Efficacy and toxicity of asparaginases during prospective drug monitoring in patients with childhood acute lymphoblastic leukemia

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Efficacy and Toxicity of Asparaginases During Prospective Drug Monitoring in Patients With Childhood Acute Lymphoblastic Leukemia

Werkzaamheid en veiligheid van asparaginases bij kinderen met acute lymfatische leukemie

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CONTENTS

Chapter 1

General introduction

GENERAL INTRODUCTION

Childhood acute lymphoblastic leukemia

Acute lymphoblastic leukemia (ALL) is the most common type of childhood cancer.[1] ALL is a heterogeneous disease in which many genetic lesions result in the development of multiple biologic subtypes. The etiology of ALL can best be described by the acquisition of multiple consecutive genetic alterations in the (pre)leukemic cells. B-lineage ALL (approximately 85% of the cases) is more frequent than T-lineage ALL (approximately 15% of the cases).[2]

The peak incidence of ALL is observed in children aged 2 to 6 years.[3] Annually, 120 new cases of childhood ALL are diagnosed in the Netherlands. These children are treated with combination chemotherapy, existing of induction, consolidation, intensification and continuation phases. With significant refinements in the treatment of childhood ALL, the survival has increased dramatically from 0-5% in the 1960's to 80-85% nowadays.[3]

Asparaginase

Asparaginase is a non-human enzyme and this drug is an essential component of remission induction and intensification treatment in childhood ALL.[4] Currently, several asparaginase preparations are available on the market. These are derived from *Escherichia coli* in its native form (native *E.coli* asparaginase) or as a pegylated enzyme from *Escherichia coli* (PEGasparaginase) or extracted from *Erwinia chrysanthemi* (*Erwinia* asparaginase). Asparaginase mediates the breakdown of asparagine into aspartic acid and ammonia (Figure 1). Given that leukemic blasts depend heavily on asparagine, deprived of this amino acid, they undergo apoptosis.[5] Also, asparaginase has glutaminase activity,[6] which differs between preparations. *Erwinia* asparaginase has got a higher glutaminase activity (10%) as compared to PEGasparaginase (2%).[7] Glutamine depletion has been suggested to enhance asparaginase efficacy and might augment asparagine depletion.[8,9]

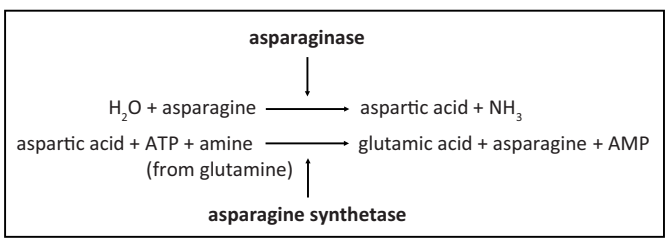

Figure 1: Asparagine breakdown mediated by asparaginase and asparagine synthesis. H_20, water; NH_3, ammonia; ATP, adenosine triphosphate; AMP, adenosine monophosphate.

All types of asparaginase have the same mode of action. However, the dose schedules for native *E.coli* asparaginase, PEGasparaginase and *Erwinia* asparaginase differ based upon pharmacokinetics. The dose and the dose schedule are, therefore, not interchangable.[10,11]

Asparaginase is used to achieve asparagine depletion, but no minimum value of asparaginase activity level for efficacy has yet been established.[12-14] A serum asparaginase activity level of ≥100 U/L is associated with complete asparagine depletion and is often considered as the target trough asparaginase activity level.[15]

Efficacy of asparaginase

Many studies have shown that intensification of asparaginase is essential to improve event-free survival (EFS) of children with ALL.[16-20] Table 1 depicts the benefit of intensive asparaginase treatment compared with less intensive regimens.

Table 1: Benefit of intensive asparaginase treatment compared with less intensive regimens.

	EFS with less intensive ASP	EFS with more intensive ASP	Difference	Reference
Erwinase versus *E.coli* ASP* (EORTC-CLG 58881)	60%	73%	**p<0.001**	Duval[16]
Erwinase versus *E.coli* ASP* (DFCI 95-01)	78%	89%	**p<0.05**	Moghrabi[17]
20 extra weeks of ASP in T-ALL (POG 8704)	55%	68%	**p<0.01**	Amylon[18]
20 extra weeks of ASP in T-NHL (POG 8704)	64%	78%	**p<0.05**	Amylon[18]
Shorter or longer than 25 weeks of ASP (DFCI 91-01)	73%	90%	**p<0.01**	Silverman[19]
20 extra weeks of ASP (iBFM/ IDH-ALL-91)	89%	94%	**p=0.05**	Pession[20]

* Same dose, same frequency.

The Dana-Farber Cancer Institute (DFCI) ALL Consortium showed that children who tolerated more than 25 weeks of asparaginase had a better EFS than those who received 25 weeks or fewer.[19] Improvement in EFS was also demonstrated in a randomized study with or without prolonged use of "high-dose" native *E.coli* asparaginase (25,000 IU/m^2 weekly for 20 weeks). Patients with standard risk ALL who received 20 weeks of native *E.coli* asparaginase had an increased 10-year EFS (94% *versus* 89%) compared with patients who were not treated with asparaginase.[20] This finding supported previous data from Amylon *et al.*(18) showing that additional "high-dose" native *E.coli* asparaginase during consolidation for 20 weeks significantly improved continuous complete remission rate in childhood T-cell ALL and lymphoblastic lymphoma compared to the patients without asparaginase therapy during consolidation. Finally, the randomized studies carried out by Duval *et al.*[16] and Moghrabi *et al.*[17] have shown that asparaginase preparations

with a shorter half-life result in a poorer EFS compared with the use of a asparaginase drug with a longer half-life given at the same dose and frequency.

Asparaginase therapy in the DCOG ALL-10 protocol

From November 2004 to April 2012, children with ALL aged 1-19 years were treated according to the Dutch Childhood Oncology Group (DCOG) ALL-10 protocol (see attachment). Patients were stratified into three risk groups: standard risk (SR), medium risk (MR) and high risk (HR) based on t(4;11) translocation, initial central nervous system and testis involvement, prednisone response at day 8, remission status at day 33 and minimal residual disease level at days 33 and 79. Infants and patients with t(9;22) translocation were excluded from the ALL-10 protocol. Twenty-five percent of all the patients were stratified as SR. Of the remaining patients, 64% were defined as MR and 11% as HR.

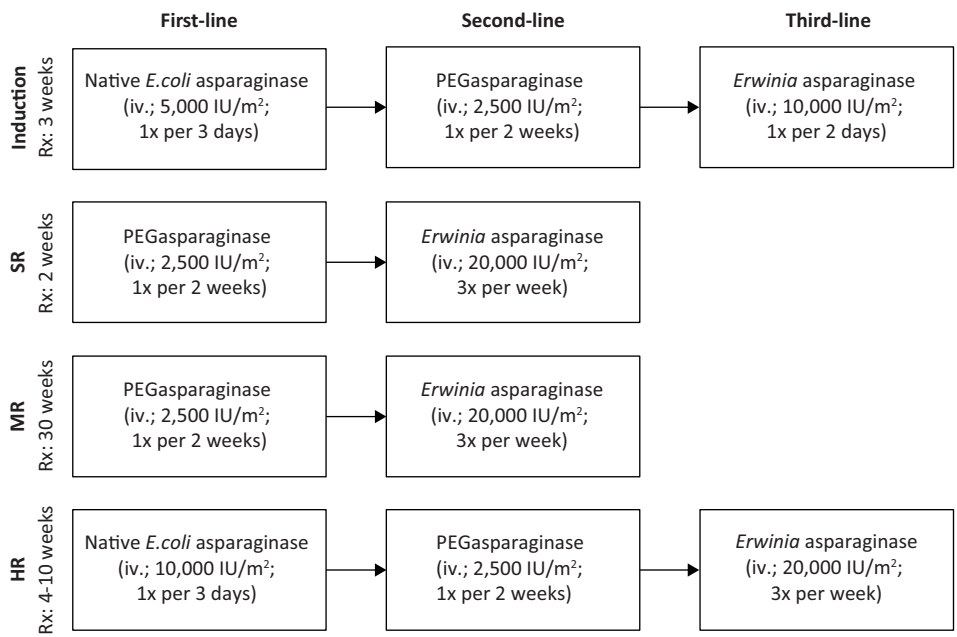

Figure 2: Overview of asparaginase doses in the DCOG ALL-10 protocol. SR; standard risk, MR; medium risk, HR; high risk, Rx; duration of asparaginase therapy.

Asparaginase was used in the induction and in the intensification phases. The induction (phase 1-A) which was based on the Berlin-Frankfurt-Munster (BFM) backbone included eight doses native *E.coli* asparaginase (5,000 IU/m^2 per dose) every three days. In case of an allergy to native *E.coli* asparaginase, PEGasparaginase (2,500 IU/m^2) was administered. If a subsequent allergy to PEGasparaginase occurred, the patients were switched to *Erwinia* asparaginase (10,000 IU/m^2 per dose).

After the asparaginase break during consolidation phases 1-B and high-dose methotrexate courses, asparaginase infusions were reintroduced. Children who are stratified as standard risk received one dose of PEGasparaginase (2,500 IU/m^2). In case of an allergy to PEGasparaginase, 6 doses of *Erwinia* asparaginase (20,000 IU/m^2) were administered. Medium risk patients receive PEGasparaginase (2,500 IU/m^2 per dose) every two weeks during the first 30 weeks of the intensification/continuation. This intensification phase was based on the intensified course of the DFCI 91-01 schedule.[19] In case of an allergy to PEGasparaginase, the patient was switched to *Erwinia* asparaginase (20,000 IU/m^2 per dose) 2-3 times per week to complete 30 weeks of therapy. In contrast, high risk patients received native *E.coli* asparaginase (10,000 IU/m^2 per dose) every three days. PEGasparaginase (2,500 IU/m^2) was used as second-line treatment in case of an allergy to native *E.coli* asparaginase. As third-line, the patients were switched to *Erwinia* asparaginase (20,000 IU/m^2 per dose) (Figure 2).

This thesis focused on the efficacy and toxicity of very prolonged use of PEGasparaginase and *Erwinia* asparaginase in children with newly diagnosed ALL. Therefore, we studied the patients who were stratified as medium risk.

Resistance to asparaginase

Asparaginase has shown to be an effective drug against acute lymphoblastic leukemia, however some patients show diminished effect of this drug (asparaginase resistance). Two putative mechanisms, which can lead to resistance to asparaginase are studied. The first mechanism is rescue of the leukemia cells via asparagine production by mesenchymal cells (MSCs) in the bone marrow. The second mechanism is the production of asparaginase antibodies which may cause rapid inactivation of asparaginase, resulting in suboptimal asparagine depletion.

Mesenchymal cells

A suggested mechanism of asparaginase resistance is the production of excess asparagine by MSCs in the bone marrow, rather than by leukemic cells, which may protect the leukemic cells of asparagine depletion mediated by asparaginase.[21] Iwamoto *et al.* showed that in contrast to leukemic cells, MSCs had a high activity of asparagine synthetase. *In vitro*, this led to high levels of asparagine, and therefore resistance of ALL cells to asparaginase.[22] However, it is unknown if this holds true for the clinical *in vivo* situation.

Asparaginase antibodies and silent inactivation

Asparagine depletion may be inhibited by asparaginase antibodies.[23-25] These antibodies may induce a faster clearance rate of asparaginase and may be associated with low asparaginase activity levels.[26] In a randomized study, patients treated with native *E.coli* asparaginase developed 13 times more antibodies compared to PEGasparaginase (26% *versus* 2% of the patients after induction therapy).[8] Asparaginase antibodies can occur with or without signs of clinical allergy,[24] the latter is called silent inactivation.

Detection of silent inactivation is important to prevent useless continuation of an inactive asparaginase product which leads to a worse event-free survival.[25,27] Monitoring of asparaginase activity levels is the only way to detect silent inactivation. Only a few studies reported the incidence of silent inactivation of asparaginase. Panosyan *et al.* showed that 29% of the patients had silent inactivation of native *E.coli* asparaginase in re-induction.[25] Vrooman *et al.* found that 10% had silent inactivation of native *E.coli* asparaginase which might be lower than in the study of Panosyan *et al*., as these patients only received 1 dose of native *E.coli* asparaginase during induction.[27]

No studies have been performed studying silent inactivation of PEGasparaginase if this drug was used for a very prolonged period. Two NOPHO studies reported 13% silent inactivation (intravenous) and 27% silent inactivation (intramuscular) of *Erwinia* asparaginase in the re-induction after receiving *Erwinia* asparaginase in induction.[8,28] We determined the occurrence of silent inactivation of asparaginase preparations during very extensive use in the intensification phase of the ALL-10 protocol.

Toxicity of asparaginase

A major side effect of asparaginase is the occurrence of a clinical allergy. Allergy is a common reason to discontinue asparaginase therapy. Other side effects are dyslipidemia, pancreatitis, hyperammonemia, thrombosis and central neurotoxicity.

Allergy

The symptoms of clinical allergy include anaphylaxis, pain, edema, urticaria, erythema, rash and pruritis.[24] The Common Terminology Criteria for Adverse Events (CTCAE) version 3.0 defines an allergic reaction as a disorder characterized by an adverse local or general response from exposure to an allergen.

Five grades of allergy are defined (CTCAE version 3.0);

- Grade 1: transient flushing or rash; drug fever <38°C.
- Grade 2: rash; flushing; urticaria; dyspnea; drug fever; ≥38°C.
- Grade 3: symptomatic bronchospasm, with or without urticaria; parenteral medication(s) indicated; allergy-related edema/angioedema; hypotension.
- Grade 4: anaphylaxis.
- Grade 5: death.

Table 2 shows the allergy rates of three asparaginase preparations. The occurrence of allergies differs among clinical studies, for instance due to the asparaginase preparation used in different treatment phases. In induction low allergy rates were reported, for example 1.5% using a single dose of PEGasparaginase[29] and approximately 3% for *Erwinia* and native *E.coli* asparaginase.[16,30]

Table 2: Allergy rates of different asparaginase preparations.

	Route of administration	Number of patients	%	Reference
Induction				
EORTC-CLG 58881 (*E.coli*)	IV	9/352	3%	Duval[16]
EORTC-CLG 58881 (*Erwinase*)	IV	9/346	3%	Duval[16]
BFM-2000 (*E.coli*)	IV/IM	4/124	3%	Schrey[30]
DFCI 05-01 (1 PEGasp dose)	IV	3/197	1.5%	Silverman[29]
Short reinduction with *E.coli* after *E.coli* in induction				
EORTC-CLG 58881	IV	86/300	29%	Duval[16]
CCG/augmented BFM	IM	35/101	35%	Nachman[31]
Short reinduction with *Erwinase* after *Erwinase* in induction				
EORTC-CLG 58881	IV	80/277	29%	Duval1[6]
Extended reinduction with *E.coli* after *E.coli* in induction				
DCOG ALL-9 HR	IV	32/49	65%	Appel[34]
POG 8602	IM	408/540	75%	Wacker[33]
SJCRH protocols	IM	18/24	75%	Wang[32]
CCG-1961	IM	142/280	51%	Panosyan[25]
DFCI 95-01	IM	21/147	14%	Moghrabi[17]
During prolonged *Erwinase* in intensification after *Erwinase* in induction				
DFCI 95-01	IM	8/139	6%	Moghrabi[17]
During prolonged asparaginase in intensification and NO asparaginase in induction				
DFCI 91-01 (*E.coli* or PEGasp)	IM	56/377	15%	Silverman[19]
DFCI 00-01*	IM	42/215	20%	Vrooman[35]
During prolonged *Erwinase* in intensification and NO asparaginase in induction				
DFCI 81-01	IM	7 out of 31	23%	Billett[14]
DFCI 00-01*	IM	14/42	33%	Vrooman[35]

IV; intravenous, IM; intramuscular, PEGasp; PEGasparaginase.
* Native *E.coli* asparaginase as first-line and *Erwinia* asparaginase as second-line therapy.

Short re-induction of native *E.coli* asparaginase after prior native *E.coli* asparaginase courses in induction led to allergy rates of 29-35%.[16,31] Also 29% was found when using *Erwinia* asparaginase in induction and subsequently in the short re-induction.[16] If extended re-induction with *E.coli* asparaginase was used with previously native *E.coli* asparaginase in induction, the allergy rates differed from 14-75%.[17,25,32-34] The percentage of 14% was based on a single native *E.coli*

asparaginase dose in induction and 20 weeks of native *E.coli* asparaginase in the re-induction.[17] Otherwise, if no asparaginase was administered in induction, an allergy percentage of 15% to 20% was found in intensification.[19,35] For children treated with *Erwinia* asparaginase, allergy rates of 23% and 33% were reported during very prolonged use without *Erwinia* asparaginase infusion in induction.[14,35]

It is unknown what the allergy rates are in intensification protocols with very prolonged PEGasparaginase or *Erwinia* asparaginase after native *E.coli* asparaginase in induction as used in the DCOG ALL-10 protocol.

Dyslipidemia

Dyslipidemia, like hypertriglyceridemia or hypercholesterolemia, is a common side effect of asparaginase. Parsons *et al.* described hypertriglyceridemia (grade 1-2) in 42% of the patients during intensified asparaginase therapy and the incidence did not vary by asparaginase type.[36] Cohen *et al*. also reported triglyceride levels grade 1 in 43% of the patients during a short re-induction with native *E.coli* asparaginase.[37]

The natural course of dyslipidemia during very prolonged PEGasparaginase and *Erwinia* asparaginase courses is unknown. Also, it is unclear whether asparaginase therapy should be interrupted or stopped. To our knowledge, the association between dyslipidemia and asparaginase activity levels has never been studied before.

Pancreatitis

Pancreatitis may occur during asparaginase therapy. The pathophysiology of asparaginase-associated pancreatitis is unknown, but is regarded to reflect asparagine depletion with a subsequent reduction of protein synthesis, for instance in organs with high protein turnover, like the liver and pancreas.[38]

It is suggested that high triglyceride levels may cause pancreatitis[39,40] but given the high incidence of hypertriglyceridemia and very low incidence of pancreatitis this relationship is doubtful.

The incidence of pancreatitis during intensified asparaginase courses is measured in the DCOG ALL-10 medium risk group. Also, the relation between triglyceride levels and pancreatitis and between asparaginase activity levels and pancreatitis during this asparaginase regimen is unknown and is studied in this thesis.

Thrombosis

The prevalence of symptomatic (venous) thrombosis has been reported to range from 0% to 36% and depended on the chemotherapy protocols containing asparaginase alone or in combination with corticosteroids.[41] Our group previously showed that during asparaginase therapy anticoagulants proteins (antithrombin, protein C and S levels) declined significantly and that the fibrinolytic potential was decreased (decreased alpha-2-antiplasmin and plasminogen

levels) due to asparagine depletion.[42] The sum of these changes in coagulation proteins induced by asparaginase contributed to the increased risk for thrombosis.

The incidence of thrombosis during extensive use of PEGasparaginase and *Erwinia* asparaginase is studied prospectively.

Hyperammonemia and central neurotoxicity

Finally, asparaginase causes hyperammonemia by the breakdown of asparagine into ammonia and aspartic acid. Central neurotoxicity has been reported during asparaginase therapy.[43-45] However, it is unknown whether hyperammonemia is associated with central neurotoxicity during very intensified asparaginase courses.

Costs of asparaginase preparations

The costs of asparaginases per patient are high, therefore more insight into costs of asparaginase preparations was desired. In a Dutch study, it was shown that medication (mainly attributed to asparaginase) and diagnostics were the major contributors to the increased costs of the ALL-10 protocol compared to the ALL-9 protocol.[46] A comparison of the costs of three asparaginase preparations was not available yet.

Only two earlier studies investigated the costs of PEGasparaginase doses, but not according to a very intensified regimen. Both found that treatment costs with PEGasparaginase were similar or slightly less than those with native *E.coli* asparaginase.[47,48]

AIMS

The aim of this research project is to study efficacy, toxicity, and possible mechanisms of asparaginase resistance during intensive PEGasparaginase and *Erwinia* asparaginase therapy in the DCOG ALL-10 medium risk intensification protocol in order to improve the use of these asparaginase preparations.

