cancer treatment in children were addressed.

Part 1: Clinical aspects

Chemotherapy induced neutropenia is a common side effect of cancer treatment. These neutropenic cancer patients have an increased risk for serious life threatening infections. Fever during neutropenia can be the first sign of a serious life threatening infections. However, patients with febrile neutropenia are a heterogeneous group with respect to the causes of fever and thus the risk for invasive bacterial infections and/or the occurrence of life-threatening complications. This led to research groups studying risk parameters to distinguish between patients at high risk and patients at low risk for invasive bacterial infections and/or complications. High risk patients should get the regular hospitalized treatment with parental antibiotics. In low risk patients shorter intravenous antibiotic treatment, oral antibiotics, or even no antibiotics at all might not be inferior to regular treatment. Moreover, low risk patients might be safely discharged earlier than high risk patients. **Chapter 2** reviews the literature on parameters in risk assessment for early discharge in fever during neutropenia in paediatric cancer patients. The historical overview showed the changing approach to fever during neutropenia in children with a low risk for invasive bacterial infection; over the years there is a tendency to earlier discharge and to use oral antibiotic treatment or no antibiotics at all.[6-15] There have been many attempts to find clear and objective parameters for risk assessment strategies to identify patients at high or low risk for invasive bacterial infections. Distinguishing between patients at high and low risk for invasive bacterial infections or adverse events is crucial when we want to reduce use of (intravenous) antibiotics. The relevance of restrictive antibiotic use lies in that misuse of broad spectrum antibiotics can cause opportunistic infections and resistant microorganisms.[16,17] This is of importance for the individual patient as well as the local environment. Moreover, risk stratification can result in, lower health care costs (e.g a reduction of the occupancy of hospital beds), and an increased quality of life for patients and their families.[10,14] Some parameters and risk assessment strategies have shown to be valuable in trials.[10,14,18] However, whether they are replicable in standard patient groups needs to be studied. Potential problems with this replication might be due to differences in treatment protocols, genetic background of the

patient group and environmental factors.[19] This is why large, preferably, randomized controlled trials are needed, like the multicenter study with interleukin-8 as the decisive parameter for withholding antibiotic treatment and early discharge in paediatric cancer patients with febrile neutropenia currently being performed by our research group.

The tendency towards earlier discharge from in-hospital treatment of patients at low risk for invasive bacterial infections triggered us to execute a literature search to determine whether (very) early discharge was as safe as non-early discharge (in other words in-hospital treatment) in this patient group. Moreover, we wanted to study whether very early discharge was as safe as early discharge. The Cochrane review that addressed this question is shown in **Chapter 3**. As there are multiple papers[10,11,15] addressing early discharge in paediatric cancer patients with low risk febrile neutropenia, we were surprised to find only one randomized controlled trial that compared early discharge to non-early discharge in paediatric cancer patients with fever during neutropenia and a low risk for invasive bacterial infection. No cohort controlled trials were found. In the only randomized controlled trial, performed by Santolaya et al, treatment results in the early discharge group were not inferior to non-early discharge.[14] The paper describing a trial performed by Ammann et al is in preparation. The risk prediction model for high risk for invasive bacterial infection used by Santolaya et al consists of the following clear and objective parameters; serum level of C-reactive protein (CRP) ≥90 mg/L, hypotension, relapse of leukemia as cancer type, platelet count of ≤50,000 platelets/mm^3, and recent (<8 days since) chemotherapy. Children were considered to be at low risk when they had none of the risk factors or either a platelet count of ≤50,000 platelets/mm^3 or recent chemotherapy as a sole risk factor.[18] This prediction model appeared to work well in the studied population.[14] However, as described above, this risk prediction model needs to be validated prospectively in other populations before broad clinical application. In these future trial settings close monitoring of patients discharged early by well trained health care professionals needs to be guaranteed. Moreover, it would also be valuable to have more information on quality of life, costs and duration of treatment. In a few years time there will probably be more evidence on whether (very) early discharge is as safe as non-early discharge in paediatric cancer patients with fever during neutropenia and a low risk for invasive bacterial infection. This is important as early discharge reduces health care costs, reduces hospital-acquired infections and might improve quality of life for patients and their family.