The specific aims are:

- To study the efficacy of PEGasparaginase and *Erwinia* asparaginase by assessing asparaginase activity, asparaginase antibodies and asparagine levels.
- To determine the occurrence of silent inactivation of asparaginase preparations during very extensive use in the intensification phase of the ALL-10 protocol.
- To determine if asparagine production by mesenchymal cells in the bone marrow plays a role in clinical asparaginase resistance of leukemic cells.
- To assess the incidence and profile of clinical hypersensitivity reactions and non-allergic side effects of asparaginase, especially the effects on lipid metabolism and determine whether it is safe to continue the use of asparaginase.

- To determine if side effects are related to asparaginase activity levels, age and gender.
- To study if the use of intensive PEGasparaginase is more expensive compared to the use of intensive native *E.coli* asparaginase in the intensification phase.

OUTLINE OF THE THESIS

Part 1 EFFICACY AND DRUG RESISTANCE

In **chapter two**, the efficacy of PEGasparaginase or *Erwinia* asparaginase is studied during intensification of the Dutch Childhood Oncology Group (DCOG) ALL-10 medium risk protocol. In **chapter three**, we analyzed whether mesenchymal cells or other cells in the bone marrow indeed produce such significant amounts of asparagine *in vivo* as shown *in vitro*. In **chapter four**, we analyse whether continuation of asparaginase in case of silent inactivation may result in desensitization, disappearance of asparaginase antibodies and recovery of asparaginase activity levels.

Part 2 TOXICITY

In **chapter five**, dyslipidemia is investigated in children with ALL and this toxicity is related to asparaginase activity levels, age and gender. Furthermore, the asparaginase-associated toxicities pancreatitis, hyperglycemia, hyperammonemia, thrombosis and central neurotoxicity are studied prospectively within the DCOG ALL-10 medium risk intensification protocol. In **chapter six**, we report a patient with extreme hypertriglyceridemia during the intensification phase of the medium risk group managed successfully by omitting corticosteroids temporarily, while asparaginase infusions were still continued.

Part 3 COSTS

In order to compare costs of PEGasparaginase, *Erwinia* asparaginase and native *E.coli* asparaginase, a cost-analysis is performed in DCOG ALL-10 medium risk intensification protocol (**chapter seven**). Also, we assess whether there are savings from using PEGasparaginase as the first-line drug rather than the native *E.coli* asparaginase. Both aims are studied during the intensification phase of the ALL-10 medium risk protocol.

Part 4

Finally, all results are discussed in Part 4, **chapter eight**.

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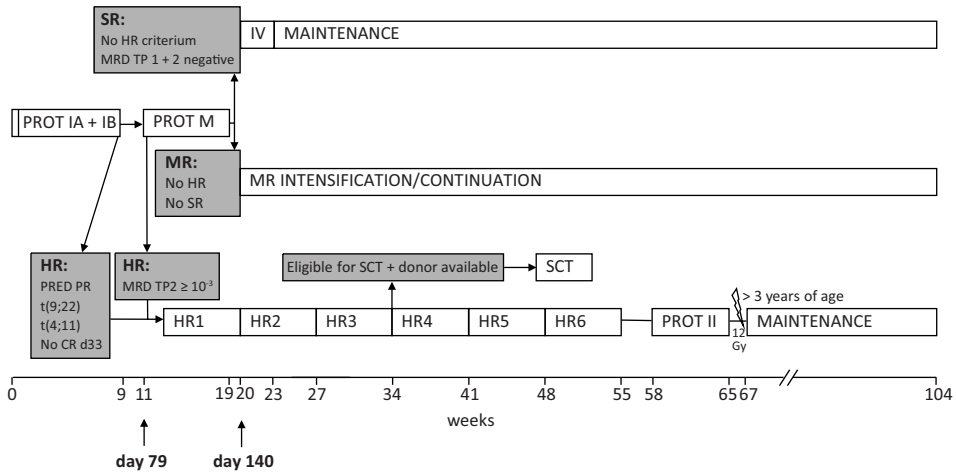
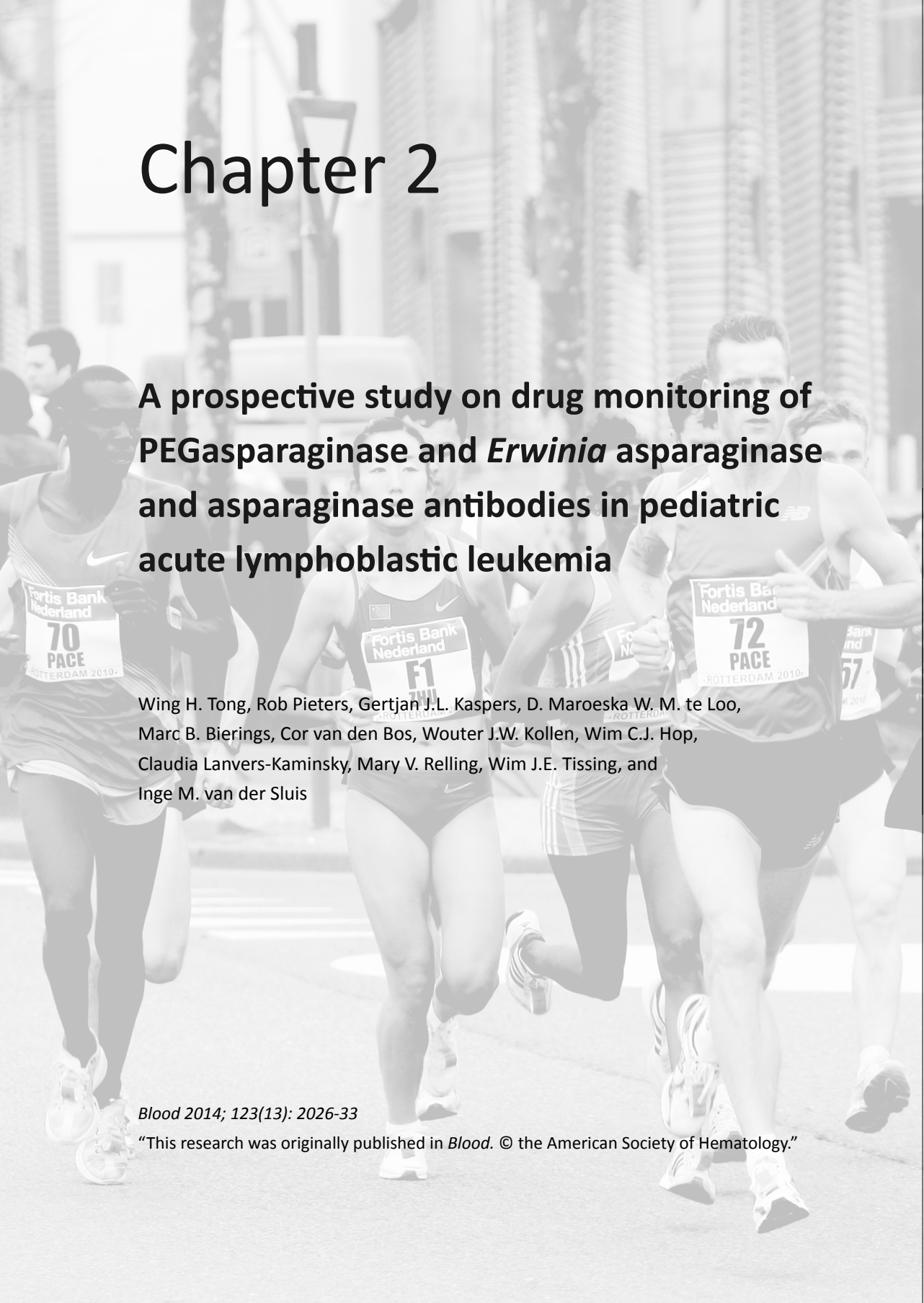
ATTACHMENT

The DCOG ALL-10 protocol

ALL-10 OVERVIEW





Chapter 2

A prospective study on drug monitoring of PEGasparaginase and *Erwinia* asparaginase and asparaginase antibodies in pediatric acute lymphoblastic leukemia

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ABSTRACT

This study prospectively analyzed the efficacy of very prolonged PEGasparaginase and *Erwinia* asparaginase courses in pediatric acute lymphoblastic leukemia (ALL) patients.

Patients received 15 PEGasparaginase infusions (2,500IU/m^2 q2 weeks) in intensification after receiving native *E.coli* asparaginase in induction. In case of allergy to or silent inactivation of PEGasparaginase, *Erwinia* asparaginase (20,000IU/m^2 2-3 times weekly) was given. Eighty-nine patients were enrolled in the "PEGasparaginase study". Twenty (22%) of the PEGasparaginase treated patients developed an allergy; seven (8%) showed silent inactivation. PEGasparaginase level was zero in all allergic patients (grade 1-4). Patients without hypersensitivity to PEGasparaginase had serum mean trough levels of 899 U/L.

Fifty-nine patients were included in the "*Erwinia* asparaginase study", two (3%) developed an allergy and none silent inactivation. Ninety-six percent had at least one *Erwinia* asparaginase trough level of ≥100 U/L. Serum asparagine level was not always completely depleted with *Erwinia* asparaginase in contrast to PEGasparaginase. Presence of asparaginase antibodies was related to allergies and silent inactivation, but with low specificity (64%).

Use of native *E.coli* asparaginase in induction leads to high hypersensitivity rates to PEGasparaginase in intensification. Therefore, PEGasparaginase should be used already in induction and we suggest that the dose could be lowered. Switching to *Erwinia* asparaginase leads to effective asparaginase levels in most patients. Therapeutic drug monitoring has been added to our ALL-11 protocol to individualize asparaginase therapy.

INTRODUCTION

Asparaginase is an enzymatic drug and an essential component of the combination chemotherapy of childhood acute lymphoblastic leukemia (ALL).[1] This drug depletes asparagine in blood and the malignant lymphoid cells which depend on extracellular asparagine will thus go in apoptosis.[2,3] Currently, several asparaginase agents are available on the market. Either these are derived from *Escherichia coli* in its native form (native *E.coli* asparaginase) or as a pegylated enzyme (PEGasparaginase). Otherwise, asparaginase is extracted from *Erwinia chrysanthemi* (*Erwinia* asparaginase).

It has been shown that intensified use of asparaginase increases event-free survival (EFS) for children with ALL by 10-15%.[4-7] Administration of asparaginase can be limited by the occurrence of hypersensitivity reactions to asparaginase, like allergic or anaphylactic reactions.[8] Patients with these reactions are switched to another asparaginase product to ensure that they are exposed to asparaginase according to treatment plan and to ensure an optimal EFS.[9] Clinical allergy is associated with inactivation of asparaginase by antibodies.[10,11] Formation of asparaginase antibodies can also neutralize asparaginase without any clinical signs of hypersensitivity, so called silent inactivation. Panosyan *et al.* and Vrooman *et al.* showed that children with silent inactivation of native *E.coli* asparaginase had poorer outcome as they were not switched to alternative asparaginase agents whereas those with clinically overt allergy were switched and had no poorer outcome.[8,12] In most protocols asparaginase is given during the induction course, followed by asparaginase-free consolidation courses and after that asparaginase is again given during the intensification/reinduction course. The far majority of hypersensitivity reactions occur during the intensification phase.

The Dutch Childhood Oncology Group (DCOG) ALL-10 protocol used native *E.coli* asparaginase in induction and the less immunogenic PEGasparaginase in the intensification phase in an attempt to prevent hypersensitivity reactions.[13] In case of either hypersensitivity to PEGasparaginase or silent inactivation, children were switched to *Erwinia* asparaginase as second-line agent in intensification. Only a few studies have been performed on silent inactivation using intensive PEGasparaginase[14] or intensive *Erwinia* asparaginase.[15,16]

The aim of this prospective drug monitoring study was to analyse the efficacy of very prolonged use of PEGasparaginase and *Erwinia* asparaginase by assessing asparaginase activity, asparagine, glutamine levels and asparaginase antibodies.

METHODS

Patients

Children between 1 and 18 years of age with newly diagnosed ALL and stratified as medium risk patients were included in the prospective "PEGasparaginase study" from May 2009 until October 2012 in two pediatric oncology centers. Patients were assigned to the medium risk group based on a prednisone good response at day 8 and cytomorphological complete remission at day 33 and minimal residual disease (MRD) positivity at day 33 and/or day 79 (before the start of protocol M), but MRD level at day 79<10^{-3} and no presence of the t(4;11)(q11;q23) translocation or the corresponding fusion gene MLL/AF4 in the leukemia cells at diagnosis. Children who had an allergy to PEGasparaginase or silent inactivation were switched to *Erwinia* asparaginase and included in the prospective "*Erwinia* asparaginase study". Because of the expected low number of allergic reactions to PEGasparaginase, the latter study was carried out in all seven pediatric oncology centers in the same study period.

The Institutional Review Board approved this study before patient enrollment. Informed consent was obtained from parents or their guardians and from patients ≥12 years of age. This study was in accordance with the Declaration of Helsinki.

DCOG ALL-10 treatment protocol

Patients were stratified into three risk groups after induction treatment: standard risk (SR), medium risk (MR) and high risk (HR).[17] The treatment scheme of the ALL-10 protocol and in more detail, the intensification phase of the medium risk patients are displayed in the Supplemental Figure 1.

All patients received eight doses native *E.coli* asparaginase (5,000 IU/ m^2 per dose) every three days in induction. If a patient was stratified as MR, PEGasparaginase as first-line agent (2,500 IU/ m^2 per dose every other week) was given for a total of 15 doses during the first 30 weeks of intensification. In case of an allergy to PEGasparaginase or silent inactivation, the drug was replaced by *Erwinia* asparaginase as second-line agent (20,000 IU/ m^2 per dose) three times per week to complete 30 weeks of asparaginase therapy. All asparaginase agents were administered intravenously.

Study design

In the "PEGasparaginase study", the PEGasparaginase activity trough levels and asparaginase antibodies (AAA) were measured in serum at start of intensification (week 0), in week 2, 4, 6, 8, 10, 14, 16, 24, 26, 28 and also 1 week after administration in week 3, 9, 15 and 25. Serum asparagine, aspartic acid, glutamine, and glutamic acid levels were measured at week 0, 2, 4, 14 and 24 during PEGasparaginase therapy.

Children who had an allergy to PEGasparaginase or silent inactivation were included in the "*Erwinia* asparaginase study". For this study, six blood samples of *Erwinia* asparaginase

activity levels during the first two weeks of therapy were obtained from children who received *Erwinia* asparaginase (Monday-Wednesday-Friday). In case of high activity levels (72-hour levels ≥100 U/L), the frequency of administration of *Erwinia* asparaginase was reduced to two times per week. Thereafter, every four weeks a blood sample was assessed.

Allergy was graded according to the National Cancer Institute (NCI) Common Terminology Criteria for Adverse Events (CTCAE) version 3.0. Silent inactivation was defined as serum PEGasparaginase activity level below 100 U/L at day 7±1 or below 20 U/L at day 14±1 after administration in a patient without clinical symptoms of allergy. Silent inactivation of *Erwinia* asparaginase was defined as serum asparaginase activity level below 20 U/L at day 2 after administration of *Erwinia* asparaginase in patients without clinical allergy.

Laboratory measurements

Serum asparaginase activity and amino acid levels were processed and assessed as described earlier.[18,19] Antibodies against native *E.coli* asparaginase (Asparaginase medac®) (Coli-AAA); against PEGasparaginase (Oncaspar®) (PEG-AAA), and against *Erwinia* asparaginase (Erwinase®) (*Erwinia*-AAA) were measured by enzyme-linked immunosorbent assays (see Supplemental methods). All asparaginase antibodies were expressed as optical densities (OD) readings. Samples were defined as positive for asparaginase antibodies if the Coli-AAA OD >0.13; if the PEG-AAA OD >0.25, and for *Erwinia*-AAA if the OD >1.96 standard deviations above the negative control processed mean (using Westgard rules).[20]

To evaluate if Coli-AAA in induction predict an allergy or silent inactivation in intensification, we measured Coli-AAA after induction in a single center (Rotterdam).[21]

Statistical analysis

The data were analysed with the software package SPSS version 20.0.0.1 (SPSS, Chicago, IL, USA). Repeated measurements of the asparaginase activity levels were evaluated using mixed models analysis of variance (see Supplemental methods). The antibody levels were compared using non-parametric tests. The role of Coli-AAA at day 79 (at the start of consolidation; protocol M) was analysed to predict an allergy or silent inactivation at day 140 (start of intensification); for this, sensitivity and specificity were given. Sensitivity and specificity were calculated with the Fisher's exact test for two-by-two contingency tables.

The occurrence of allergy to or silent inactivation of PEGasparaginase related to age and gender was investigated with the Chi square (χ^2) test/ Fisher's exact tests where appropriate. A two-sided p-value <0.05 was considered statistically significant. Data are presented as mean ± SEM or specified otherwise.

RESULTS

"PEGasparaginase study"

Supplemental Table 1 displays the characteristics of 89 patients included in the "PEGasparaginase study". 20/89 (22%) of the patients had clinical allergic reactions to PEGasparaginase (6 grade I, 7 grade II, 7 grade III) and 7/89 (8%) showed silent inactivation. 18/20 (90%) of allergic reactions occurred on the second PEGasparaginase dose. Age, gender and ALL immunophenotype did not differ between patients with or without allergy/silent inactivation of PEGasparaginase.

Serum PEGasparaginase activity levels were measured in 592 samples of 89 children (Figure 1-A).

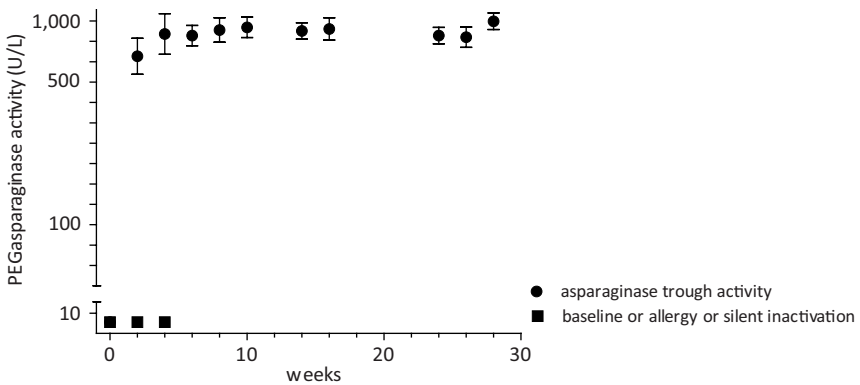

Figure 1-A: Serum PEGasparaginase activity levels (mean ± SEM) of children with or without allergy or silent inactivation of PEGasparaginase; (2,500 IU/m^2 every other week) (n=62).

PEGasparaginase activity levels were not related to age (<10 years or ≥10 years) or gender (both p=0.2). Patients without allergy to and without silent inactivation of PEGasparaginase had mean trough activity level of 899 U/L (see also Supplemental Figure 2). All 20/89 (22%) allergic patients (including grade I-IV CTCAE) showed PEGasparaginase activity levels of zero. This was not due to the fact that the infusion was stopped as 18 patients showed their allergic reactions at the second dose whereas the serum asparaginase activity level after the first full dose already appeared to be zero in all 18 cases. Moreover, in 4 cases the second full PEGasparaginase dose was given with clemastine and hydrocortisone, also resulting in unmeasurable serum activity levels of PEGasparaginase after the second dose. All allergic patients were switched to *Erwinia* asparaginase. 2/7 children with silent inactivation were switched to *Erwinia* asparaginase. The other five patients continued with PEGasparaginase, because real-time asparaginase measurements were not available at that moment. Those were excluded from further analysis.

At the start of the intensification phase, the asparagine levels were normal in almost all patients (normal range 40-80 µM). All children without an allergy to PEGasparaginase and without silent inactivation had complete asparagine depletion over time with a lower level of quantification (LLQ) of 0.2 µM (Figure 2-A).

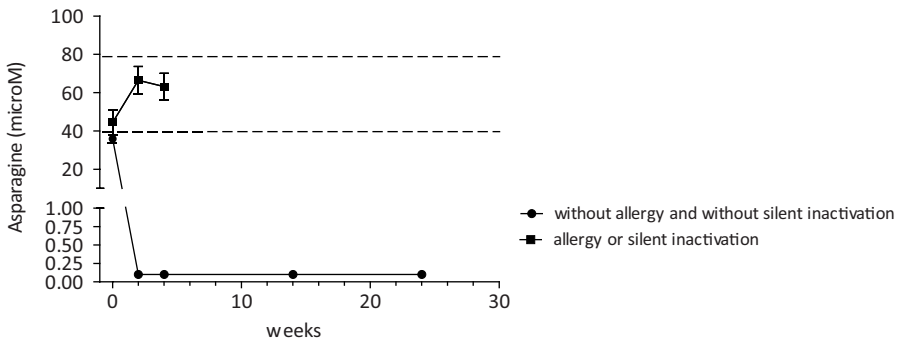

Figure 2-A: Serum asparagine (**A**) levels (mean ± SEM) during PEGasparaginase (2,500 IU/m²) in children without allergy to PEGasparaginase and without silent inactivation.
Dashed lines show normal values of asparagine (40-80 µM).
The lower level of quantification of asparagine is 0.2 µM.

Children with an allergy to PEGasparaginase or silent inactivation showed no asparagine depletion (Figure 2-A). Supplemental Figure 3-A shows PEGasparaginase activity levels in relation to the asparagine levels. Figure 2 B-D displays aspartic acid, glutamine, and glutamic acid levels.

Aspartic acid levels increased after one PEGasparaginase infusion in children without allergy and without silent inactivation in line with the asparagine depletion. Thereafter, no changes were seen. No glutamine depletion was seen during PEGasparaginase therapy. Glutamic acid levels increased after the first two PEGasparaginase infusions in children without allergy and without silent inactivation, no changes were seen thereafter.

Predictive value of Coli-AAA in induction for an allergy or silent inactivation in intensification

Coli-AAA were measured serially in 40 patients; 11/40 (27.5%) had allergy and 4/40 (10%) had silent inactivation, respectively, in intensification. The sensitivity of Coli-AAA at day 79 to detect allergy or silent inactivation in intensification (day 140) was 87% (95%-CI: 60%-98%) and specificity was 64% (95%-CI: 43%-82%).

At day 79, the Coli-AAA levels of children with allergy to or silent inactivation of PEGasparaginase in the subsequent intensification phase were significantly higher than those in children without (p=0.02). The same was found at day 140 (p=0.006) (Figure 3 A-B).

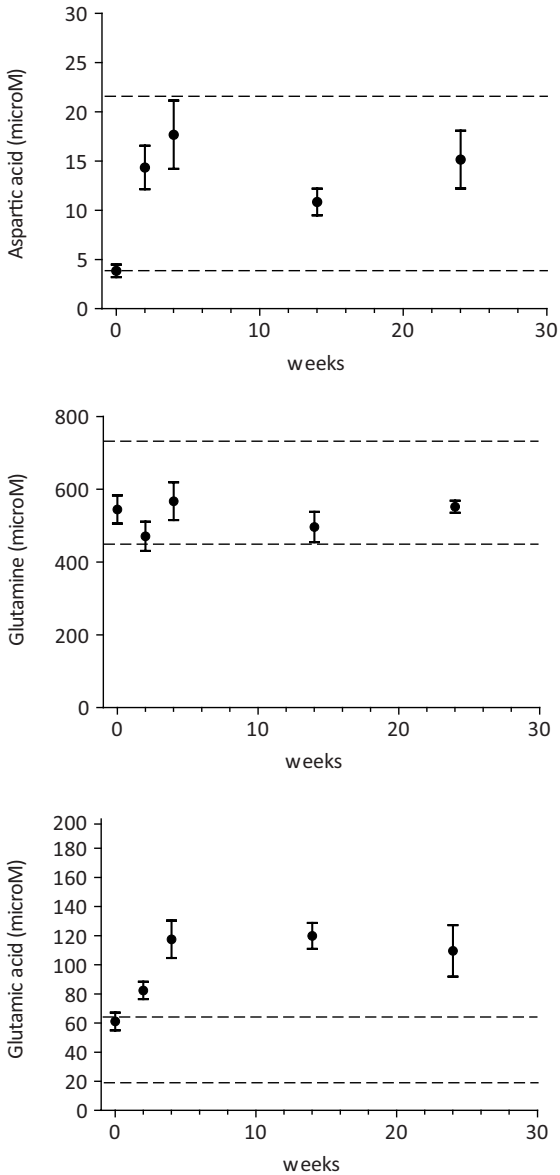

Figure 2-B,C,D: Serum aspartic acid (**B**), glutamine (**C**), glutamic acid (**D**) levels (mean ± SEM) during PEGasparaginase (2,500 IU/m^2) in children without allergy to PEGasparaginase and without silent inactivation.

Dashed lines show normal values of aspartic acid (4-22 µM); glutamine (457-738 µM), and glutamic acid (18-65 µM).

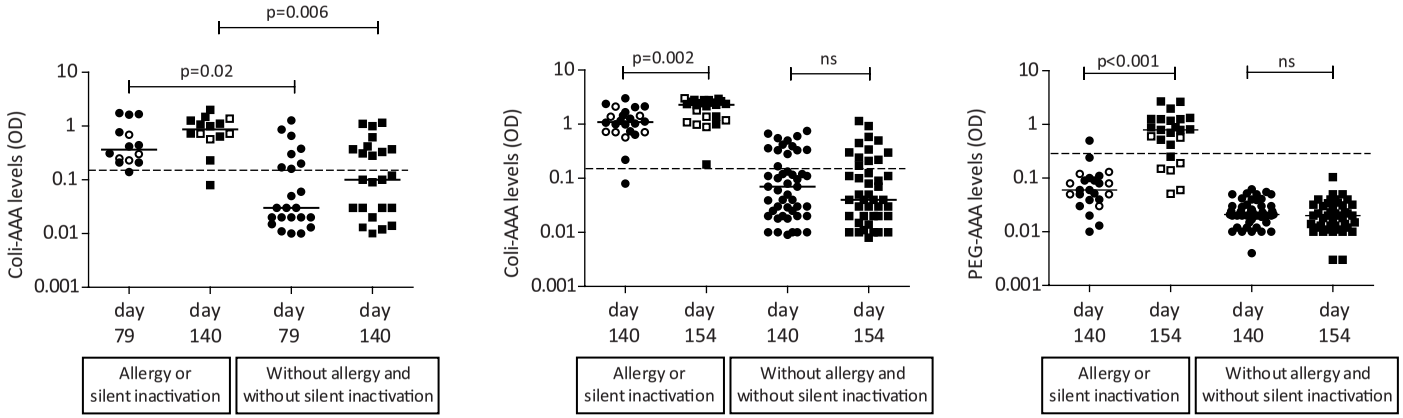

Figure 3-A,B,C,: Serum asparaginase antibodies against native *E.coli* asparaginase (Coli-AAA levels) of patients at day 79 (start of the consolidation phase) and at day 140 (start of the intensification) (**A**) and Coli-AAA levels and serum asparaginase antibodies against PEGasparaginase (PEG-AAA levels) at day 140 (before first PEGasparaginase dose) and at day 154 (14 days after first PEGasparaginase dose) (**B-C**).

Dashed lines: samples were defined as positive for asparaginase antibodies if the Coli-AAA OD > 0.13, and if the PEG-AAA OD > 0.25.

Closed circles and blocks show cases with allergy or cases without allergy and without silent inactivation; silent inactivation cases are shown by open circles and blocks.

Median Coli-AAA and PEG-AAA levels are indicated by bars.

NS: not significant.

All children allergic to PEGasparaginase, except one, had Coli-AAA at start of intensification (day 140) (Figure 3-B). Only one patient had PEG-AAA at day 140 (Figure 3-C). The levels of Coli-AAA and PEG-AAA increased significantly from day 140 to day 154 after the first PEGasparaginase infusion in allergic patients ($p=0.002$ and $p<0.001$, respectively). All allergic children except one developed PEG-AAA after the first PEGasparaginase infusion. All children with silent inactivation had Coli-AAA before (day 140) and after (day 154) the first PEGasparaginase infusion. Only 2/7 silent inactivation patients had PEG-AAA after the first PEGasparaginase infusion (day 154) and patients with silent inactivation had lower PEG-AAA at day 154 than patients with clinical allergy ($p=0.001$).

"*Erwinia* asparaginase study"

Supplemental Table 1 presents the characteristics of the 59 patients with an allergy to or silent inactivation of PEGasparaginase in this 7-center study; 25 patients already participated in the "PEGasparaginase study".

2/59 patients (3%) developed an allergy to *Erwinia* asparaginase; one showed an allergy grade III at the fourth infusion, the other an allergy grade II at the sixth infusion. These two patients did not receive further asparaginase therapy. No patients with silent inactivation of *Erwinia* asparaginase were seen.

Serum *Erwinia* asparaginase activity levels were measured in 444 samples of 57 patients without allergy to *Erwinia* asparaginase to complete 30 weeks of exposure (Figure 1-B).

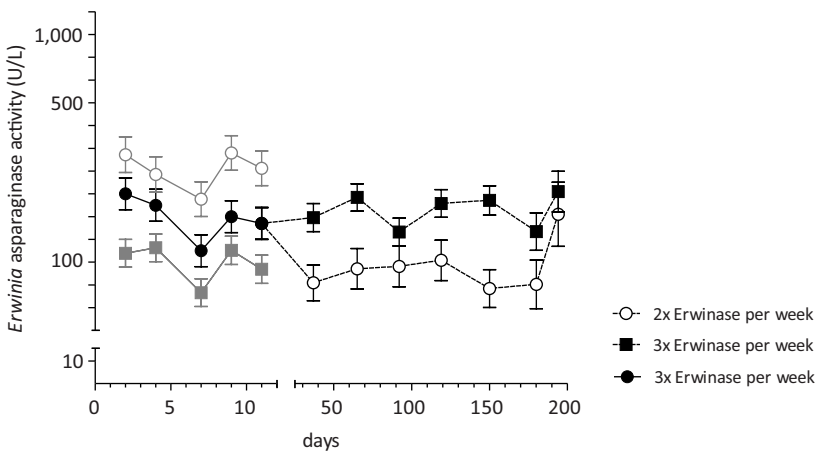

Figure 1-B: Serum *Erwinia* asparaginase activity levels (mean ± SEM) of children without allergy to *Erwinia* asparaginase (20,000 IU/m^2 2-3 times per week) (n=57).

In panel B, in the first two weeks of *Erwinia* asparaginase therapy, three curves are shown: The upper curve (white open circles): 19 children who had "high" *Erwinia* asparaginase levels (72-hour ≥100 U/L) who were switched to 2 times per week infusions after the first 2 weeks of *Erwinia* asparaginase therapy.

The lower curve (grey blocks): 38 children who had "low" *Erwinia* asparaginase levels (72-hour <100 U/L) who continued 3 times per week infusions after the first 2 weeks of *Erwinia* asparaginase therapy.

The middle curve (closed circles): ANOVA estimates of all 57 children receiving 3 times per week *Erwinia* asparaginase in the first two weeks.

The two children with an allergy to *Erwinia* asparaginase had asparaginase activity levels of zero. This was not due to the fact that the infusion was stopped as the asparaginase level of the previous dose already appeared to be zero in both patients. Also, in both cases the full next *Erwinia* asparaginase dose was given with clemastine and hydrocortisone, also resulting in unmeasurable serum *Erwinia* asparaginase activity levels. Table 1 shows the *Erwinia* asparaginase activity levels during intensification therapy. *Erwinia* asparaginase activity levels were not related to age (<10 years or ≥10 years) or gender (p=0.7 and p=0.4, respectively).

Table 1: Serum *Erwinia* asparaginase trough levels ≥20 U/L, ≥50 U/L or ³ 100 U/L.

first 2 weeks of *Erwinia* asparaginase therapy	***Erwinase* activity** median (range)	**samples (n=231)**			**patients (n=57)**		
		≥20	**≥50**	**≥100**	**≥20**	**≥50**	**≥100**
3 times per week	157 (11-913)	98%	85%	65%	95%*	60%*	32%*
at least one sample above 20, 50 or 100 U/L					100%	100%	96%
week 6 to week 30 of *Erwinia* asparaginase therapy	***Erwinase* activity** median (range)	**samples (n=213)**			**patients (n=57)**		
		≥20	**≥50**	**≥100**	**≥20**	**≥50**	**≥100**
3 times per week 48-hours interval	182 (22-737)	100%	94%	77%	100%*	87%*	47%*
at least one sample above 20, 50 or 100 U/L					100%	100%	100%
2 times per week 72-hours interval	83 (14-908)	96%	86%	34%	95%*	68%*	11%*
at least one sample above 20, 50 or 100 U/L					100%	100%	68%

Please note that Table 1 is based on 57 patients without allergy to *Erwinia* asparaginase. The two patients with clinical allergy to *Erwinia* asparaginase were removed from this group. Table 1 shows the data of the period week 6 to week 30 of *Erwinia* asparaginase therapy of 142 samples of 38 patients who continued 3 times per week infusions after the first two weeks of *Erwinia* asparaginase therapy and the data of 71 samples of 19 patients who were switched to 2 times per week infusions after the first two weeks of therapy.

* Number of patients with all samples above 20 U/L, 50 U/L or 100 U/L.

In the first two weeks, all children received 6 doses of *Erwinia* asparaginase. Of the non-allergic *Erwinia* asparaginase patients in the first 2 weeks, 55/57 (96%) had at least one *Erwinia* asparaginase activity level ≥100 U/L and 57/57 (100%) ≥50 U/L. In 65% and 85% of all patients, all *Erwinia* asparaginase activity levels in the first 2 weeks were ≥100 U/L and ≥50 U/L, respectively. Median trough levels were 183 U/L at 48-hour and 93 U/L at 72-hour. 19 children (33%) were switched to twice-weekly infusions, because “high” activity levels (72-hour levels ≥100 U/L) were measured in the first two weeks.

Children receiving 2x per week therapy showed a significant decrease of *Erwinia* asparaginase activity levels at day 37 (week 6) as compared to day 11 (week 2) (p=0.002), while children continuing 3x per week *Erwinia* asparaginase showed a significant increase of activity levels (p=0.004) (Figure 1-B). The *Erwinia* asparaginase activity levels remained stable thereafter for all patients. Concerning the non-allergic patients during *Erwinia* asparaginase therapy, 57/57 (100%) had at least one *Erwinia* asparaginase activity level ≥100 U/L after the first two weeks. Median trough levels were 177 U/L at 48-hour and 86 U/L at 72-hour.

Before start of *Erwinia* asparaginase, the asparagine levels were normal (normal range 40-80 µM). During repeated asparagine measurements, these levels were strongly depleted but not always fully depleted over time with a LLQ of 0.2 µM (Figure 4-A).

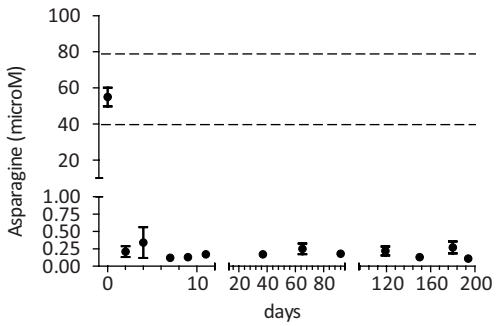

Figure 4: Serum asparagine (A) levels (mean ± SEM) during during *Erwinia* asparaginase (20,000 IU/m^2) in children without allergy to *Erwinia* asparaginase and without silent inactivation.
Dashed lines show normal values of asparagine (40-80 µM).
The lower level of quantification of asparagine is 0.2 µM.

The two children with an allergy to *Erwinia* asparaginase showed no asparagine depletion. Supplemental Figure 3-B shows *Erwinia* asparaginase activity levels in relation to the asparagine levels and Figure 4 B-D displays aspartic acid, glutamine, and glutamic acid levels.

No glutamine depletion was seen during therapy, however, after 6 weeks of *Erwinia* asparaginase therapy the glutamine levels were decreased, but not significantly.

Figure 5 presents the *Erwinia*-AAA and *Erwinia* asparaginase activity levels over time. In total, 38% of patients developed *Erwinia*-AAA during therapy, all at days 37, 65 and 92. Thereafter, the levels dropped again to baseline values. Presence of *Erwinia*-AAA did not influence *Erwinia* asparaginase activity levels. No silent inactivation was seen during therapy. Both children (3%) with an allergy to *Erwinia* asparaginase had detectable *Erwinia*-AAA.

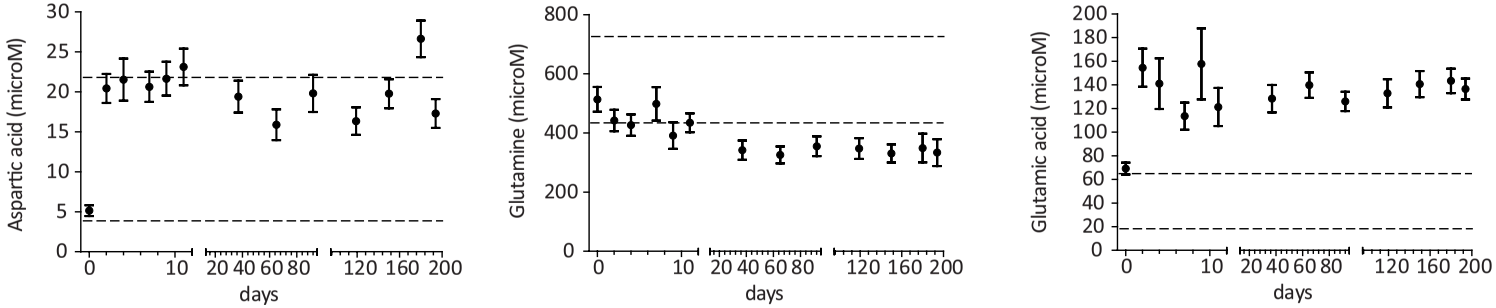

Figure 4-A,B,C: Serum aspartic acid (**B**), glutamine (**C**), glutamic acid (**D**) levels (mean ± SEM) during *Erwinia* asparaginase (20,000 IU/m²) in children without allergy to *Erwinia* asparaginase and without silent inactivation.

Dashed lines show normal values of aspartic acid (4-22 µM); glutamine (457-738 µM), and glutamic acid (18-65 µM).

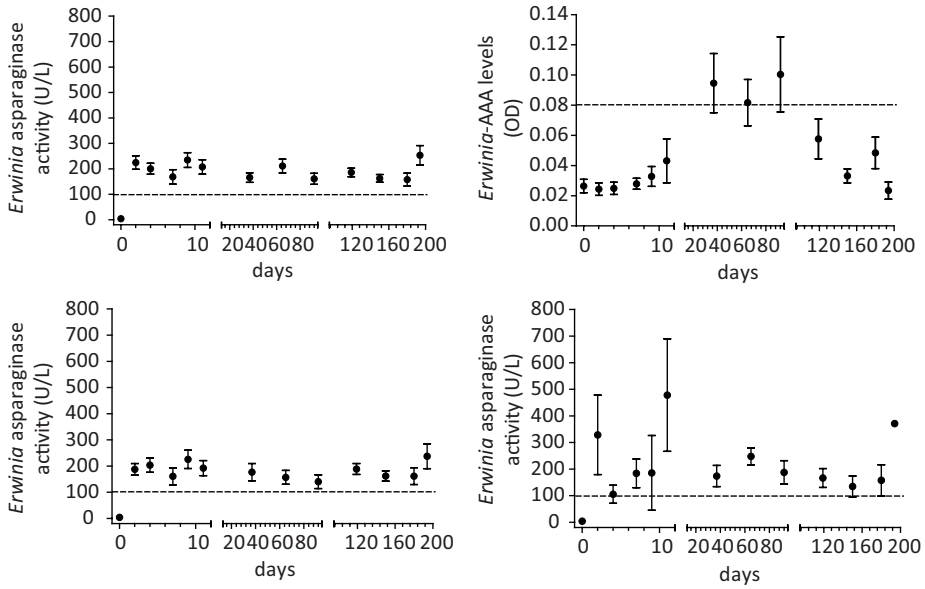

Figure 5-A,B,C,D: Serum *Erwinia* asparaginase activity (**A**) and serum asparaginase antibodies (**B**) against *Erwinia* asparaginase (*Erwinia*-AAA) over time. Serum *Erwinia* asparaginase activity levels of patients without *Erwinia*-AAA (**C**) and with *Erwinia*-AAA (**D**) (mean ± SEM).

Dashed lines in Figure 5-A, C-D: *Erwinia* asparaginase activity level of 100 U/L.