Specific treatment elements can increase the risk for severe infectious complications, such as prolonged use of corticosteroids. Since the start of the ninth treatment protocol for ALL treatment of the Dutch Childhood Oncology Group (DCOG-ALL-9), an increase in lethal infections in children with ALL during the maintenance treatment was noticed. The main difference in maintenance treatment between DCOG-ALL-9[5] and the DCOG-ALL-7[2] and DCOG-ALL-8[20] protocols is the interruption of methotrexate and 6-mercaptopurine

by vincristine (2 mg/m^2 weekly) and dexamethasone (6 mg/m^2 daily) for 14 days every 7 weeks in the DCOG-ALL-9 protocol. In **Chapter 4** we studied 1107 children included in and treated according to the DCOG-ALL-7, DCOG-ALL-8 or DCOG-ALL-9 protocol with respect to infectious death during maintenance treatment (July 1988–July 2002). Seven of the 510 children died of severe infections during the maintenance phase of DCOG-ALL-9, compared to none of the 597 patients during the DCOG-ALL-7 and DCOG-ALL-8 protocols (1.37% versus 0.0%; p = 0.013). Results from our research suggest that repeated, prolonged exposure to dexamethasone results in an increase of lethal infections. In the dosing-schedule used, the disadvantage of dexamethasone may outweigh the therapeutic effects of dexamethasone. Especially in the ALL non-high risk group it is questionable whether dexamethasone should be administered in this dosing schedule, as the risk for death by recurrent disease might be less than the risk for lethal infections and other unwanted side effects. A factor possibly contributing to lethal infections was that the usual surge of granulocytes during the dexamethasone block was not seen in patients who died of infections. Also, hypogammaglobulinaemia is found in patients after chemotherapy[21]; this was found frequently, in the patients included in the DCOG ALL-9 protocol and probably contributed to infectious complications. That is why the advice was given to check IgG concentrations and to supplement when applicable. Whether or not hypogammaglobulinaemie was also found in patients of the DCOG ALL-7 and 8 protocols was not analysed in these studies. As hypocortisolism could also be a cause of the fast deterioration of these patients, doctors were advised to give stress doses of hydrocortisone when patients presented with fever.[5] Dexamethasone can cause inhibition of the hypothalamic-pituitary-adrenal axis and a strong anti-inflammatory effect, which results in a diminished production of cortisol and possibly a slow inflammatory response when patients have an infection. This combination can lead to a relatively late presentation and an inadequate stress reaction of the body resulting in a fast deterioration and sometimes death. One can imagine that the relatively long dexamethasone pulses used in the DCOG ALL-9 maintenance treatment increase the risk of a fulminant course. It is difficult to state whether or not dexamethasone should be used in the maintenance treatment of ALL. Recently a large meta-analysis on the addition of vincristine plus steroid pulses in maintenance treatment of ALL was published. Several trials were included of which only one was randomized for dexamethasone or prednisone pulses, both in combination with vincristine pulses. The other trials randomized patients for treatment with or without either dexamethasone or prednisone in combination with vincristine. This analysis did not show benefit of dexamethasone over prednisone on event free survival and total survival.[1] However earlier randomized controlled trials showed significantly higher event free survival in ALL patients treated with dexamethasone compared to patients treated with prednisone.[22,23] Based on the three mentioned studies it is difficult to decide whether or not dexamethasone pulses in maintenance treatment should be given. The benefit of dexamethasone pulses depend on the individual risk for relapse, but also on the

pretreatment; when pretreatment is intensive and/or the relapse risk is small, treatment with dexamethasone might be unnecessary.[1] In the current DCOG ALL-10 different treatment-schedules are used and only medium risk patients receive five days of dexamethasone (6 mg/m2 daily) and one vincristine (2 mg/m2 weekly) pulse every 3 weeks. This results in shorter periods of dexamethasone use and shorter periods without dexamethasone in between. Standard risk patients are not receiving dexamethasone and thus are not exposed to possible adverse effects of dexamethasone during maintenance treatment. High risk patients receive a very intensive treatment and are not comparable. Results including cure rate, relapse and adverse effects such as infectious deaths during DCOG-ALL-10 will follow.