Dashed line in Figure 5-B: samples were defined as positive for *Erwinia*-AAA if the OD >1.96 standard deviations above the negative control processed mean (using Westgard rules).[20]

DISCUSSION

This prospective drug monitoring of asparaginases has resulted in several important findings.

First, if patients show no clinical allergy to or silent inactivation of PEGasparaginase, the serum levels are too high using a schedule of 2,500 IU/m^2 every other week. Based on this finding we suggest that the administered PEGasparaginase dose can be lowered. This dose reduction seems feasible as different re-induction protocols used 1,000 IU/m^2 PEGasparaginase with adequate trough levels of ≥ 100 U/L in approximately 80% of the patients.[22,23] Appel *et al.* showed earlier that 1,000 IU/m^2 PEGasparaginase in induction resulted in asparaginase levels above 100 U/L for at least two weeks with complete asparagine depletion in all patients.[24] However, this dose reduction of PEGasparaginase should be used and guided by careful monitoring of asparaginase activity levels. There is need in the future to evaluate this dose reduction with

respect to outcome and toxicity data. Of interest, a dose reduction of PEGasparaginase could lead to cost savings, this should also be studied.

Second, we found a high incidence of inactivation of PEGasparaginase (22% clinical allergy and 8% silent inactivation) in the intensification phase due to antibody development against native *E.coli* asparaginase which was used in induction. This implies that PEGasparaginase should be used upfront already during the induction course instead of native *E.coli* asparaginase, since this has been shown to result in less antibody formation.[16] Similar inactivation rate of PEGasparaginase (36%) was found one week after the PEGasparaginase course by Muller *et al.* using the same asparaginase regimen in induction and a single PEGasparaginase in re-induction.[25] However, they reported no allergies in re-induction. If a subsequent second PEGasparaginase course was administered, we believe that the allergy rate of PEGasparaginase would be increased. An important finding is that even very mild allergic reactions (grade I) also necessitate a switch to *Erwinia* asparaginase. Continuing with PEGasparaginase combined with pre-treatment of clemastine and hydrocortisone is not useful as PEGasparaginase activity levels remain zero (Figure 1-A).

Third, drug monitoring is useful as it detects too high levels in part of the patients and even more important, silent inactivation in another part of the patients. Detection of silent inactivation is important to prevent useless continuation of an inactive asparaginase product which may lead to a worse event-free survival as shown by Panosyan *et al.* and Vrooman *et al*.[8,12] Nevertheless, drug monitoring is the only way to detect cases of silent inactivation of asparaginase agents.

Fourth, in case of allergy to or silent inactivation of PEGasparaginase, patients can be treated effectively with *Erwinia* asparaginase. The far majority of the patients that switched to *Erwinia* asparaginase showed effective asparaginase activity levels during the first two weeks of *Erwinia* asparaginase, namely median trough activity level of 183 U/L (48-hour) and 93 U/L (72-hour) and asparaginase activity level ≥100 U/L in 100% (48-hour) and 33% (72-hour) of the patients. Similar rates were found by Vrooman *et al*. (83% (48-hour) and 45% (72-hour)) using a dose of 25,000 IU/m^2 three times per week intravenously.[26] These activity levels were lower compared to that of the study of Salzer *et al.*, they found 645 U/L (48-hour) and 248 U/L (72-hour) as median trough activity levels and asparaginase activity levels ≥100 U/L in 93% (48-hour) and 88% (72-hour) of the patients using intramuscular *Erwinia* asparaginase in a dose of 25,000 IU/m^2 three times per week.[27] The route of *Erwinia* asparaginase administration might explain the higher median asparaginase activity levels which were found by Salzer *et al*.[27] However, previous studies have shown that no differences in mean asparaginase activity levels, asparagine depletion and asparaginase antibodies were found after intravenous or intramuscular administration of *Erwinia* asparaginase.[16,28,29] Only 2/59 (3%) developed an allergy to *Erwinia* asparaginase in our study. Please note that this study does not allow comparison of allergy rates to *E.coli* asparaginase (including the pegylated form) and *Erwinia* asparaginase as *E.coli* asparaginases were given in induction and intensification with an asparaginase-free interval of 4 months whereas *Erwinia* asparaginase was administered continuously without such interval.

Fifth, the mean asparagine levels for both preparations were below the detection level of 0.5 µM as used by others.[14,30-32] We observed not always completely depleted asparagine levels with our detection level of 0.2 µM[21] in *Erwinia* asparaginase treated patients in contrast to PEGasparaginase. This may simply reflect the differences in serum drug levels of these two compounds. With our very low LLQ of 0.2 µM[21], 19/57 patients (33%) showed no complete asparagine depletion. If the threshold levels are used from literature (mean of 0.5 µM) 9/57 patients (16%) showed incomplete asparagine depletion.[14,30-32] Samples with *Erwinia* asparaginase activity levels >100 U/L and asparagine >0.2 µM were also found in 9/57 patients (16%).

Why this difference in asparagine levels during asparaginase therapy exists between *Erwinia* asparaginase and PEGasparaginase is unclear. Measuring asparaginase activity levels is preferred over measuring asparagine levels to monitor the efficacy of asparaginase therapy because if not properly handled, asparagine is very rapidly degraded *ex vivo* in the tube by asparaginase leading to false low asparagine levels (Lanvers *et al*. in press).[33] It is remarkable that the glutamine levels of *Erwinia* asparaginase treated patients were lower as compared to those receiving PEGasparaginase irrespective of the much lower serum activity levels of *Erwinia* asparaginase. This can be explained by higher glutaminase activity of *Erwinia* asparaginase.[34] Although glutamine is broken down to glutamic acid by asparaginase, this does not lead to glutamine depletion as this is supplemented from other organ stocks *in vivo*.

Sixth, our study shows that the presence of asparaginase antibodies is related to allergy to and silent inactivation of asparaginase as shown by others,[10,20] but predicting asparaginase allergy or silent inactivation based upon antibody formation is hampered by low specificity (64%) of the test. The specificity of Coli-AAA test in intensification was higher, 73%, but still not perfect. The levels of PEG-AAA were above the cut-off in almost all allergic patients after the first PEGasparaginase infusion and negative in children without an allergy. The specificity of the PEG-AAA test is 100% to predict an allergy to or silent inactivation of PEGasparaginase in intensification. However, the two patients with an allergy at the first PEGasparaginase infusion had no detectable PEG-AAA (day 0 of intensification). And *Erwinia*-AAA were found in 38% of the patients, but were not associated with inactivation of asparaginase. So, measuring asparaginase levels is a more direct and more accurate way of drug monitoring than measuring asparaginase antibodies. It has been reported that females and toddlers are more prone to develop allergies.[35] However, we found no relation between age or gender and allergy or antibody formation.

In conclusion, the use of native *E.coli* asparaginase in induction leads to a significant rate of allergy and silent inactivation of PEGasparaginase in intensification. Switching to *Erwinia* asparaginase in case of allergy to or silent inactivation of PEGasparaginase leads to effective asparaginase activity levels in most patients, but close drug monitoring remains necessary to ensure adequate drug levels. The relevance of asparaginase antibodies in clinical practice appears to be limited. It is more useful to monitor the serum asparaginase activity levels. In the absence of allergy or silent inactivation, PEGasparaginase activity levels are too high with a dose schedule of 2,500 IU/m^2 every other week.

This study has therefore resulted in significant changes in the use of asparaginase in the DCOG ALL-11 protocol. PEGasparaginase is used instead of native *E.coli* asparaginase already in the induction and the starting dose of PEGasparaginase has been lowered to 1,500 IU/m^2. Also, a therapeutic drug monitoring program is now used to individualize the PEGasparaginase dose and to detect silent inactivation. In case of allergy or silent inactivation, patients are switched to *Erwinia* asparaginase with therapeutic drug monitoring to allow individualized dosing of *Erwinia* asparaginase.

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Supplementals accompanying Chapter 2

SUPPLEMENTAL METHODS

Antibodies against PEGasparaginase (Oncaspar®) and native *E.coli* asparaginase (medac®, Germany)

Briefly, Nunc MaxiSorp™ microplates were coated with native *E.coli* asparaginase or PEGasparaginase (diluted to protein concentrations of 2 or 1 µg/mL in PBS, respectively) over night at 5°C. After one blocking step applying 15% (v/v) foetal calf serum in PBS for 3 hours at 37°C and two washing steps with PBST (PBS/0.05% Tween 20), the plates were preserved with an in-house preservation buffer (a sugar-containing PBS), dried, sealed under vacuum and stored at 5°C. Serum samples were diluted 1:50 and 1:300 (for anti-PEGasparaginase and anti-*E.coli* antibodies detection, respectively) in sample diluent (PBST with new-born calf serum and preservatives). On each microplate a blank (sample diluent), one negative and two positive controls (consisting of human sera) were measured to confirm validity of each test run (see below). A 50 µL/well (duplicate determination) of each sample and control were incubated for 1 hour at 37°C. All wells were washed three times with PBST.

The presence of bound immunoglobulins was detected using horseradish peroxidase-labelled goat anti-human IgG/M F(ab')2 antiserum (Dianova, Hamburg, Germany; final dilution depending on lot reactivity about 1:50,000) and a chromogen/substrate solution containing tetramethylbenzidine/peroxide (TMB, medac, Hamburg, Germany). A 50 µL conjugate per well were incubated for 1 hour at 37°C, followed by three washing steps and a 30-min incubation of 50 µL/well TMB at 37°C. The chromogenic reaction was stopped with 100 µL/well 0.5 M sulphuric acid. Photometric reading of optical densities (OD) was done at 450 nm (reference wavelength 620 nm). In valid test runs OD values of blank had to be <0.05, of negative control <0.1 and of positive controls within appropriately predefined lot-specific ranges. Cut-off values were defined during assay validation as OD of the negative control plus 0.25 (anti-PEGasparaginase assay) or plus 0.13 (anti-*E.coli* asparaginase assay) by investigation of 128 presumably negative human sera allowing less than 5% samples above cut-off. Sixty-four samples of blood donors from Medical School Hannover (Germany) and 64 samples of newly diagnosed asparaginase naive ALL patients served as negative controls. All samples were assessed twice independently by two persons yielding highly similar results. Within this assumed negative panel 5 samples each (4%) revealed OD values above these cut-offs. To account for regular assay variations a grey zone of cut-off ± 10% was defined. Samples with ODs within this grey zone were evaluated as equivocal, those above as positive, and below as negative.

Antibodies against *Erwinia* asparaginase (Erwinase®)

Serum IgG antibodies against *Erwinia* asparaginase were measured by ELISA. For antibodies to *Erwinia* asparaginase, samples were defined positive if the natural log of its OD reading at a 1:400 dilution of serum was greater than 1.96 standard deviations above the negative control processed mean (using Westgard rules) as previously described.[1]

Supplemental Table 1: Characteristics of patients in the "PEGasparaginase study" and in the "*Erwinia* asparaginase study".

	PEGasparaginase study	***Erwinia* asparaginase study**
N	89	59
Gender		
Boys	44 (49%)	36 (61%)
Girls	45 (51%)	23 (39%)
Median age (range) at diagnosis (yr)	4.9 (1.2-16.2)	7.0 (1.8-17.5)
Age category 1-2 yr	4 (4%)	1 (2%)
Age category 2-6 yr	48 (55%)	27 (46%)
Age category 6-12 yr	22 (25%)	14 (24%)
Age category 12-18 yr	15 (17%)	17 (29%)
Immunophenotype		
B-ALL	78 (88%)	53 (90%)
T-ALL	11 (12%)	6 (10%)
No. of participating center	2	7
Allergy (CTCAE criteria)		
Grade 1	6 (7%)	0
Grade 2	7 (8%)	1 (2%)
Grade 3	7 (8%)	1 (2%)
Grade 4	0	0
Occurrence of allergy		
First infusion	2/20 (10%)	0
Second infusion	18/20 (90%)	0
Other	0	2/59 (3%)*
Silent inactivation		
yes	7/89 (8%)	0

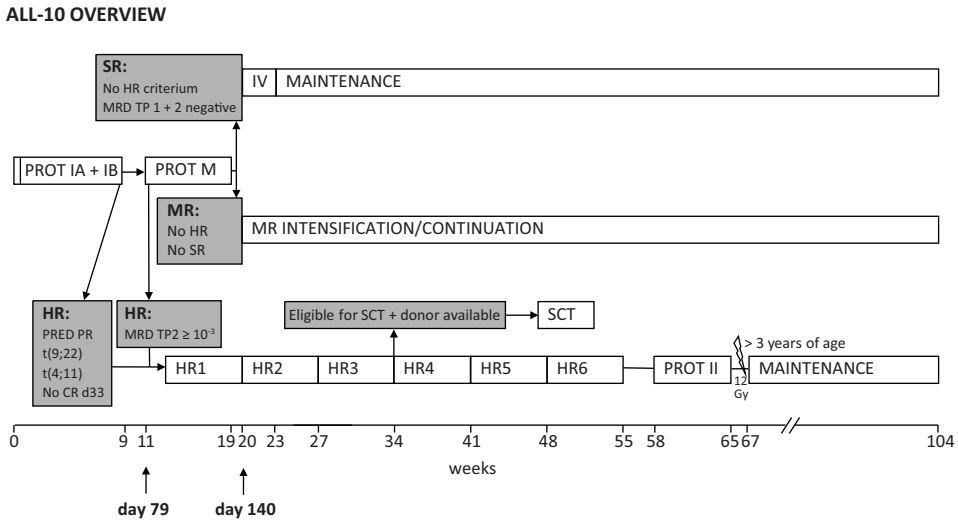
* one patient had an allergy to *Erwinia* asparaginase at the fourth infusion and the other patient at the sixth infusion.

Statistics

PEGasparaginase and *Erwinia* asparaginase activity levels had to be transformed logarithmically in order to get approximately normal distributions (as shown by the Shapiro-Wilk test). Asparaginase activity levels equal to 0 U/L were replaced by 4 U/L in order to allow the logarithmic transformation. The LLQ of asparaginase activity levels was set at <5 U/L, for further statistical calculations, we used 4 U/L in all asparaginase analysis. Pairwise comparisons of either week PEGasparaginase activity, two-week PEGasparaginase activity and *Erwinia* asparaginase activity levels were made using the paired t-test.

Changes over time of PEGasparaginase and *Erwinia* asparaginase activity levels were evaluated using mixed models analysis of variance (ANOVA), also investigating the relation with age and gender. The mean values for the asparaginase activity levels were estimated by backtransforming the mean of log-values.

We evaluated the sensitivity and specificity with 95% confidence interval of Coli-AAA at the start of the consolidation phase (at day 79) for the development of an allergy to or silent inactivation of PEGasparaginase at the start of the intensification phase (at day 140).



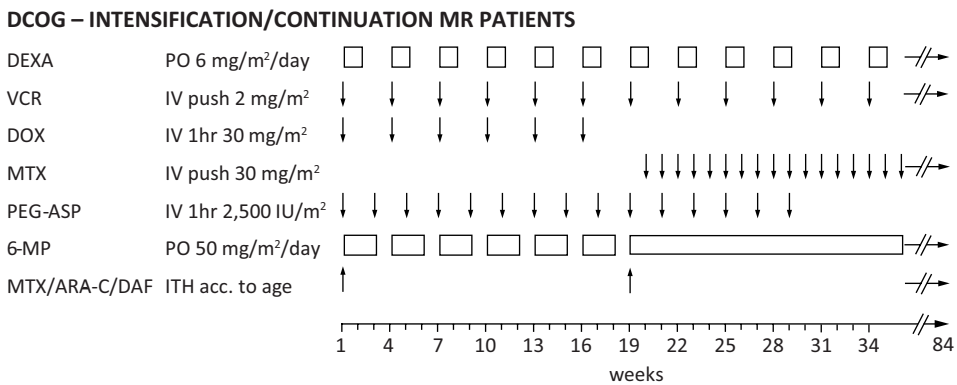
Supplemental Figure 1-A: The Dutch Childhood Oncology Group ALL-10 protocol.

SUPPLEMENTAL RESULTS

ANOVA estimates of PEGasparaginase activity levels

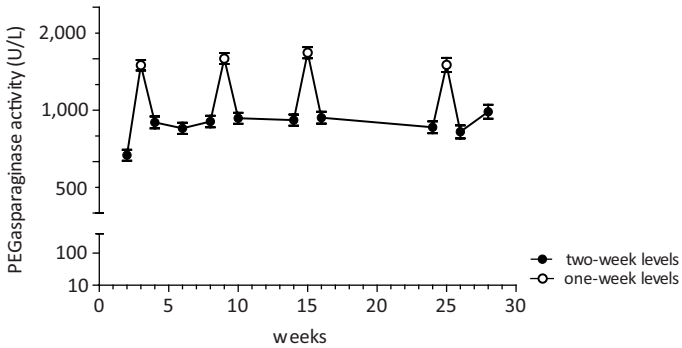
Supplemental Figure 2 presents the patients without allergy and without silent inactivation of PEGasparaginase. The mean values of PEGasparaginase activity levels were estimated at 899 U/L, much higher than 100 U/L required for complete asparagine depletion. The week PEGasparaginase activity levels were estimated at 1,679 U/L and were significantly higher as compared to the trough levels ($p<0.001$). Before start of therapy, the PEGasparaginase activity levels were zero. After the first and second PEGasparaginase dose, the mean values changed significantly ($p<0.001$).

The trough levels, thereafter, did not change significantly between week 4 till week 26 as shown by paired t-test and indicating stability after two PEGasparaginase infusions. Only before the last PEGasparaginase was given (week 28), a significant increase of the PEGasparaginase activity level after the previous infusion (week 26) was seen ($p=0.01$). The week PEGasparaginase activity levels at week 3, 9, 15 and 25 did not change significantly.

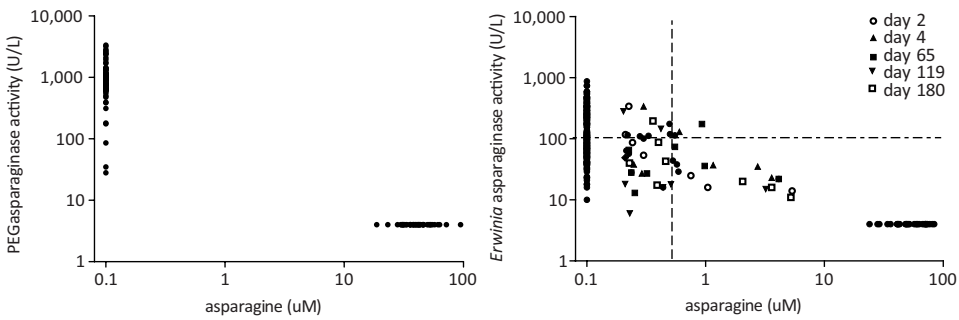


Supplemental Figure 1-B: The intensification/continuation phase of the Dutch Childhood Oncology Group ALL-10 protocol (medium risk group, MRG).

The intensification/continuation phase of the Dutch Childhood Oncology Group ALL-10 protocol (medium risk group, MRG). This material is reproduced with permission of John Wiley & Sons, Inc.



Supplemental Figure 2: Serum PEGasparaginase activity levels over time (mean ± SEM) in a dose of 2,500 IU/m2 in children without allergy to and without silent inactivation of PEGasparaginase.



Supplemental Figure 3: Relations between serum PEGasparaginase (**A**) and serum *Erwinia* asparaginase (**B**) activity levels with serum asparagine levels.

Normal values of asparagine are; 40-80 µM, the asparaginase activity levels were then below LLQ. In Figure 3-B, the horizontal dotted line indicates the *Erwinia* asparaginase activity level of 100 U/L which is associated with complete asparagine depletion. The vertical dotted line indicates the asparagine level of 0.5 µM based on different laboratory reference values in the literature to define (complete) asparagine depletion. Figure 3 A-B displays more than one sample of each study patient.

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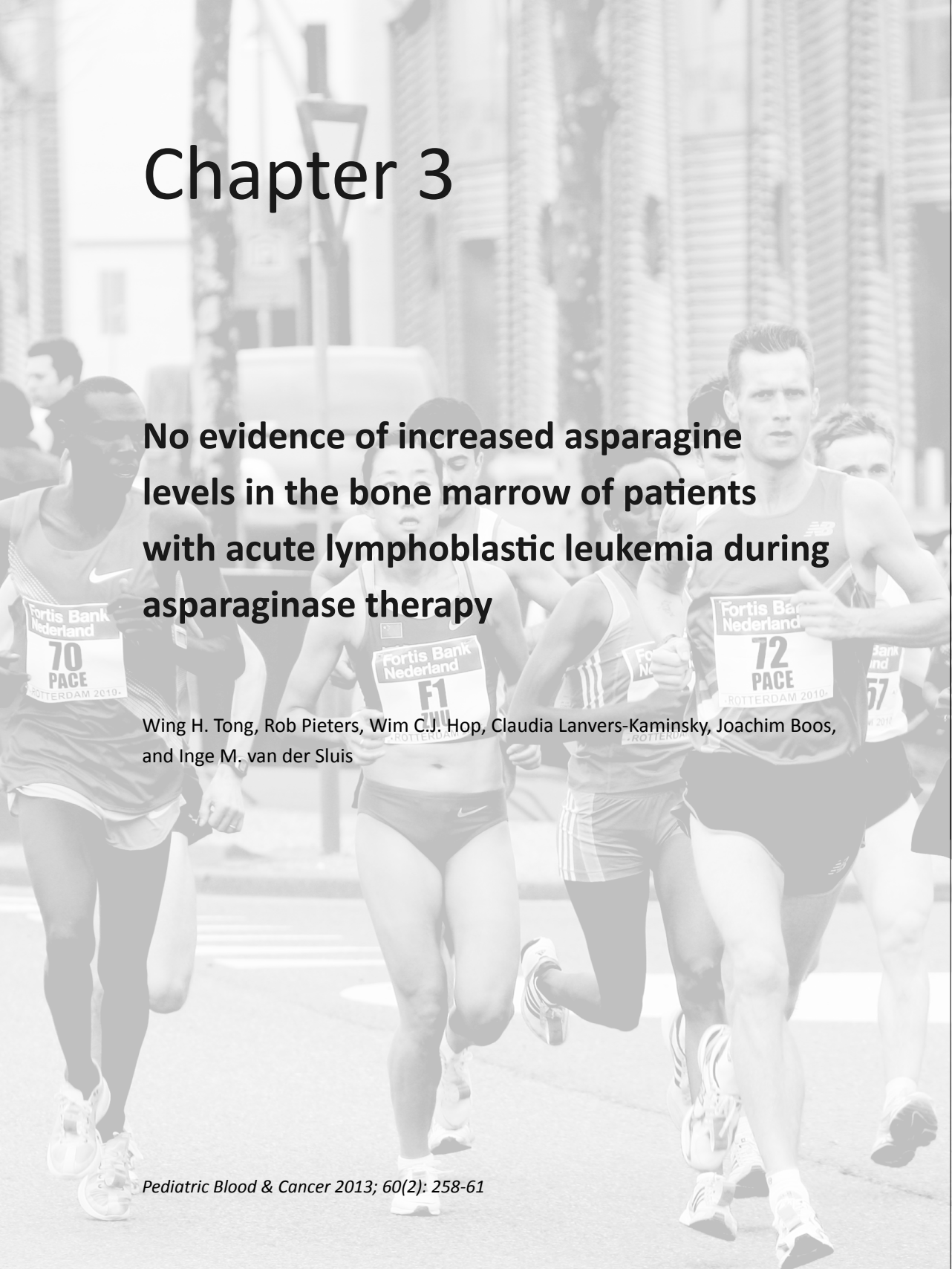
Chapter 3



No evidence of increased asparagine levels in the bone marrow of patients with acute lymphoblastic leukemia during asparaginase therapy

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ABSTRACT

Background: Mesenchymal cells (MSCs) in bone marrow (BM) may produce asparagine and form protective niches for leukemic cells. *In vitro*, this led to high levels of asparagine and conferred asparaginase resistance to acute lymphoblastic leukemia (ALL) cells. The aim of this study was to investigate whether MSCs or other cells in BM indeed produce such significant amounts of asparagine *in vivo* as to result in clinical asparaginase resistance.

Procedure: Twenty-six patients with newly diagnosed ALL were enrolled. All children received induction chemotherapy according to Dutch Childhood Oncology Group (DCOG) ALL-10 protocol. Asparaginase was administered from days 12 to 33. Asparaginase, asparagine, aspartic acid, glutamine and glutamic acid levels were measured in BM and blood at diagnosis, days 15, 33 and 79.

Results: Median asparaginase trough levels were not significantly different at days 15 and 33. Only at diagnosis, asparagine level was significantly higher in BM than in blood ($p=0.001$). Asparagine levels were all below the lower limit of quantification in BM and blood at days 15 and 33. However, aspartic acid level in BM was significantly higher than in blood ($p<0.001$) at diagnosis, and also at days 15, 33 and day 79.

Conclusions: We demonstrate higher aspartic acid levels in BM compared to blood; however, no increased asparagine levels were seen during induction therapy containing asparaginase in BM when compared to blood. Therefore, increased asparagine synthesis by MSCs is of relevance for resistance to asparaginase of leukemic cells *in vitro*, but it is questionable whether this leads to asparaginase resistance in childhood ALL patients.

INTRODUCTION

Asparaginase is an essential component of the combination chemotherapy of acute lymphoblastic leukemia (ALL)[1] and intensified use of asparaginase has been shown to significantly improve survival of children with ALL.[2-4] Mechanistically, asparaginase mediates the breakdown of asparagine into aspartic acid and ammonia (Figure 1).

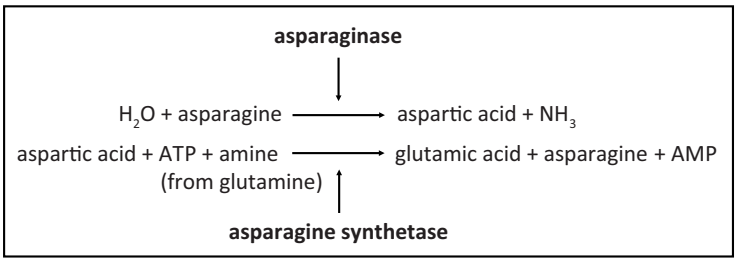

Figure 1: Asparagine breakdown mediated by asparaginase and asparagine synthesis. H_20= water; NH_3= ammonia; ATP= adenosine triphosphate; AMP= adenosine monophosphate.

Because asparagine is necessary for protein synthesis, its depletion results in cell death. Asparagine depletion may be inhibited by asparaginase-neutralizing antibodies.[5-7] A recently suggested mechanism of asparaginase resistance is the production of excess asparagine by mesenchymal cells (MSCs) in the BM, rather than by leukemic cells, which may protect the leukemic cells of asparagine depletion mediated by asparaginase.[8] Iwamoto *et al.* showed that in contrast to leukemic cells, MSCs have a high activity of asparagine synthetase. *In vitro*, this led to high levels of asparagine, and consequentially, asparaginase resistance to the ALL cells.[9] However, it is unknown if this holds true for the clinical *in vivo* situation. The aim of this study was to investigate whether MSCs or other cells in the BM indeed produce such significant amounts of asparagine *in vivo* as to result in clinical asparaginase resistance.

METHODS

Patients and treatment

This study included patients between 1 and 18 years of age with newly diagnosed ALL and on induction treatment of the Dutch Childhood Oncology Group (DCOG) ALL-10 protocol (study approved by the Institutional Review Board, informed consent was obtained from parents or children's guardians in accordance with the Declaration of Helsinki).

All children received chemotherapy according to protocol 1-A [10], consisting of prednisolone (60 mg/m^2 divided into three single doses per day; days 1-28 and tapering to zero in nine days); vincristine (1.5 mg/m^2; days 8, 15, 22 and 29); daunorubicin (30 mg/m^2; days 8, 15, 22 and 29); *E-coli* L-asparaginase (5,000 IU/m^2; days 12, 15, 18, 21, 24, 27, 30 and 33); intrathecal injection with methotrexate (MTX) at day 1 and intrathecal injections with MTX, cytosine arabinoside and prednisolone (days 15 and 33). Protocol 1-B [10] starts at day 36 with: 6-mercaptopurine (60 mg/m^2 per day; days 36-63); cyclophosphamide (1,000 mg/m^2; days 36 and 63); cytosine arabinoside (75 mg/m^2 per dose in four courses of four days) and intrathecal injections with MTX, cytosine arabinoside and prednisolone (days 45 and 59).

Laboratory procedure and processing guidelines

Serum asparaginase levels of the BM and blood were determined by incubating the samples with an excess amount of L-aspartic β-hydroxamate (AHA) at 37°C. Asparaginase hydrolyzed AHA to L-aspartic acid and hydroxylamine, which was detected at 710 nm after condensation with 8-hydroxychinoline and oxidation to indooxine.[11] Amino acid levels in the serum were measured using reversed-phase high-performance liquid chromatography (RP-HPLC) after precolumn derivation with o-phthalaldehyde (OPA) and using fluorescence detection.[12] BM and blood samples were put in an ice bath and immediately transferred to the laboratory for serum processing within 15 minutes after BM puncture or blood was drawn. These samples were centrifuged at 800g to 1,600g, and 4°C for 10 minutes. For determination of amino acid levels all samples were deproteinized to minimize the problem of ongoing asparaginase activity by adding 1 part of 10% sulphosalicyclic acid to 4 parts of the serum, thoroughly mixed and centrifuged (4°C for 5 minutes) at 10,000g to 13,000g, and the supernatant was harvested.

Asparaginase levels and amino acid levels (asparagine, aspartic acid, glutamine and glutamic acid) were measured in the BM and blood at diagnosis (day 1), day 15, day 33 and day 79. On days that asparaginase was administered (days 15 and 33) it was ensured that study material was obtained before the *E-coli* L-asparaginase infusions.

From every BM aspirate, BM smears were made to prove this was representative material. To avoid blood dilution, the first BM aspirate contained only 0.5 ml at days 15 and 33 in a subset of patients. Thereafter, 3 ml BM and blood were obtained. Of all patients at all time points, 3 ml BM and blood were obtained.

Efficacy assessment

Efficacy of treatment was determined by evaluating minimal residual disease (MRD) status at protocol day 33 (after induction phase 1-A) and at day 79 (after induction phase 1-B). MRD status was determined by the real-time quantitative polymerase chain reaction (RQ-PCR) method.

Statistics

The data were analyzed with the software packages SPSS for Windows version 17.0.2 (SPSS, Chicago, IL, USA) and GraphPad Prism version 5.01 (GraphPad Prism Inc., San Diego, CA, USA). Changes over time of asparaginase trough levels in the BM and blood were evaluated using mixed models analysis of variance (ANOVA). The amino acid levels in 0.5 ml BM, 3 ml BM and blood at days 15 and 33 were also compared using mixed models ANOVA. All these analyses were done after log-transformation of measured values to get approximate normal distributions. Median values were estimated by backtransforming mean log-values. A two-sided p-value <0.05 was considered statistically significant. Data are presented as medians (ranges) unless otherwise specified.

RESULTS

Patients

Twenty-six patients with newly diagnosed ALL (11 female) were included. Twenty-one patients had B-cell lineage ALL, five patients had T-ALL. The median age was 5.3 years (range 1.8-16.4 years). The initial median white blood count (WBC) was 14.8 x 10^9 per liter (range 1.8-569 x 10^9 per liter).

Asparaginase activity levels

Asparaginase activity levels were all below detection limit (<5 U/L) in the BM and blood at days 1 and 79. The median asparaginase trough levels in the BM and blood were not significantly different at days 15 and 33. In Figure 2, the individual asparaginase trough levels of BM and blood are shown at days 15 and 33.

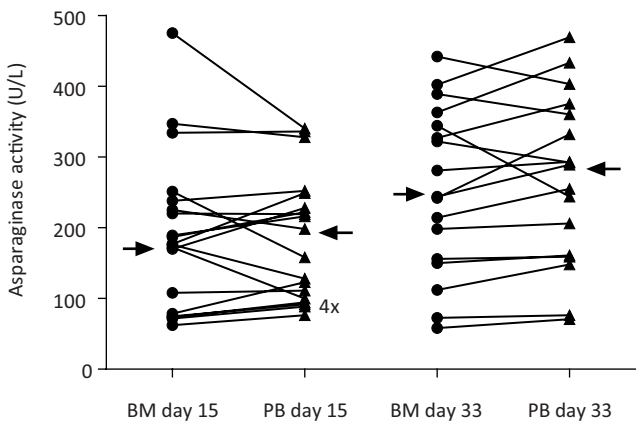

Figure 2: Individual asparaginase trough levels in the bone marrow (BM) and peripheral blood (PB) of children with acute lymphoblastic leukemia at days 15 and 33.
Individual data are connected by straight lines.
Median values are indicated by arrows.
The second lowest line labelled '4x' in the left panel represents 4 different patients.

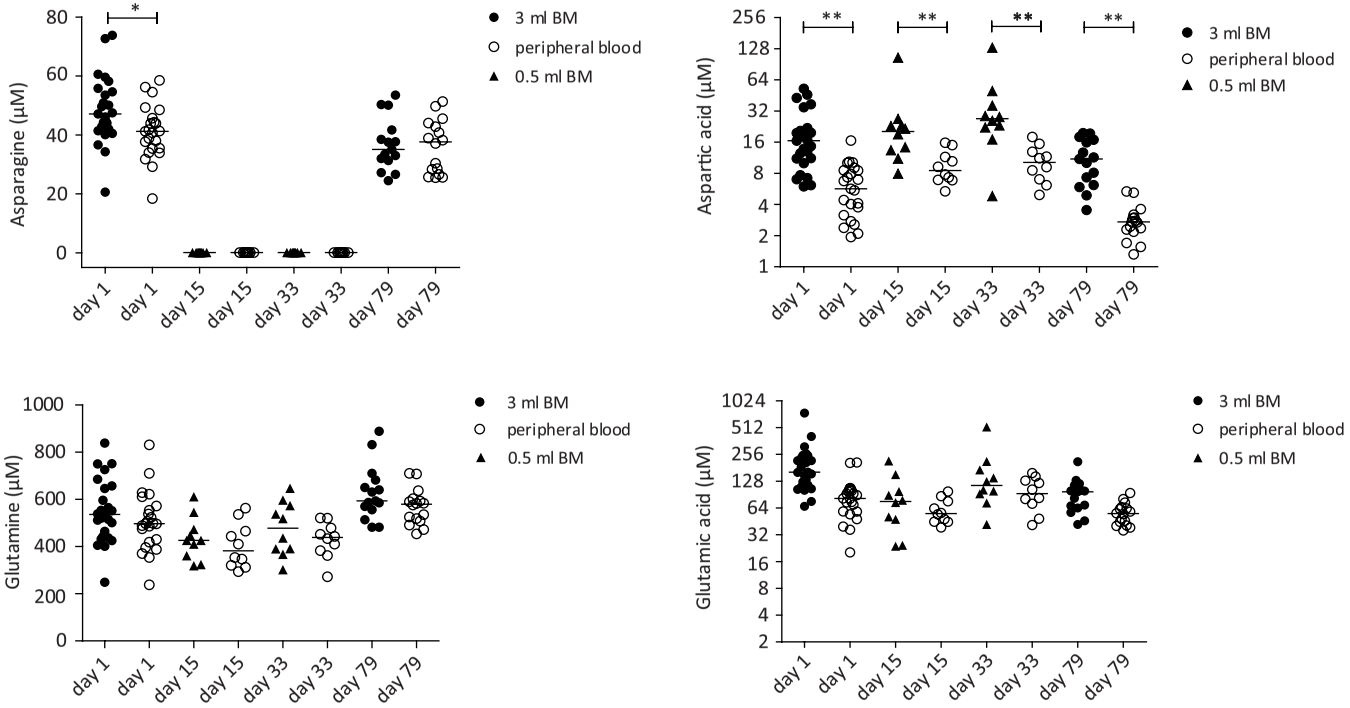

Figure 3-A,B,C,D: Individual levels of: asparagine (**A**), aspartic acid (**B**), glutamine (**C**) and glutamic acid (**D**) of children with acute lymphoblastic leukemia at days 1, 15, 33 and 79.

Bars indicate median values. * p-value = 0.001. ** p-value < 0.001.

Asparagine, aspartic acid, glutamine, glutamic acid levels

At days 1 and 79, paired data (3 ml BM, blood) were obtained from 24 and 16 patients, respectively. In total 10 paired (0.5 ml BM versus 3 ml BM versus blood) amino acids measurements were available for analysis at days 15 and 33.

At diagnosis, a significant difference (p=0.001) in asparagine levels was found between 3 ml BM and blood (median: 47.1 μM (range 20.6-73.8 μM) and 41.2 μM (range 18.4-58.5 μM), respectively). After the first asparaginase infusion at day 12 complete asparagine depletion (lower limit of quantification <0.2 μM) was reached in the BM and blood in all patients at days 15 and 33 (Figure 3A). At day 79, the median level of asparagine was 35.1 μM (range 24.5-53.5 μM) in BM and 37.7 μM (range 25.5-51.3 μM) in blood (no statistically significant difference).

However, compared to blood, higher aspartic acid levels were found in the BM at all time points (Figure 3B). At diagnosis (day 1), the median level of aspartic acid in 3 ml BM (16.5 μM; range 6.0-52.6 μM) was significantly higher when compared to the median level of blood (5.7 μM; range 1.9-16.6 μM) (p<0.001). ANOVA showed that the median aspartic acid levels did not significantly change between days 15 and 33. This applied to 0.5 ml BM, 3 ml BM and blood. At days 15 and 33 combined, the median aspartic acid level in 0.5 ml BM (23.6 μM) was significantly higher than in 3 ml BM (13.6 μM) (p=0.001) and blood (9.4 μM) (p<0.001). Aspartic acid levels in blood at day 1 were significantly higher than the levels at days 15 and 33 (both p≤0.01). Aspartic acid levels in 3 ml BM did not show significant differences between day 1 versus day 15 and day 33. At day 79, aspartic acid levels in BM (median: 11 μM; range 3.5-19.6 μM) were significantly higher (p<0.001) than in blood (median: 2.7 μM; range 1.3-5.4 μM). The glutamine and glutamic acid levels are also displayed in Figures 3C and 3D. At days 1, 15, 33 and 79, no significant changes in glutamine and glutamic acid levels were seen.

Correlations of asparagine and aspartic acid levels *versus* MRD levels

No significant correlations were found between aspartic acid levels at days 1, 15, 33 and 79 *versus* MRD levels at days 33 or 79. Similarly, asparagine levels at days 1 and 79 did not show a correlation with MRD levels. Because of complete asparagine depletion during asparaginase therapy, asparagine levels at days 15 and 33 could not be correlated with MRD levels.

DISCUSSION

These data demonstrate that levels of aspartic acid are higher in the BM compared to blood. The higher levels of aspartic acid in the BM were found at diagnosis as well as at days 15, 33 and 79. The fact that aspartic acid levels were higher in 0.5 ml BM versus 3 ml BM suggests dilution with blood when taking higher volumes of BM aspirates.

Iwamoto *et al.* showed increased asparagine production in TERT-immortalized MSCs.[9] We found high asparagine levels in the BM compared to blood, but only at diagnosis. This could indicate an increased asparagine synthesis in the BM *in vivo* by MSCs, leukemic cells or other

cells. The clinical relevance of asparagine production by MSCs during chemotherapy at days 15, 33 and 79 seems to be marginal as an increase in asparagine synthesis could not be detected at these time points. Moreover, we were able to culture MSCs successfully at diagnosis (day 1) and at complete remission (day 79), indicating that the MSCs remain viable during chemotherapy, however apparently these MSCs do not lead to increased asparagine levels *in vivo* in the BM at day 79. Asparagine levels were all below the lower limit of quantification (LLQ <0.2 µM) in 0.5 ml BM, 3 ml BM and blood during asparaginase treatment at days 15 and 33. Thus, we can conclude that asparagine synthesis in the BM by MSCs did not lead to measurable asparagine levels under asparaginase therapy.

On the other hand, it might be possible that the leukemic blasts themselves produce asparagine, at diagnosis. At diagnosis (day 1), many leukemic blasts were present in the BM, but not at days 15, 33 and 79, which is consistent with the high asparagine levels found in the BM compared to blood.