The previously described and other life-threatening events in paediatric cancer patients lead to paediatric intensive care unit (PICU) admissions. In the past mortality rates of paediatric oncology patients admitted to the PICU have been reported to be more than 84% in patients needing respiratory and circulatory support.[24,25] More recent studies of paediatric oncology patients admitted to the PICU have shown improved mortality rates up to 25-34%.[26,27] In **Chapter 5** we showed that PICU survival in non-elective PICU admissions in oncology patients was as high as 87%. We identified mechanical ventilation, inotropic support and transfusion of blood products as independent variables associated with mortality. One should realize that these variables are treatment-epiphenomena of the sickest PICU patients and as such are not predictors of mortality. We also evaluated predictive mortality risk scores (Paediatric Index of Mortality and Paediatric Risk of Mortality (PIM and PRISM)) in this PICU population. These scores help to predict mortality in cohorts of patients. We saw that PRISM was a better predictor of mortality than PIM. Our results underscore that admission to the PICU for a child with cancer should not be denied based solely on the diagnosis or severity of illness, as most children are expected to survive until PICU discharge. Like cancer treatment, intensive care treatment is improving. It will be interesting to see survival-rates in the coming years.

Chemotherapy-induced neutropenia is a major dose limiting side effect of intensive chemotherapy in paediatric cancer patients. To reduce the depth and/or length of the neutropenia granulocyte colony stimulating factors can be administered. Pegfilgrastim (a once-per-cycle dosage) granulocyte colony stimulating factor is available for adults, but not registered for children. In **Chapter 6** we reported 32 episodes of pegfilgrastim use to diminish chemotherapy-induced neutropenia in seven paediatric cancer patients with various kinds of cancer. We found that neutropenia alone, or neutropenia in combination with fever and thrombocytopenia was responsible for treatment delay in two cases (6%). Short-term adverse effects, like musculo-skeletal pain, were not recorded. In our patient group pegfilgrastim appears to be feasible and safe. In the time since our study, two small randomized trials were published which concluded that pegfilgrastim is comparable to

filgrastim.[28,29] In our opinion, large randomized controlled trials comparing paediatric cancer patients treated with filgrastim to patients treated with pegfilgrastim are needed to accurately assess differences in frequency of neutropenia and febrile neutropenia, adherence to treatment protocol, adverse effects, costs and quality of life.

Part 2: Genetic aspects

In the second part of this thesis we studied genetic aspects: gene polymorphisms of the innate immunity and the risk of developing neutropenia, fever during neutropenia, and childhood ALL.

As stated before, the susceptibility to infections increases when the granulocyte counts decrease.[30] It is remarkable that patients vary considerably in the number of (febrile) neutropenic episodes, despite receiving identical treatment[31, chapter 7 and chapter 8]. We hypothesized that single nucleotide polymorphisms (SNPs) of innate immunity genes play a role in this observed difference. Toll-Like Receptor 4 (TLR4) has been shown to play a role in increased cell survival of neutrophils.[32] In **Chapter 7** we studied the association of eight SNPs in the *TLR4* gene with the risk of developing chemotherapy-induced neutropenia in 194 children with acute lymphoblastic leukaemia. This resulted in four SNPs (rs10759931, rs11536889, rs1927911 and rs6478317) that were associated with an increased risk of developing chemotherapy-induced neutropenia. The *TLR4* SNPs explained 8.1 % of the variation in the number of neutropenic episodes. Other factors associated with an increased risk for neutropenic episodes were age, ALL risk group and type of ALL. Other genetic factors might influence the frequency and duration of neutropenic episodes. To elucidate this other large(r) studies are needed. We found *TLR4* to be important in whether or not patients develop neutropenia, this might be due to the role TLR4 plays in neutrophil survival.[32]

In paediatric cancer patients the risk for fever during neutropenia is even more important than the risk for neutropenia. SNPs in genes involved in immunity defence might influence the risk for fever during neutropenia episodes. Mannose binding lectin (coded by *MBL2*) levels play a central role in innate immunity defence and low levels of MBL predispose to infectious illness.[33-36] We hypothesized that *MBL2* low-producing genotypes and haplotypes would increase the risk of fever during neutropenia in children with ALL. However, in **Chapter 8**, we demonstrated that *MBL2* low producing genotypes and haplotypes were not associated with fever during neutropenia in the maintenance treatment of children with ALL. This study confirms the results of previous studies in which no significant association between *MBL2* genotypes and febrile neutropenia was found.[37-39] The previous studies had a relatively small sample size, a short observation period or were done in children with various kinds of cancer. As our study consists of a relatively large, homogenous group of patients, which were studied for more than a year

and our results confirm the results of previous studies, it can be concluded that *MBL2* SNPs are not of clinical significance for developing fever during neutropenia in paediatric patients with ALL.