The aspartic acid levels at days 1 and 79 were higher in the BM compared to blood. At initial diagnosis, despite increased purine synthesis of the ALL cells[13] which may consume aspartic acid, the aspartic acid levels were already higher in the BM. This suggests *de novo* synthesis of aspartic acid in the BM. At complete remission (day 79), the aspartic acid levels remained higher in the BM. These data together suggest that the increased aspartic acid levels in the BM do neither originate from the leukemia cells nor from asparagine breakdown by asparaginase, but from cells in the microenvironment of the BM.

It is known that asparaginase has 3-4% glutaminase activity.[14] However, asparaginase treatment did not result in glutamine depletion in our patients. Theoretically, the glutamine levels would decrease and the glutamic acid levels would increase by asparaginase in the tube *ex vivo* if a blood or BM sample was processed too late. However, the glutamine and glutamic acid levels did not show this pattern in our study as we strictly followed the sampling procedures which consisted of immediately transfer in an ice bath and processing within 15 minutes after BM puncture or blood was drawn. Also, to minimise the problem of ongoing asparaginase activity in the tube, we rapidly deproteinized the BM and blood samples for amino acid analysis.[15] Therefore, the absence of asparagine at days 15 and 33 is not due to processing errors.

This study has some limitations. The number of patients is small. Furthermore, we have not measured amino acid levels in cultured MSCs. So, no correlations could be made between MSCs and asparagine production.

In conclusion, our data showed higher aspartic acid levels in the BM compared to blood. These levels neither originate from the leukemia cells nor from asparagine breakdown by asparaginase, but from cells in the microenvironment of the BM. Asparagine levels were higher in the BM compared to blood only at diagnosis, a finding consistent with increased asparagine synthesis within the BM of ALL patients. However, asparagine was not detectable in the BM during therapy with asparaginase. Therefore, increased asparagine synthesis by MSCs is of relevance for resistance to asparaginase of leukemic cells *in vitro*, but it is questionable whether this leads to asparaginase resistance *in vivo* in ALL patients.

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Chapter 4

Should we use a desensitization protocol in acute lymphoblastic leukemia patients with silent inactivation of PEGasparaginase?

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ABSTRACT

We analyzed whether continuation of asparaginase in case of silent inactivation may result in desensitization, disappearance of asparaginase antibodies and recovery of asparaginase activity levels in children with newly diagnosed acute lymphoblastic leukemia.

Patients received PEGasparaginase or *Erwinia* asparaginase in intensification after native *E.coli* asparaginase in induction. Serum asparaginase antibodies and asparaginase activity levels were analyzed.

7/89 patients had silent inactivation of PEGasparaginase. 2 were detected by real-time measurements and switched to *Erwinia* asparaginase. 5 patients continued PEGasparaginase as their drug levels became available later in time and recovered after 2-7 PEGasparaginase infusions once every 2 weeks. In all 5 patients, anti-*Coli*-asparaginase-antibodies were present at start of intensification and declined coinciding with rise of PEGasparaginase activity levels. Anti-PEGasparaginase-antibodies were absent at start, increased after 1-2 PEGasparaginase doses, and declined thereafter also coinciding with recovery of drug levels.

In a different cohort of 59 patients treated with *Erwinia* asparaginase, there was 0% silent inactivation and 3% allergy. Anti-*Erwinia*-asparaginase-antibodies were absent at start of therapy. In 50% of patients, anti-*Erwinia*-asparaginase-antibodies gradually increased and again decreased to absent baseline values during therapy with *Erwinia* asparaginase 2-3x weekly.

This unintended desensitization program applied in 5 patients with silent inactivation of PEGasparaginase leads to recovery of drug levels. However, as this recovery takes an unpredictable time period, we do not advise such desensitization approaches, but recommend switching to *Erwinia* asparaginase.

INTRODUCTION

Asparaginase is one of the most important drugs against acute lymphoblastic leukemia (ALL).[1] Intensified asparaginase therapy has shown to increase event-free survival (EFS) in childhood ALL by 10-15%.[2-5] Asparaginase breaks down asparagine into aspartic acid and ammonia resulting in apoptosis of ALL cells, that depend on asparagine for its protein synthesis. Formation of asparaginase antibodies neutralizes asparaginase which is accompanied by a clinically overt allergic reaction, but in part of the patients without any clinical signs of hypersensitivity, termed as silent inactivation.[6,7]

Vrooman *et al.* and Panosyan *et al.* showed that children with silent inactivation of native *E.coli* asparaginase had poorer outcomes as they were not switched to another asparaginase preparation that retained its activity.[8,9] Recently, a case report described the successful use of desensitization courses in a patient with severe hypersensitivity reaction to native *E.coli* asparaginase.[10] Therefore, we analyzed childhood ALL patients from our drug monitoring program to see whether continuation of asparaginase in case of silent inactivation may have resulted in desensitization, disappearance of asparaginase antibodies and recovery of asparaginase activity levels.

We present asparaginase antibodies and asparaginase activity levels of five silent inactivation patients who continued with PEGasparaginase because the results of the drug monitoring program were available only in a later phase. We compared these data with those of patients without an allergy and without silent inactivation of PEGasparaginase. Finally, we re-evaluated the course of *Erwinia* asparaginase antibodies and asparaginase activity levels of patients who were switched to long-term *Erwinia* asparaginase from the perspective of desensitization.

DESIGN AND METHODS

Patients and DCOG ALL-10 protocol

Children with newly diagnosed ALL were enrolled in the Dutch Childhood Oncology Group (DCOG)-ALL-10 protocol and were stratified into three risk groups after induction treatment: standard risk (SR), medium risk (MR) and high risk (HR). The Institutional Review Board approved this ALL-10 protocol. Informed consent was obtained from parents or their children's guardians in accordance with the Declaration of Helsinki.

All patients received eight doses native *E.coli* asparaginase (5,000 IU/m^2, every three days) in induction. In the MR group, PEGasparaginase (2,500 IU/m^2, every other week) was given during the first 30 weeks of the intensification/reinduction course. In case of an allergy to PEGasparaginase, the patient was switched to *Erwinia* asparaginase (20,000 IU/m^2, per dose) 2-3 times per week to complete 30 weeks of *Erwinia* asparaginase therapy.[11] All asparaginase preparations were administered intravenously.

In the starting phase of our drug monitoring program real-time asparaginase measurements were not available, and patients with silent inactivation were not recognized in time and continued with PEGasparaginase.

Study design and definitions

Serum asparaginase activity trough levels and serum asparaginase antibodies were measured at 15 and 13 different time points for PEGasparaginase and *Erwinia* asparaginase analysis, respectively.

Allergy was graded according to the National Cancer Institute (NCI) Common Terminology Criteria for Adverse Events (CTCAE) version 3.0. Silent inactivation was defined as serum PEGasparaginase activity level below 100 U/L at day 7 ± 1 or below 20 U/L at day 14 ± 1 after PEGasparaginase infusion in absence of a clinical allergy. Silent inactivation of *Erwinia* asparaginase was defined as serum asparaginase activity level below 20 U/L at day 2 after administration of *Erwinia* asparaginase in absence of a clinical allergy.

Asparaginase activity levels and asparaginase antibodies

Serum asparaginase activity levels were processed and assessed as described earlier.[12,13] Serum antibodies against native *E.coli* asparaginase (Asparaginase medac®) (Coli-AAA), against PEGasparaginase (Oncaspar®) (PEG-AAA), and against *Erwinia* asparaginase (*Erwinia*-AAA) were measured by enzyme-linked immunosorbent assays (submitted). All asparaginase antibodies were expressed as optical densities (OD) readings. Samples were defined as positive for asparaginase antibodies if the Coli-AAA OD >0.13; if the PEG-AAA OD >0.25, and for *Erwinia*-AAA if the OD >1.96 standard deviations above the negative control processed mean (using Westgard rules).[14]

Statistical analysis

The data were analyzed with the software packages SPSS for Windows version 20.0.0.1 (SPSS, Chicago, IL, USA) and GraphPad Prism version 5.01 (GraphPad Prism Inc., San Diego, CA, USA). The Coli-AAA, PEG-AAA, and PEGasparaginase activity levels of five patients with silent inactivation of PEGasparaginase were analyzed with individual curves. The *Erwinia*-AAA and *Erwinia* asparaginase activity levels of all patients without an allergy to *Erwinia* asparaginase were also analyzed with individual curves. Changes over time of PEGasparaginase activity levels were evaluated using mixed models analysis of variance (ANOVA). The mean values were estimated by backtransforming the mean of log-values. PEGasparaginase activity levels are presented as mean ± SEM.

RESULTS

Seven out of 89 patients (8%) had silent inactivation of PEGasparaginase. Two out of seven silent inactivation patients were switched to *Erwinia* asparaginase based upon real-time asparaginase measurements after two PEGasparaginase infusions showing asparaginase activity levels of zero. The other five patients continued with PEGasparaginase, because real-time asparaginase measurements were not available at that moment. All five patients achieved sufficient PEGasparaginase activity levels while continuing with PEGasparaginase.

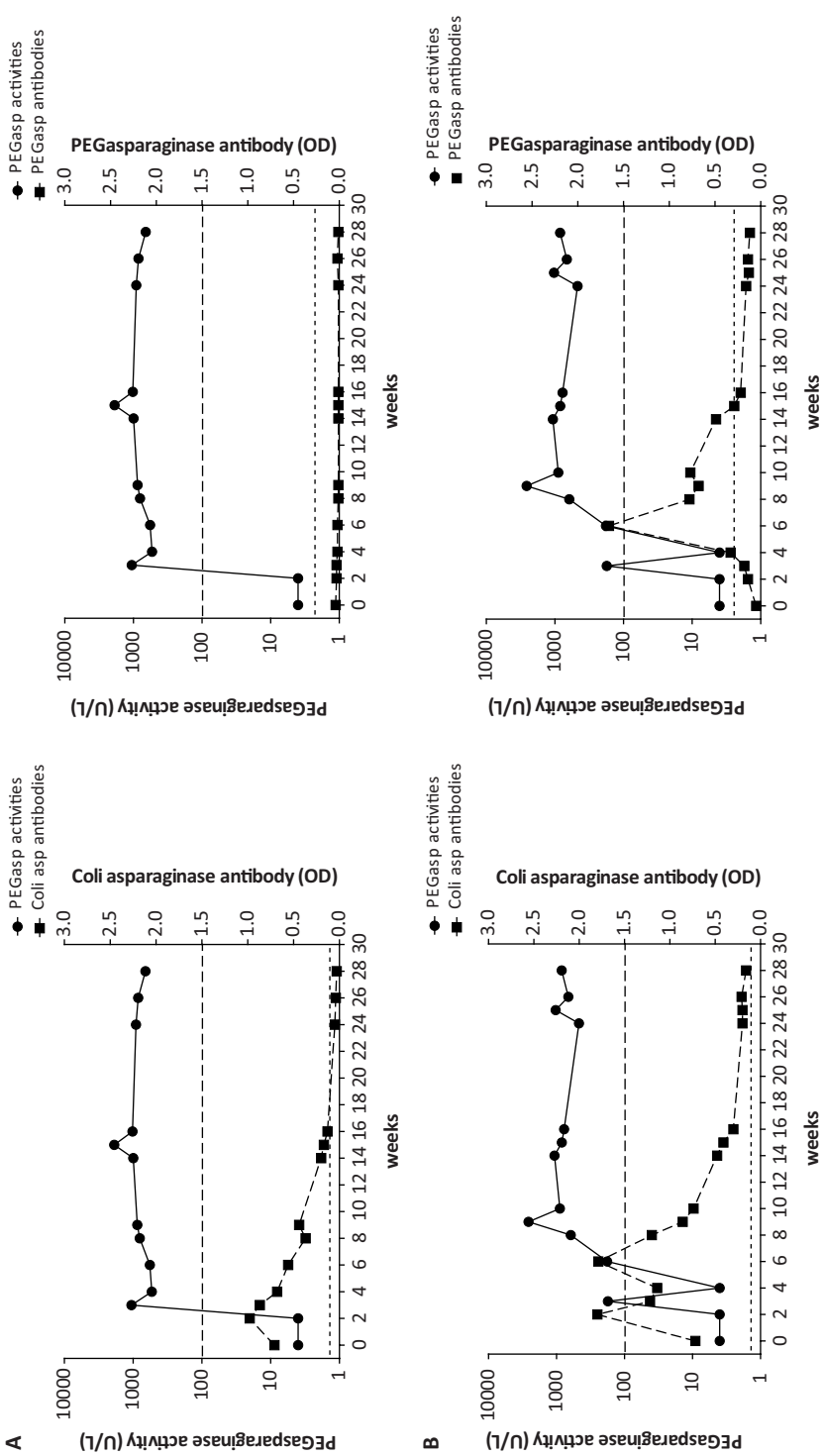
Figure 1 shows the Coli-AAA and PEG-AAA per patient in relation to the PEGasparaginase activity levels over time in these five patients.

Patient (A) had PEGasparaginase trough activity level of 536 U/L after the second dose of PEGasparaginase. Three patients (B-D) had PEGasparaginase trough activity levels ≥100 U/L after the third PEGasparaginase infusion (181 U/L; 1485 U/L; 587 U/L, respectively). Patient (E) showed a PEGasparaginase trough activity level of 581 U/L after the seventh dose of PEGasparaginase. All PEGasparaginase trough activity levels were zero in all five patients till these became measurable. In all five patients, Coli-AAA were present already before the start of the first PEGasparaginase infusion. Thereafter, the Coli-AAA declined in all five patients during PEGasparaginase therapy coinciding with the rise of asparaginase activity levels. PEG-AAA were absent in all 5 patients at the start of the intensification phase. In one patient (A) these PEG-AAA remained absent, in two patients (C-D) there was a mild increase followed by a decrease to zero and in two patients (B, E) a strong increase and decrease to zero. The decrease coincided with the rise of asparaginase activity levels.

For comparison, Figure 2 shows the Coli-AAA, PEG-AAA and PEGasparaginase activity levels of patients without an allergy and without silent inactivation.

29% of the patients were positive for Coli-AAA before the start of intensification. Also in this group of patients, the Coli-AAA gradually decreased and after five PEGasparaginase infusions, the Coli-AAA levels were below the cut-off. PEG-AAA were absent at the start, gradually increased but remained below the cut-off level. After four doses of PEGasparaginase, the PEG-AAA levels gradually decreased to absent baseline values during therapy. Both asparaginase antibodies did not affect the PEGasparaginase activity levels which remained stable after two PEGasparaginase infusions until end of therapy, but it should be noted that patients with silent inactivation were removed from this group as well as the patients with clinical allergy (Figure 2-C).

59 patients were treated to complete 30 weeks of exposure with *Erwinia* asparaginase 2-3 times per week, after having experienced allergy to or silent inactivation of PEGasparaginase. Two patients developed allergic reactions to *Erwinia* asparaginase and no patient developed silent inactivation of *Erwinia* asparaginase. In Figure 3, the *Erwinia*-AAA and *Erwinia* asparaginase activity levels of patients without an allergy to *Erwinia* asparaginase are shown as individual curves.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B
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Figure 1-A,B

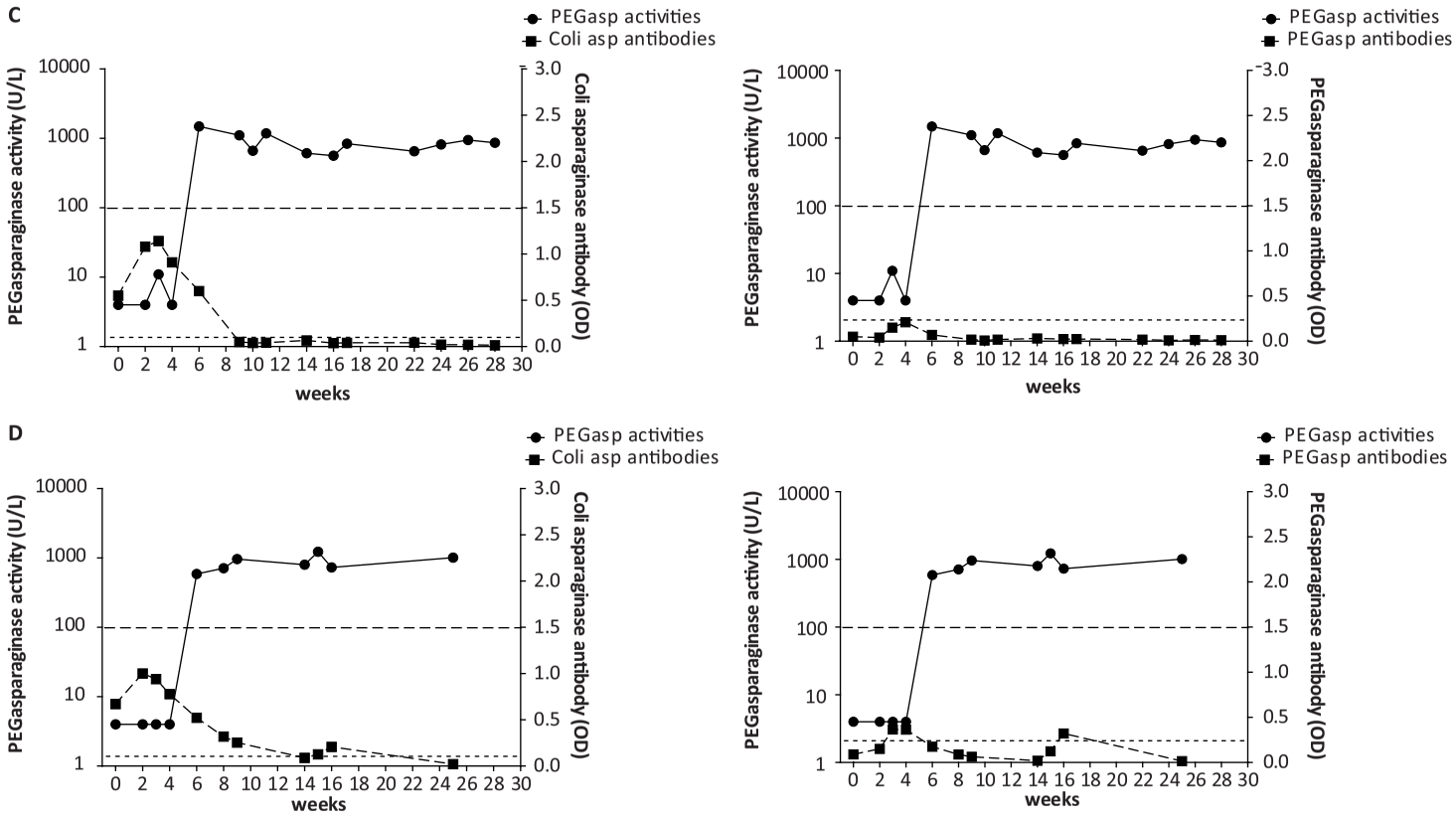
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Figure 1-C,D

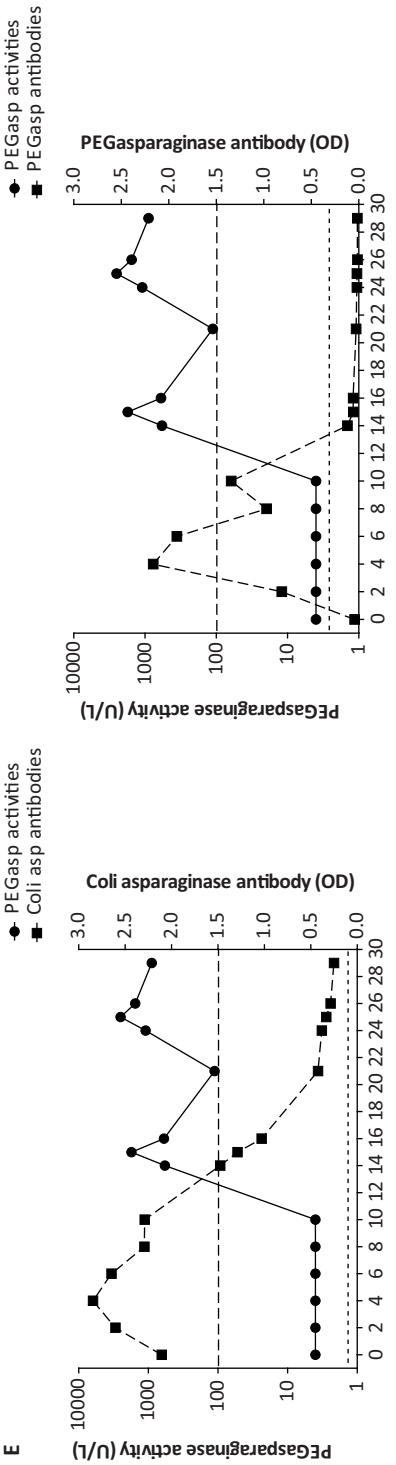

Figure 1-A,B,C,D,E: Individual Coli-AAA levels and PEG-AAA levels (OD) *versus* PEGasparaginase activity levels (U/L) of children with silent inactivation of PEGasparaginase.

Upper horizontal dotted line; PEGasparaginase activity level of 100 U/L which is associated with complete asparagine depletion (lower level of quantification of 0.2 μM).
Lower horizontal dotted line; above the cut-off: Coli-AAA and PEG-AAA positive.

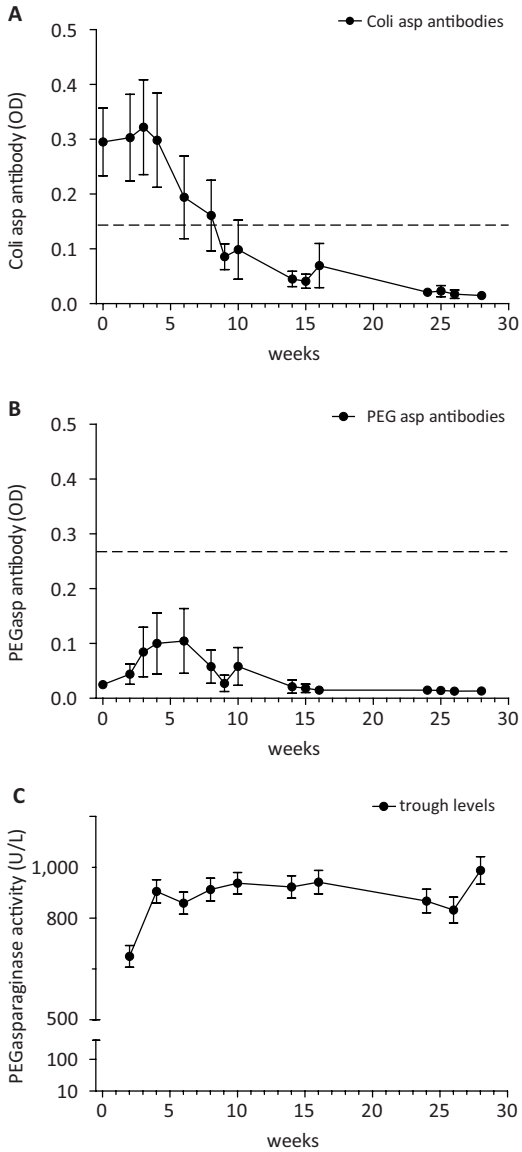

Figure 2-A,B,C: Coli-AAA levels (**A**), PEG-AAA levels (**B**), and PEGasparaginase activity levels (**C**) of children without an allergy and without silent inactivation of PEGasparaginase.
Panels A and B; mean ± SEM. Dotted horizontal lines; above the cut-offs: Coli-AAA and PEG-AAA positive.
Panel C; mean ± SEM.

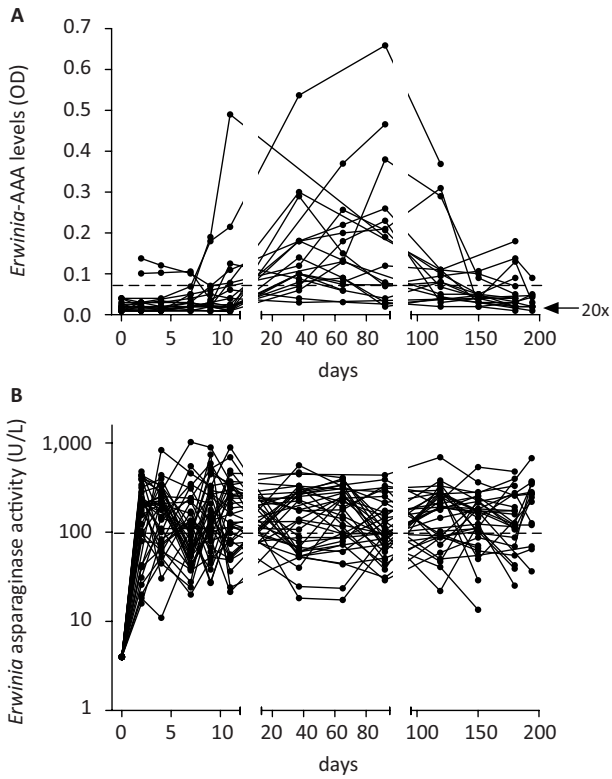

Figure 3-A,B: Individual *Erwinia*-AAA levels (**A**) and individual *Erwinia* asparaginase activity levels (**B**) of children without an allergy and without silent inactivation of *Erwinia* asparaginase.
Panel A; dotted horizontal line; above the cut-offs: *Erwinia*-AAA positive.
Panel B; mean ± SEM. The lowest line indicated by an arrow and labelled '20x' represents 20 different *Erwinia* asparaginase patients.

None of these patients were positive for *Erwinia*-AAA before the start of *Erwinia* asparaginase therapy. Four patients developed *Erwinia*-AAA in the first two weeks of therapy. The *Erwinia*-AAA were mainly present during therapy at days 37, 65 and 92 of therapy. In total 50% of the non-allergic patients, the *Erwinia*-AAA again gradually decreased to absent baseline values for nearly all patients. The *Erwinia*-AAA did not affect *Erwinia* asparaginase activity levels until end of therapy, but again it should be noted that the two patients with a clinical allergy to *Erwinia* asparaginase were removed from this group (Figure 3-B).

DISCUSSION

These data demonstrate that asparaginase antibodies decline over time, in patients with silent inactivation of PEGasparaginase, and also in patients without an allergy to PEGasparaginase or *Erwinia* asparaginase. Also in patients without an allergy and without silent inactivation, antibodies against PEGasparaginase and *Erwinia* asparaginase developed and decreased back to normal levels when continuing asparaginase for (very) prolonged time periods.

Previous reports described desensitization of patients with clinical allergies to asparaginase. Desensitization protocols were designed to administer asparaginase in a much lower starting dose and prolonged infusion time.[10,15,16] The dose needs to be increased gradually, for instance within a few days, till the total dose is given.[10] Continuous administration of the antigen leads to a decrease of T-helper 2 cells (secreting IgE which is associated with an allergic reaction) and increase of T-helper 1 cells (responsible for delayed hypersensitivity and synthesis of IgG) resulting in less allergic symptoms.[17] It has been suggested that immunosuppression induced by combined dexamethasone and asparaginase may lower antibody formation.[18] The influence of subsequent dexamethasone courses as used in this therapy schedule for 5 days every 3 weeks could play an additional role in PEGasparaginase desensitization.

In contrast to the allergic patients, PEGasparaginase was continued without any clinical signs in our patients with silent inactivation. This, finally, resulted in a decrease of asparaginase antibodies and recovery of therapeutic PEGasparaginase activity levels (≥100 U/L) in all patients. We thereby, unintentionally, desensitized the patients by continuing the full PEGasparaginase dose every two weeks. Sufficient PEGasparaginase activity levels were found for the first time after 2-7 PEGasparaginase infusions, so after 2-12 weeks in these patients with silent inactivation who continued PEGasparaginase.

Of note, it is questionable if patient (A) indeed showed a truly silent inactivation. This patient had a PEGasparaginase trough activity level of 536 U/L after two PEGasparagnase infusions. To avoid unintended silent inactivation diagnosis, we suggest performing two independent sampling to confirm silent inactivation if this is suspected. Also, it should be ensured that the correct asparaginase preparation is administered, not native *E.coli* asparaginase with a short half-life instead of the less immunogenic PEGasparaginase as we reported earlier.[19]

The important question is how to handle in case of allergy to or silent inactivation of PEGasparaginase: use a desensitization protocol or switch preparation to *Erwinia* asparaginase? Most childhood ALL protocols prescribe PEGasparaginase during a much shorter intensification period than 30 weeks. Therefore, no time is available to apply a “wait-and-see policy” and to wait whether desensitization occurs during an uncertain time for instance 2-12 weeks as found in the present study. As the intensification phase is of crucial importance in the treatment of ALL and adequate asparaginase therapy improves outcome, it is not worth taking the risk of a desensitization course with an uncertain outcome. Therefore, we recommend a switch to *Erwinia* asparaginase in case of allergy to or silent inactivation of PEGasparaginase.

In conclusion, our data show that five silent inactivation patients and continuing with PEGasparaginase had antibodies which declined over time. These patients had therapeutic PEGasparaginase activity levels thereafter. However, as recovery of asparaginase activity levels takes an unpredictable and sometimes long time period, we do not advise such desensitization approaches, but we recommend a switch to *Erwinia* asparaginase. A significant proportion of patients treated for prolonged period with PEGasparaginase or *Erwinia* asparaginase develops antibodies without influencing asparaginase activity levels that disappear with continued use of the same asparaginase product.

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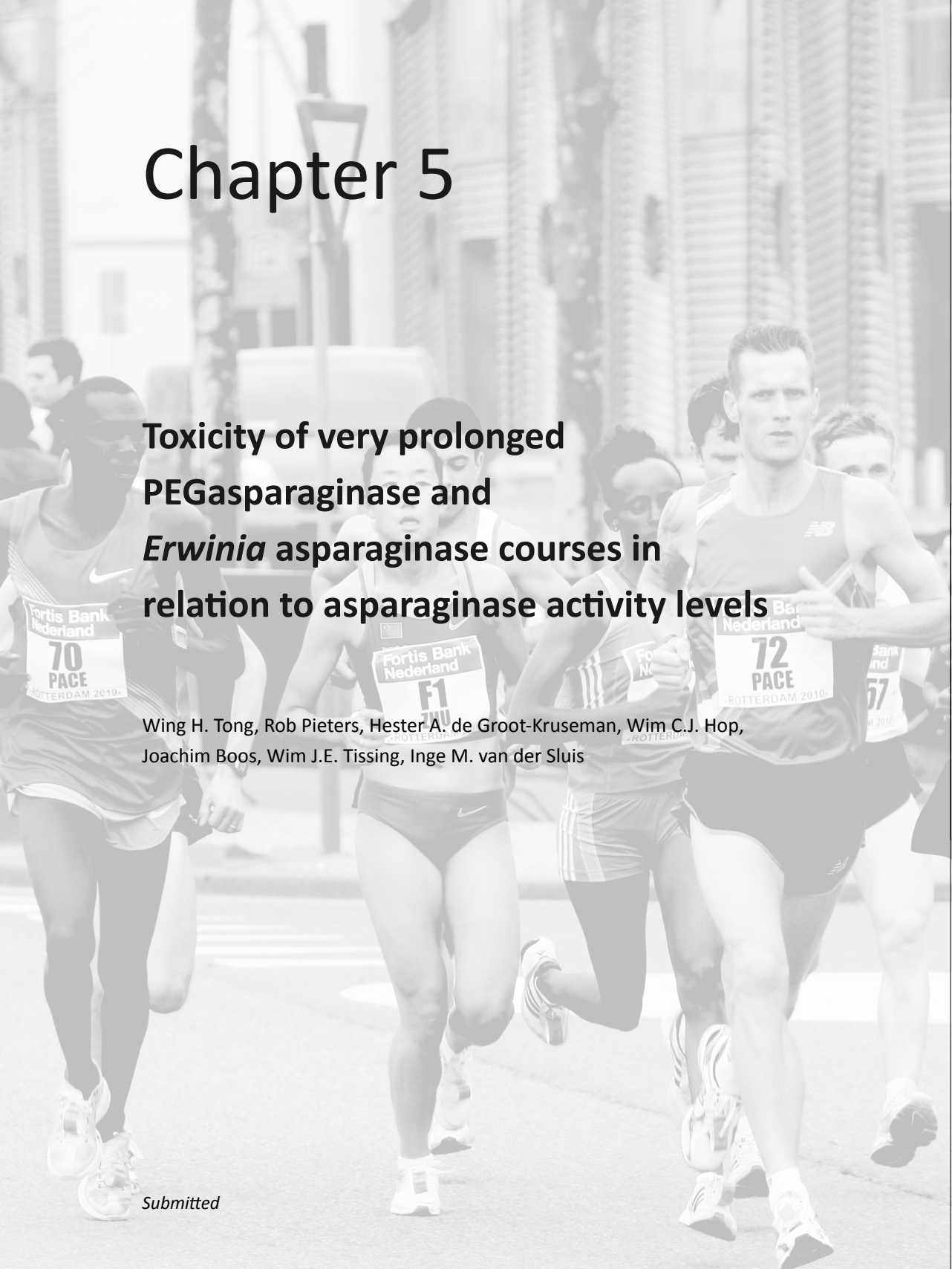
Chapter 5



Toxicity of very prolonged PEGasparaginase and *Erwinia* asparaginase courses in relation to asparaginase activity levels

Wing H. Tong, Rob Pieters, Hester A. de Groot-Kruseman, Wim C.J. Hop, Joachim Boos, Wim J.E. Tissing, Inge M. van der Sluis

Submitted

ABSTRACT

We prospectively studied the incidence and clinical course of hypertriglyceridemia and hypercholesterolemia during very prolonged use of PEGasparaginase or *Erwinia* asparaginase in relation to asparaginase activity levels in children with acute lymphoblastic leukemia (ALL). Also, the incidence of pancreatitis, thrombosis, hyperammonemia and central neurotoxicity and their association with asparaginase activities were evaluated.

Patients were treated according to Dutch Childhood Oncology Group (DCOG) ALL-10 medium risk intensification protocol, which includes 15 doses of PEGasparaginase (2,500 IU/m^2) for 30 weeks. *Erwinia* asparaginase (20,000 IU/m^2) was administered when an allergy to or silent inactivation of PEGasparaginase occurred.

89 patients were enrolled from two pediatric oncology centers. Triglyceride, cholesterol and ammonia levels increased rapidly and remained temporary elevated using each of both agents, but normalized after finishing the last asparaginase dose. Hypertriglyceridemia and hypercholesterolemia grade 3/4 were found in 47% and in 25%, respectively, of the patients treated with PEGasparaginase. No grade 3/4 dyslipidemia was found in patients receiving *Erwinia* asparaginase. Hyperammonemia grade 3/4 was only found in *Erwinia* asparaginase treated patients (10%). Triglyceride and cholesterol levels were significantly related to asparaginase activities. No associations were found between pancreatitis and hypertriglyceridemia nor between ammonia and central neurotoxicity. Thrombosis occurred in 4.5%, pancreatitis in 6%, and central neurotoxicity in 10% of the patients; these toxicities were not related to asparaginase activity levels.

In conclusion, severe dyslipidemia occurred frequently, but was temporary and is no clinical reason to interrupt asparaginase. Only dyslipidemia was related to asparaginase activities. The toxicities; pancreatitis, thrombosis, and central neurotoxicity were not related to asparaginase activities.

INTRODUCTION

Asparaginase is a key component in the treatment of acute lymphoblastic leukemia (ALL). Many studies have shown that intensification of asparaginase treatment is essential to improve event-free survival of children with ALL.[1-5]

The use of asparaginase is associated with multiple toxicities including thrombosis, pancreatitis, hyperammonemia, central neurotoxicity, and relatively common, hypertriglyceridemia and hypercholesterolemia (dyslipidemia). Although these are well-known side effects, the incidence and natural course of dyslipidemia during very prolonged PEGasparaginase and *Erwinia* asparaginase courses are unknown. Also, it is unclear whether asparaginase therapy should be interrupted or stopped in case of these toxicities and whether the different toxicities are related to asparaginase activity levels.

Therefore, we analyzed the incidence of dyslipidemia and its natural course during very prolonged PEGasparaginase or *Erwinia* asparaginase courses in children with ALL. Furthermore, other asparaginase-associated toxicities such as pancreatitis, hyperammonemia, thrombosis and central neurotoxicity were studied prospectively and related to asparaginase activity levels.

METHODS

Patients and ALL-10 treatment protocol

From July 2009 till October 2012, ALL-10 medium risk (MR) patients from two pediatric oncology centers (Rotterdam and Groningen, the Netherlands) were included in this study. The Institutional Review Board approved this study before patient enrollment. Informed consent was obtained from parents or their guardians and from patients ≥12 years of age in accordance with the Declaration of Helsinki.

All patients received eight doses native *E.coli* asparaginase (5,000 IU/m^2 per dose) every three days in induction. The intensification/continuation phase of the ALL-10 MR treatment included PEGasparaginase as first-line agent (2,500 IU/m^2 per dose) every two weeks for a total of 15 doses (Supplemental Figure 1). In case of an allergy to or silent inactivation of PEGasparaginase, the patient was switched to *Erwinia* asparaginase as second-line agent (20,000 IU/m^2 per dose) 2-3 times per week to complete 30 weeks of asparaginase therapy. Asparaginase was administered intravenously in one hour.

Study design and definitions

For practical reasons, the measurements of triglyceride, total cholesterol, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, glucose, amylase and ammonia were non-fasting. Blood samples were taken at week 1 (baseline), week 3, 5, 7, 9, 15 and 25 during the intensification phase including asparaginase and week 37, eight weeks after the final PEGasparaginase or six weeks after the final *Erwinia* asparaginase infusion. Additionally,

in a subset of patients, we measured lipoprotein lipase (LPL) and hepatic lipase (HL) activities at weeks 1, 19 and 37.

Hypertriglyceridemia, hypercholesterolemia, hyperammonemia, pancreatitis, thrombosis and central neurotoxicity were graded prospectively according to the National Cancer Institute (NCI) Common Terminology Criteria for Adverse Events (CTCAE) version 3.0. Thrombosis was scored only if it was not related to a central venous line. To study the relation between ammonia level and central neurotoxicity, we prospectively collected central neurotoxicity items on structured case record forms assessed by the pediatric oncologists.

These items were ataxia, somnolence or depressed level of consciousness, mood alteration and seizures. Central neurotoxicity included posterior reversible encephalopathy syndrome (PRES) which was not mentioned in the CTCAE.

Laboratory measurements

Asparaginase activity levels were processed and assessed as described earlier.[6] Triglyceride, cholesterol, LDL, HDL, glucose, amylase and ammonia levels were analyzed in both medical centres. LPL and HL activities were only analyzed in Rotterdam. Samples for ammonia levels, LPL, and HL determinations were put in an ice bath and were immediately processed at the laboratory. LPL and HL activities were assessed as described earlier.[7]

Statistical analysis

The data were analyzed with the software packages SPSS for Windows version 20.0.0.1 (SPSS, Chicago, IL, USA) and GraphPad Prism version 5.01 (GraphPad Prism Inc., San Diego, CA, USA). Changes over time of triglyceride, cholesterol, LDL, HDL, glucose, amylase and ammonia levels were evaluated using mixed models analysis of variance (ANOVA). Changes related to age and gender were also investigated using mixed models ANOVA. All analyses were done after log-transformation of measured values to get approximate normal distributions. Mean values were estimated by backtransforming the mean of log-values. Spearman correlation coefficients were used to evaluate the relations between triglyceride, cholesterol and asparaginase activity levels. A two-sided p-value <0.05 was considered statistically significant. Data are presented as mean ± SEM or specified otherwise.

RESULTS

We enrolled 89 patients (49% boys). The median age was 4.9 years (range 1.2-16.2 years), 78 (88%) patients had precursor B-ALL and 11 (12%) patients had T-ALL. 22 out of 89 (25%) patients were switched to *Erwinia* asparaginase because of either allergy to or silent inactivation of PEGasparaginase.

Table 1 summarizes the toxicity data.

Table 1: Toxicity of PEGasparaginase and *Erwinia* asparaginase.

	PEGasparaginase (n=67)				*Erwinia* asparaginase (n=22)			
	Grade 1/2		Grade 3/4		Grade 1/2		Grade 3/4	
	n	%	n	%	n	%	n	%
Pancreatitis	0	0	3	5	1	5	2	9
Hypertriglyceridemia	15	22	31	47	7	32	0	0
Hypercholesterolemia	6	9	17	25	8	37	0	0
Hyperammonemia	34	51	0	0	9	41	2	9
Thrombosis[1]	0	0	2	3	0	0	2	9
Central neurotoxicity	0	0	7	10	0	0	1	5

[1] Non-vascular access-related.