SNPs in innate immunity genes are associated with atopic disease.[40-44] The presence of atopic disease has been shown to have an inverse association with childhood ALL.[45-47] In **Chapter 9** we showed that two SNPs (rs5743798 and rs6531666) of the *TLR6* differed significantly between the group of children with ALL, the group of children with atopic disease and the control group. These risk alleles for atopic disease were seen less in children with ALL than in the control group. This inverse association supports the immune surveillance hypothesis, which can be an explanation of the inverse association of atopic disease and childhood ALL. Future studies in large cohorts are needed to confirm or contradict our results and look for other genetic determinants of the innate immunity associated with childhood ALL. In this way we may add to unravel the aetiology of ALL.

Future perspectives

Serious, and even life threatening, infectious complications are seen more often in the immunocompromised host, such as patients with neutropenia or prolonged use of corticosteroids. Fever can be the only symptom in the neutropenic cancer patient, as for other inflammatory phenomena like, pain, redness, swelling and pus, neutrophilic granulocytes are needed. Patients with febrile neutropenia are a heterogeneous group with respect to the causes of fever and it is known that we probably overtreat more than 50% of these patients. For this reason risk stratification for patients at high or at low risk for invasive infections or serious infectious complications is interesting and essential to accomplish adequate treatment for the individual patient with fever during neutropenia. Patients at low risk do not need the same intravenous antibiotic courses as high risk patients; for low risk patients oral antibiotics or even no antibiotics at all and early discharge from in-hospital treatment with good monitoring might be more adequate. This can result in lower health care costs, less resistant microorganisms and maybe most importantly an increased quality of life. To accomplish this the currently published risk assessment strategies, with items like relapse of leukemia as the cancer type, and interleukin-8 and CRP serum levels, need to be tested in preferably large randomized controlled trials in various countries as differences in environmental factors, treatment protocols and genetic background might influence the results. These risk assessment strategies thus need to prove their reliability before broad clinical application. To further improve the current risk assessment strategies more objective parameters need to be found and also prove their value in large, preferably international, randomized, controlled trials.

Chemotherapy induced neutropenia leads to postponing chemotherapy treatment, dosage reduction and an increased risk for infections. To reduce these negative consequences of neutropenia, granulocyte colony stimulating factors are given in certain groups of paediatric cancer patients. In these groups of patients large randomized controlled trials need to be executed to compare effectiveness and possible short and long term side effects of filgrastim and pegfilgrastim. If pegfilgrastim is not inferior to filgrastim it needs to be registered for children to reduce the number of injections and increase quality of life.

To establish our results of the association of the 4 *TLR4* SNPs with an increased risk of developing chemotherapy-induced neutropenia large studies are required. Moreover it would be interesting to know whether pediatric patients with ALL with the particular SNPs in the *TLR4* gene also experience more infections. If so, a randomized controlled trial with prophylactic antibiotic treatment would be the logical next step. This could result in a reduction of morbidity and mortality due to infections, and thus an increase in quality of life and a decrease in health care costs.

The future studies mentioned above could result in better supportive care, and also more intensive chemotherapy treatment. These innovative and important studies are only possible with close cooperation of research groups.

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Chapter 11

Nederlandse samenvatting voor de geïnteresseerde leek

Chemotherapie en neutropenie

De overleving van kinderen met kanker is in de afgelopen decennia verbeterd, mede dankzij het gebruik van chemotherapeutica. Deze hebben, naast het gewenste effect tegen de kankercellen, helaas ook ongewilde bijwerkingen. Een van die bijwerkingen is de remming van de aanmaak van witte bloedcellen in het beenmerg. De witte bloedcellen zijn onderdeel van het niet-specifieke of aangeboren immuunsysteem. Het aangeboren immuunsysteem is de eerste afweer van het lichaam tegen ziekteverwekkers. Het bestaat uit barrières zoals huid en slijmvliezen die ziekteverwekkers kunnen tegenhouden. Daarnaast zijn er cellen, zoals de genoemde witte bloedcellen, die onder meer signaalstoffen kunnen afgeven bij beschadiging of nadat ze ziekteverwekkers zoals bacteriën hebben gefagocyteerd (opgegeten). Deze signaalstoffen kunnen dan een afweerreactie in gang zetten. Verder zijn er eiwitten, zoals Mannose Binding Lectine (MBL), die patronen op de buitenkant van de ziekteverwekkers kunnen herkennen en deze ziekteverwekkers hierdoor kunnen vernietigen en afweerreacties in gang kunnen zetten.

Er zijn verschillende typen witte bloedcellen; de grootste groep zijn de neutrofiele granulocyten. Als het aantal neutrofiele granulocyten te diep zakt spreekt men van neutropenie. Een neutropenie geeft een verhoogd risico op infecties en infectieuze complicaties, met name bij kinderen die chemotherapie krijgen, een patiëntengroep die al een verhoogd risico op infecties heeft, doordat de barrières kunnen zijn aangetast door de chemotherapie. Koorts bij neutropenie kan het eerste teken van een infectie zijn. Daarom is het de normale gang van zaken dat kinderen met kanker die zich in het ziekenhuis presenteren met koorts bij neutropenie, worden opgenomen en behandeld met intraveneuze (iv.) antibiotica totdat het aantal neutrofiele granulocyten hoog genoeg is en hij of zij minstens 24 uur koortsvrij is.

Koorts bij neutropenie kan echter ook door andere oorzaken ontstaan, zoals door een virusinfectie of als reactie op medicijnen of transfusie van bloedproducten. Het is echter moeilijk in te schatten of een kind een potentieel ernstige infectie heeft of niet. Daarom krijgen alle kinderen behandeling met iv. antibiotica en wordt er dus hoogstwaarschijnlijk een groep kinderen overbehandeld. Dit heeft ertoe geleid dat verschillende onderzoeksgroepen op zoek zijn gegaan naar parameters die een risico-inschatting kunnen geven van patiënten die zich presenteren met koorts bij neutropenie. Doel hiervan is, een onderscheid te maken tussen patiënten met een hoog en een laag risico op het krijgen van ernstige infecties of infectieuze complicaties. De laagrisicogroep zou dan mogelijk korter, niet, of met orale antibiotica behandeld kunnen worden en korter of zelfs helemaal niet in het ziekenhuis opgenomen hoeven te worden.

DNA, polymorfismen en SNPs

Menselijk DNA (desoxyribonucleïnezuur) bevat al onze erfelijke informatie. Ons DNA is opgeslagen in onze cellen en heeft de vorm van een dubbele helix. De bouwstenen hiervan worden nucleotiden genoemd. Een nucleotide is opgebouwd uit een suiker-fosfaat molecuul en een base. En de verbinding van twee nucleotiden tussen de dubbele helix wordt een basenpaar genoemd. Van de vier basen bindt Guanine (G) altijd aan Cytosine (C) en bindt Adenine (A) altijd aan Thymine (T). Drie basenparen op een rij vormen een codon en coderen voor een nucleïnezuur. Deze nucleïnezuren vormen eiwitten en eiwitten zijn de basis van alle celfuncties waarmee lichaamsprocessen geregeld worden.

Genetische verschillen kunnen onder andere ontstaan door genpolymorfismen. Dit zijn vaak voorkomende (>1%) varianten in genetische informatie. De polymorfismen die in dit proefschrift worden geanalyseerd zijn single nucleotide polymorphisms (SNPs). Een SNP is een genetische variatie in de DNA-volgorde die zich voordoet als één nucleotide is veranderd. Als SNPs voorkomen in een gen kan dit de hoeveelheid eiwit, de structuur van het eiwit of de functie van het eiwit beïnvloeden.

Bij verschillende SNPs is een relatie met en verhoogde vatbaarheid voor bepaalde ziekten beschreven zoals atopie (verzamelnaam voor ziekten als astma, hooikoorts en constitutioneel eczeem), colitis ulcerosa (darmontsteking), meningokokkenziekte en ernstige malaria. Er zijn bijvoorbeeld meerdere SNPs betrokken bij de vatbaarheid, ernst en afloop van meningokokkenziekte. SNPs kunnen ook geassocieerd zijn met bijvoorbeeld de functionele levensduur van neutrofiele granulocyten en verminderde reactie op bacteriën. Verder kunnen SNPs geassocieerd zijn met acute lymfatische leukemie (ALL) bij kinderen. Er werd bijvoorbeeld een SNP gevonden die geassocieerd was met leukemie bij negroïden; deze bevinding draagt bij aan de verklaring van raciale verschillen in het vóórkomen van leukemie. In dit proefschrift worden enkele SNPs van belangrijke spelers in het aangeboren immuunsysteem onderzocht als mogelijke voorspellers voor neutropenie, koorts gedurende neutropenie en ALL bij kinderen.

Deel 1: klinische aspecten

In **Deel 1** van dit proefschrift werden studies over enkele klinische aspecten van infectieuze bijwerkingen van kankerbehandeling bij kinderen besproken. In **Hoofdstuk 2** bestudeerden we de literatuur over parameters voor risico-inschatting van ernstige bacteriële infecties of infectieuze complicaties bij kinderen met kanker en koorts gedurende neutropenie. Daarbij lieten we een historisch overzicht zien van de veranderde benadering van patiënten met koorts gedurende neutropenie en een laag risico op

ernstige bacteriële infecties of infectieuze complicaties in studieverband: het moment van risico-inschatting is steeds vroeger geworden en daarnaast wordt er vaker gekozen voor een poliklinische behandeling, zodat patiënten niet per se hoeven worden opgenomen in het ziekenhuis. Daarom wordt er niet alleen meer gekeken naar parameters als een "goede klinische conditie", laboratoriumwaarden en bloedkweken, maar ook naar zogenaamde logistieke parameters. Deze geven bijvoorbeeld aan, of ouders in geval van nood de patiënt tijdig naar het ziekenhuis kunnen brengen.

Slotsom van dit hoofdstuk was dat er grote gerandomiseerde en gecontroleerde studies nodig zijn om aanvullende parameters te vinden waarmee de risico-inschatting geoptimaliseerd en de laagrisicogroep vergroot kan worden. Patiënten in deze groep kunnen in de toekomst mogelijk veilig poliklinisch behandeld worden met korte kuren, met orale antibiotica of zelfs zonder antibiotica. Dit zou kunnen bijdragen aan een betere kwaliteit van leven van patiënten en hun familie en een reductie van de kosten van de gezondheidszorg.

Dit riep bij ons de vraag op, of er studies zijn die vervroegd ontslag vergelijken met niet vervroegd ontslag bij patiënten met koorts bij neutropenie, als de risico's laag worden ingeschat. **Hoofdstuk 3** beschreef een uitgebreide systematische zoektocht naar literatuur over dit onderwerp. Uiteindelijk werd er slechts één studie gevonden, die een vergelijking maakte tussen laagrisicopatiënten die wel vervroegd werden ontslagen en patiënten bij wie dat niet gebeurde. In deze studie werden geen aanwijzingen gevonden dat vervroegd ontslag onveiliger was. De duur van de behandeling was voor beide patiëntgroepen gelijk. Vervroegd ontslag maakte de behandeling wel goedkoper. Om deze resultaten te bevestigen of ontkrachten zijn grote gerandomiseerde en gecontroleerde studies nodig.

Hoofdstuk 4 gaat over de onderhoudsbehandeling van het negende protocol voor acute lymfatische leukemie van de Stichting Kinderoncologie Nederland (SKION). De onderhoudsbehandeling heeft als doel de laatste, mogelijk aanwezige, kankercellen te doden en is in principe het afsluitende deel van de behandeling tegen kanker. Dit deel duurt het langst en is het minst intensief. De protocollen van de SKION worden met enige regelmaat aangepast en na de start van het negende protocol (ALL-9) werd een toename van het aantal dodelijke infecties bij de behandelde kinderen opgemerkt in vergelijking met ALL-7 en -8. De kans om te overlijden aan de gevolgen van een infectie tijdens de onderhoudsbehandeling van ALL-9 bleek 1,37% te zijn, tegen 0,00% voor ALL-7 of -8.

De oorzaak hebben we gezocht in het feit dat chemotherapeutica naast het gewenste effect op kankercellen, ernstige bijwerkingen kunnen hebben. Het cruciale verschil tussen de onderhoudsbehandeling van het ALL-7 en -8 en het ALL-9 protocol is de langdurige en herhaalde behandeling met dexamethason in ALL-9. Dit in combinatie met de details van de zeven overleden patiënten leverde op dat hoogstwaarschijnlijk de herhaalde, langdurige behandeling met dexamethason het risico op een dodelijke infectie doet

stijgen. Bij dit doseringsschema is het de vraag of de voordelen van dexamethason zwaarder wegen dan het hogere risico op een infectieuze dood, zeker bij de groep kinderen met ALL in een minder agressieve vorm.

In **Hoofdstuk 5** bestudeerden we de overleving van kinderen met kanker bij een niet geplande opname op de Pediatrische Intensive Care Unit (PICU). We bekeken het overlevingspercentage, de parameters die samenhangen met overlijden en we evalueerden twee systemen waarmee het risico op overlijden gescoord wordt: Paediatric Index of Mortality (PIM) en Paediatric Risk of Mortality (PRISM).

De overlevingspercentages van kinderen met kanker op de PICU zijn in de loop der jaren sterk gestegen. In onze studie bleek het overlevingspercentage 87% te zijn. Beademing, bloeddrukondersteunende medicatie en transfusie van bloedproducten zijn factoren die samenhangen met de ernst van de ziekte en deze bleken logischerwijs samen te hangen met het overlijden tijdens de opname op de PICU. Ten slotte bleek dat PIM- en PRISM-scores goede voorspellers te zijn van de overlijdenskans van de patiëntengroep als geheel, maar niet van individuele patiënten. Zij zijn met name bruikbaar om PICU's onderling of dezelfde PICU in verschillende tijdsperioden te vergelijken.

Zelfs de ziekste patiënten hebben bemoedigende overlevingskansen en alle kinderoncologische patiënten zouden dus in voorkomende gevallen intensive care-behandeling moeten ontvangen.

Als gevolg van bepaalde chemotherapeutische behandelingen komen ernstige neutropenieën voor. Om deze te voorkomen of te bekorten kan medicatie gegeven worden die zorgt voor een versnelde rijping van neutrofiele granulocyten in het beenmerg en het vervroegd vrijkomen van neutrofiele granulocyten in de bloedbaan. Hierdoor wordt het infectiegevaar kleiner en kan beter worden vastgehouden aan het chemotherapieschema. Pegfilgrastim is een geneesmiddel dat slechts eenmaal per chemotherapie-cyclus hoeft te worden toegediend. Pegfilgrastim is geregistreerd als geneesmiddel voor volwassenen en niet voor kinderen.

In **Hoofdstuk 6** hebben we de toepasbaarheid en veiligheid van Pegfilgrastim bij kinderen geëvalueerd, door 32 episodes bij zeven kinderen te bekijken waarbij Pegfilgrastim gebruikt werd. Daartoe hebben we gekeken naar de gerapporteerde bijwerkingen en het vasthouden aan het behandelingsschema. In deze studie werden er slechts twee van de in totaal elf behandelingsvertragingen veroorzaakt door neutropenie (6%). Er werden geen kortetermijnbijwerkingen door patiënten gerapporteerd. In deze kleine patiëntengroep was Pegfilgrastim toepasbaar en veilig. Er zijn grote gerandomiseerde en gecontroleerde onderzoeken nodig om nauwkeuriger te bepalen of Pegfilgrastim toepasbaar en veilig is bij kinderen.

Deel 2: genetische aspecten

De klinische aspecten van infectieuze bijwerkingen van kankerbehandeling bij kinderen speelden een belangrijke rol in deel 1 van dit proefschrift. In **Deel 2** keken we naar de genetische aspecten van het aangeboren immuunsysteem in relatie tot infectieuze bijwerkingen van kankerbehandeling en het ontstaan van leukemie bij kinderen. We bestudeerden een mogelijke relatie tussen enkele genpolymorfismen van het aangeboren immuunsysteem en het risico op de ontwikkeling van neutropenie, koorts gedurende neutropenie en ALL bij kinderen.

In de inleidende alinea's van deze samenvatting is uitgelegd dat de vatbaarheid voor infecties toeneemt als het aantal neutrofiele granulocyten door de chemotherapeutische behandeling afneemt. Het verschilt echter sterk van patiënt tot patiënt hoeveel periodes van neutropenie optreden, en dat bij (vrijwel) identieke behandelingen. Van Toll-Like Receptor 4 (TLR4) is aangetoond dat het een belangrijke rol speelt in de remming van geprogrammeerde celdood van neutrofiele granulocyten.

In **Hoofdstuk 7** is daarom onderzoek gedaan naar de samenhang van SNPs in het *TLR4*-gen met het risico op het ontwikkelen van neutropenie bij kinderen met ALL. In deze studie vergeleken we het vóórkomen van acht SNPs in het *TLR4*-gen met de frequentie van de neutropene episodes gedurende de chemotherapeutische behandeling bij 194 kinderen met ALL. Vier SNPs in het *TLR4*-gen waren geassocieerd met een hogere frequentie van de perioden met neutropenie.

Verdere onderzoeken zijn nodig om te belichten of kinderen met ALL en de betreffende SNPs in het *TLR4*-gen ook meer infecties doormaken en of zij zouden profiteren van profylactische antibiotische behandeling. De gedachte hierachter is dat infecties en als gevolg daarvan ziekteperiodes en sterfgevallen zouden kunnen worden voorkomen.

Het ontwikkelen van koorts gedurende de periode met neutropenie bij kinderen met kanker kan het eerste teken van een infectie zijn. SNPs in genen die belangrijk zijn in het aangeboren immuunsysteem, spelen mogelijk een rol bij het ontwikkelen van koorts gedurende neutropene episoden. Een voorbeeld is het *MBL2*-gen, dat onder andere de spiegels van Mannose Binding Lectine in het bloed regelt. Eerdere onderzoeken hebben hiernaar gekeken in kleine en/of heterogene patiëntengroepen en/of gedurende een relatief korte behandelingsperiode. In **Hoofdstuk 8** is in een grote, homogene patiëntengroep, gedurende de gehele onderhoudsbehandeling, bekeken of *MBL2* laag-producerende genetische varianten geassocieerd waren met koorts gedurende neutropenie in de onderhoudsbehandeling bij kinderen met ALL. In tegenstelling tot wat wij dachten bleek dit niet het geval te zijn.

In **Hoofdstuk 9** werd gekeken naar een mogelijke verklaring voor het feit dat kinderen met atopie minder kans hebben op het ontwikkelen van ALL. Er is weinig bekend over de ontstaanswijze van ALL bij kinderen. Het hebben van een atopische ziekte zoals IgE-gemedieerd astma en/of IgE-gemedieerd eczeem blijkt te beschermen tegen het ontstaan van ALL bij kinderen. IgE-antilichamen kunnen geproduceerd worden tegen veel onschuldige, niet ziekmakende, lichaamsvreemde eiwitten, als reactie op bijvoorbeeld katten, honden, huisstofmijt en pollen.

Het doel van deze studie was om te onderzoeken of SNPs van het aangeboren immuunsysteem, die eerder geassocieerd zijn met atopische ziekte, de omgekeerde associatie van atopische ziekte met ALL op de kinderleeftijd kunnen verklaren. We bestudeerden hiervoor 192 kinderen met ALL, 149 kinderen met atopie en 184 gezonde kinderen als controlegroep.

De genotype distributies van twee SNPs in het *TLR6* gen verschilden significant tussen kinderen met ALL, kinderen met atopie en gezonde controles. In het bijzonder bij kinderen met allergisch eczeem werden risicovarianten van SNPs voor atopie vaker gezien dan in de controlegroep. Daarnaast werden deze SNPs minder vaak gezien bij kinderen met ALL dan in de controlegroep.

Deze bevindingen onderschrijven de immune surveillance-hypothese: deze veronderstelt dat de atopische ziekte zorgt voor toegenomen alertheid van het immuunsysteem in de monitoring, identificatie en eliminatie van kwaadaardige cellen. Om de rol van het aangeboren immuunsysteem bij de ontwikkeling van ALL bij kinderen in meer detail te onderzoeken is verder onderzoek noodzakelijk.

In **Hoofdstuk 10** werd een samenvatting gegeven van de hoofdstukken en een beschouwing van de resultaten.

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Curriculum vitae & publications

Curriculum vitae

Esther te Poele was born on 30 May 1975 in Lievelde, the Netherlands. She attended Antonius School in Lievelde and Scholengemeenschap Marianum in Groenlo. In 1993 she started studying medicine at Nijmegen University. Parts of her study were internships in Fukushima, Japan, and Berekum, Ghana. In 2000 she graduated and briefly worked on the emergency ward of the Carolus Liduina Ziekenuis (now part of the Jeroen Bosch Ziekenhuis) in 's Hertogenbosch. After that, she worked for one year as a resident in Paediatrics in Het Spittaal (part of Gelre Ziekenhuizen) in Zutphen and from October 2001 until April 2003 in the Canisius Wilhelmina Ziekenhuis in Nijmegen. In 2003 she started working as AGIKO (combined medical training and PhD study) at the University Medical Centre Groningen (successive heads Prof. dr. P.J.J. Sauer and mr. dr. A.A.E. Verhagen). Her research was supervised by Prof. dr. W.A. Kamps and Prof. dr. E.S.J.M. de Bont. From October 2007 until April 2009 she worked at the Medisch Spectrum Twente in Enschede (head dr. B. Thio). In July 2011 she finished her training in Paediatrics and since August 2011 she has been working as chef de clinique at the Isala Klinieken in Zwolle. Esther has been in a relationship since 1996 with Bart de Goeij. They have a daughter (Laïla, 2009) and a son (Imre, 2011).

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