Three patients developed severe pancreatitis during PEGasparaginase and two patients during *Erwinia* asparaginase; these patients discontinued asparaginase permanently. No grade 3/4 dyslipidemia was found in patients receiving *Erwinia* asparaginase. However, hypertriglyceridemia and hypercholesterolemia grade 3/4 were found in 47% and in 25%, respectively, of the patients treated with PEGasparaginase. Hyperammonemia grade 3/4 occurred in two patients (9%) treated with *Erwinia* asparaginase, but in none of the PEGasparaginase treated patients.

Two patients receiving PEGasparaginase and two other patients receiving *Erwinia* asparaginase developed thrombosis, all localized in the sinus sagitallis superior. Asparaginase therapy was stopped and again reintroduced with low molecular weight heparin prophylaxis after resolution of the vascular obstruction in all four patients. No recurrence of thrombosis was seen thereafter. Central neurotoxicity grade 3/4 occurred in 10% of patients treated with PEGasparaginase and in 5% of those treated with *Erwinia* asparaginase. Central neurotoxicity included somnolence or seizures (grade 3 or 4) or posterior reversible encephalopathy syndrome (PRES).

For none of the grade 3/4 toxicities (pancreatitis, thrombosis, central neurotoxicity), a difference was found between PEGasparaginase versus *Erwinia* asparaginase (all p>0.27).

Clinical course of dyslipidaemia and associations with asparaginase activity levels

The median triglyceride (0.8 mmol/L, upper limit of normal (ULN): 1 mmol/L) and the median cholesterol 3.7 mmol/L (ULN: 5 mmol/L) levels at start of intensification were normal. Figure 1-A shows that after the first and second PEGasparaginase infusion, a significant increase in triglyceride levels was seen at weeks 3 and 5 (p<0.001).

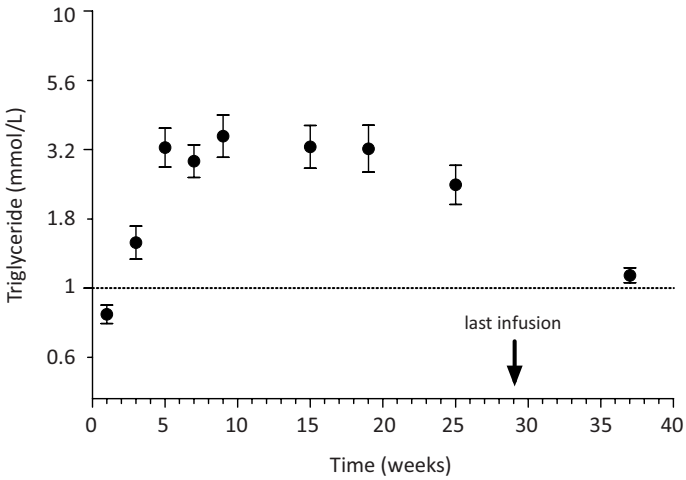


Figure 1-A: Serum triglyceride levels over time during PEGasparaginase therapy (mean ± SEM).
The dotted line; upper limit of normal.

From week 5 until the last PEGasparaginase infusion was given, triglyceride levels remained high and no significant changes were seen. Cholesterol levels increased significantly ($p<0.001$) during the first 9 weeks of PEGasparaginase therapy and remained stable thereafter (Figure 1-B). At week 37, the triglyceride and cholesterol levels normalized in all patients.

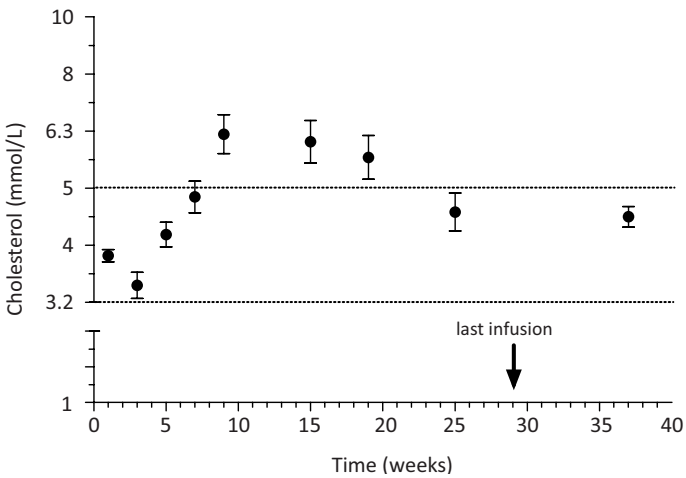


Figure 1-B: Serum cholesterol levels over time during PEGasparaginase therapy (mean ± SEM).
The dotted lines; upper and lower limit of normal.

The median triglyceride (1.3 mmol/L) and median cholesterol (4.3 mmol/L) levels were also normal at start of *Erwinia* asparaginase. Figure 1-C shows that also in the first five weeks of *Erwinia* asparaginase the triglyceride levels increased, although not significantly.

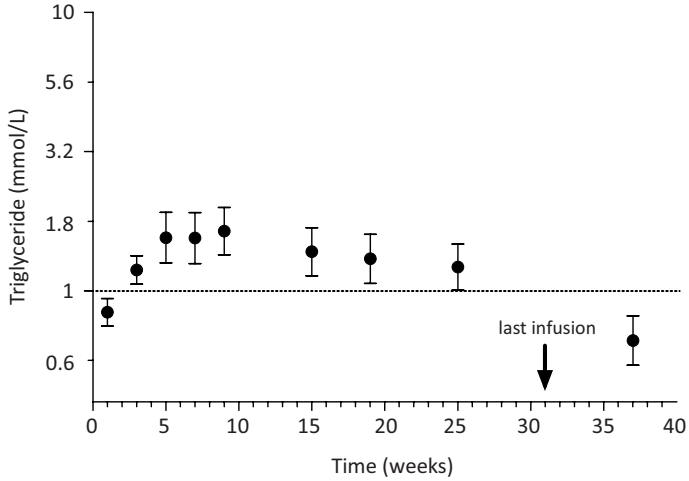


Figure 1-C: Serum triglyceride levels over time during *Erwinia* asparaginase therapy (mean ± SEM).
The dotted line; upper limit of normal.

Thereafter, the triglyceride levels remained stable, at week 37, the triglyceride levels normalized after the final *Erwinia* asparaginase infusion. Cholesterol levels were within the normal range during and after *Erwinia* asparaginase therapy (Figure 1-D).

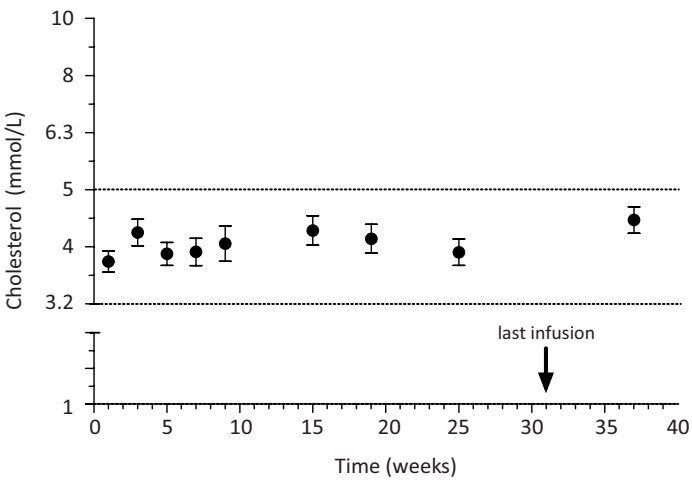


Figure 1-D: Serum cholesterol levels over time during *Erwinia* asparaginase therapy (mean ± SEM).
The dotted lines; upper and lower limit of normal.

In two children with extreme hypertriglyceridemia during PEGasparaginase treatment, dexamethasone was omitted temporarily. In both patients triglyceride levels decreased enormously, in one patient from 43.1 mmol/L to 16.7 mmol/L and the other one from 36.3 mmol/L to 3.2 mmol/L. Triglyceride levels normalized completely in both patients at week 37. In none of the patients with dyslipidemia, asparaginase courses were discontinued and 30 weeks of asparaginase exposure were completed without any other interventions.

It has to be noted that the PEGasparaginase activity levels were much higher than *Erwinia* asparaginase activity levels. Figure 1 E-F show that high triglyceride and cholesterol levels were associated with high asparaginase activity levels.

It has to be noted that the PEGasparaginase activity levels were much higher than *Erwinia* asparaginase activity levels. Figure 1 E-F show that high triglyceride and cholesterol levels were associated with high asparaginase activity levels.

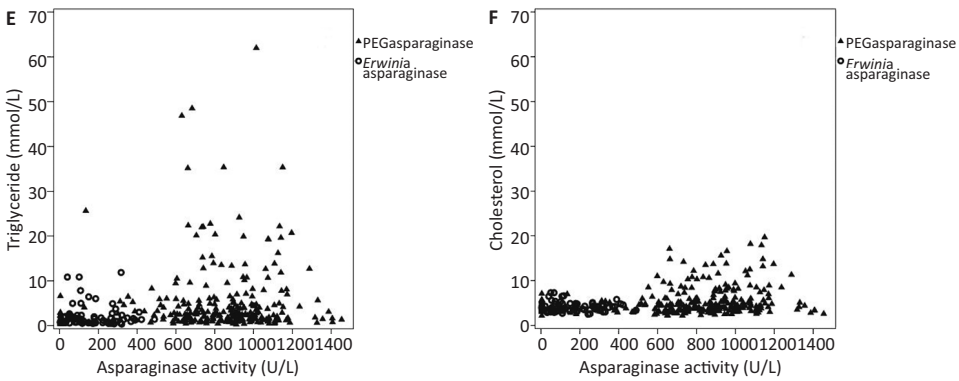


Figure 1 E-F: Serum triglyceride, cholesterol levels and relations to serum asparaginase activity levels. Association between triglyceride and asparaginase activity levels (E) and association between cholesterol and asparaginase activity levels (F).

Studying the correlations between PEGasparaginase activities and triglyceride levels at the various treatment weeks showed the strongest correlation at week 5 (Rs=0.36, p=0.005). PEGasparaginase activities *versus* cholesterol levels correlated strongest at week 9 (Rs=0.35, p=0.01). These associations did not differ between PEGasparaginase and *Erwinia* asparaginase therapy.

Using mixed model analysis, it was found that children ≥10 years had higher triglyceride levels as compared to younger patients (<10 years) adjusted for asparaginase preparations: median 4.9 mmol/L *versus* 1.6 mmol/L (p<0.001). The same applied to children for cholesterol levels with median values of 6.2 mmol/L *versus* 4.2 mmol/L (p<0.001).

Pancreatitis, thrombosis, central neurotoxicity

Figure 2 A-B show the mean ammonia levels during PEGasparaginase or *Erwinia* asparaginase therapy along time.

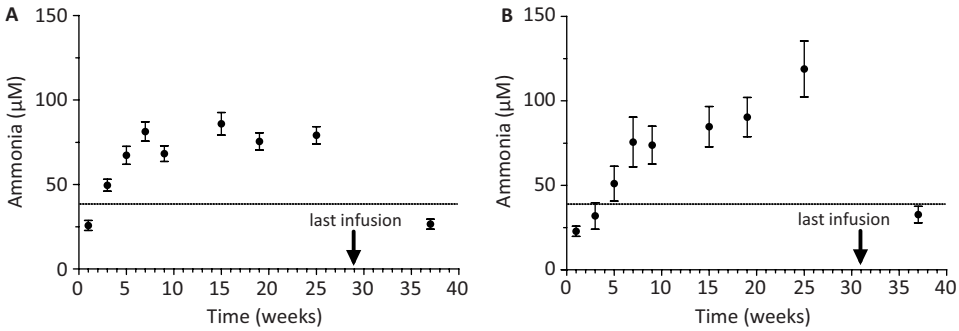


Figure 2 A-B: Ammonia levels over time (mean ± SEM).
Ammonia levels (A: PEGasparaginase and B: *Erwinia* asparaginase) during asparaginase therapy. Mean ± SEM.
The dotted line; upper limit of normal.

Figure 2 A-B show the mean ammonia levels during PEGasparaginase or *Erwinia* asparaginase therapy along time.

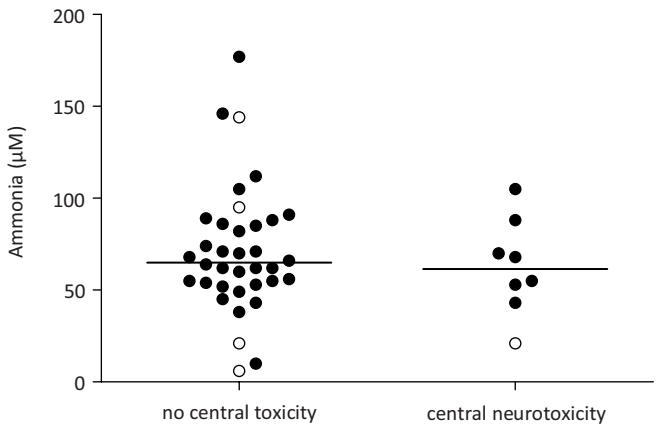


Figure 2-C: Ammonia levels and associations with central neurotoxicity.
Ammonia levels related to central neurotoxicity (C). The ammonia levels in week 5 of the PEGasparaginase courses and in week 7 of the *Erwinia* asparaginase courses (both steady state of asparaginase activity levels) were used as controls for the ammonia levels closest to the occurrence of central neurotoxicity.
Bars indicate median.
Open circles indicate patients treated with *Erwinia* asparaginase.

Asparaginase activity levels were not related to the occurrence of pancreatitis, thrombosis or central neurotoxicity (Figure 3-A). Triglyceride levels were also not related to pancreatitis or thrombosis (Figure 3-B). However, due to the small numbers of patients with toxicity, no statistical comparisons were done.

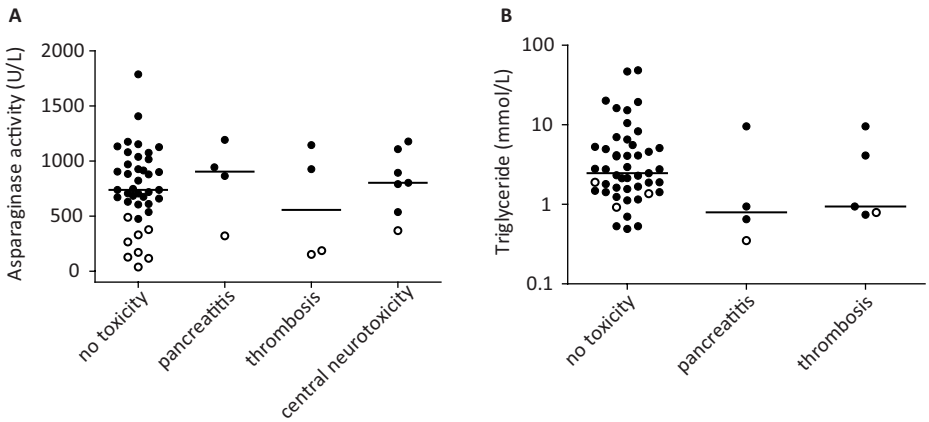


Figure 3: Serum asparaginase activity and triglyceride levels related to pancreatitis, thrombosis or central neurotoxicity.

Serum asparaginase activity levels in patients without toxicity and patients with pancreatitis, thrombosis or central neurotoxicity (A).

Serum triglyceride levels in patients without toxicity and patients with pancreatitis or thrombosis (B).

The samples in week 5 of the PEGasparaginase courses and in week 7 of the *Erwinia* asparaginase courses (both steady state of asparaginase activity levels) were used as controls for the asparaginase activity and triglyceride levels closest to the occurrence of pancreatitis/ thrombosis and central neurotoxicity.

Bars indicate median. Open circles indicate patients treated with *Erwinia* asparaginase.

LPL and HL activities, amylase, glucose

The amylase, glucose, HDL and LDL levels were within the normal range before start, during and after asparaginase therapy, except for two patients who developed hyperglycemia during *Erwinia* asparaginase combined with dexamethasone courses and needed insulin therapy temporarily. *Erwinia* asparaginase therapy was continued temporarily in both patients.

In 10 patients, lipoprotein lipase (LPL) and hepatic lipase (HL) activities were measured at different time points (Supplemental results and Supplemental Table 1). No association was found with triglyceride levels.

DISCUSSION

Our study showed that 6% of the patients developed pancreatitis and 4.5% thrombosis when using very prolonged courses of asparaginases. Recently, the Dana Farber Consortium Institute (DFCI) group reported a comparable incidence of pancreatitis (5%) and thrombosis (8%) with the same schedule.[8] We demonstrate that asparaginase activity levels are related to hypertriglyceridemia and hypercholesterolemia (dyslipidemia). Important observation is that dyslipidemia, even grade 3/4, is temporary and there is no clinical reason to interrupt or discontinue asparaginase courses.

Triglyceride levels were increased using PEGasparaginase as well as *Erwinia* asparaginase, but hypertriglyceridemia was more frequent and more severe during PEGasparaginase. This may be caused by the much higher levels of PEGasparaginase compared to *Erwinia* asparaginase. It has been suggested that high triglyceride levels cause severe pancreatitis.[9] However, in the few patients with pancreatitis in our study the highest triglyceride level was only 4 mmol/L (grade 2). Of note, we found the same rate of pancreatitis in *Erwinia* asparaginase treated patients and none of these patients had hypertriglyceridemia. Also, the asparaginase activity levels of patients with pancreatitis were similar to the levels in patients without pancreatitis (Figure 3-A). Therefore, we conclude that the development of pancreatitis is not associated with hypertriglyceridemia or asparaginase activity levels. In addition, we show that triglyceride levels and asparaginase activity levels of patients with thrombosis were in the same range as those of patients without thrombosis. This suggests that the development of thrombosis is also independent of the asparaginase activity levels or triglyceride levels.

Hypertriglyceridemia occurs especially when corticosteroids and asparaginase are combined.[10-11] The decline in triglyceride levels in the two patients in our study after stopping dexamethasone while continuing asparaginase illustrates this. Corticosteroids are known to induce the production of triglyceride-rich particles[12-13] and asparaginase might decrease the activity of lipoprotein lipase (LPL),[10] an enzyme that removes triglyceride from plasma.[14-15] However, normal LPL values have been reported in ALL patients with hypertriglyceridemia treated with asparaginase and steroids.[16] No association between LPL activity and triglyceride level was found in the present study. So, we conclude that asparaginase does not affect LPL activity, therefore the pathophysiology of asparaginase-induced hypertriglyceridemia is not mediated by decreased LPL activity.

Temporary hypertriglyceridemia grade 3/4 was seen in about half of patients receiving PEGasparaginase and this normalized completely in all patients after finalizing 30 weeks of PEGasparaginase infusions. We conclude therefore that there is no need to discontinue or interrupt asparaginase therapy in case of hypertriglyceridemia.

The ammonia levels after *Erwinia* asparaginase therapy were significantly higher compared to those after PEGasparaginase therapy ($p<0.001$) which seems paradoxal as the PEGasparaginase activity levels were much higher than the *Erwinia* asparaginase activity levels. This can be explained by higher glutaminase activity of *Erwinia* asparaginase as we showed recently.[17] In

case reports, it was suggested that ammonia release could lead to encephalopathy.[18-20] Our prospective study, however, shows that ammonia level was not related to central neurotoxicity. Even the patients with hyperammonemia grade 3/4 did not experience central neurotoxicity and the four patients with central neurotoxicity grade 3/4 did not have higher ammonia levels than those without central neurotoxicity.

The strength of our study was its prospective and longitudinal nature. We closely monitored dyslipidemia and hyperammonemia in two representative centers in the Netherlands during very prolonged asparaginase courses. The limitations were in the low number of patients experiencing pancreatitis, thrombosis or central neurotoxicity.

In conclusion, this study shows that hypertriglyceridemia and hypercholesterolemia grade 3/4 occur frequently, but are temporary and there is no clinical reasons to interrupt or discontinue asparaginase courses. We show that high asparaginase activities are associated with high triglyceride and high cholesterol levels. However, pancreatitis, thrombosis, and central neurotoxicity appear unrelated to asparaginase activity levels. Also, no associations were found between pancreatitis and hypertriglyceridemia nor between ammonia level and central neurotoxicity.

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Supplementals accompanying Chapter 5

SUPPLEMENTAL RESULTS

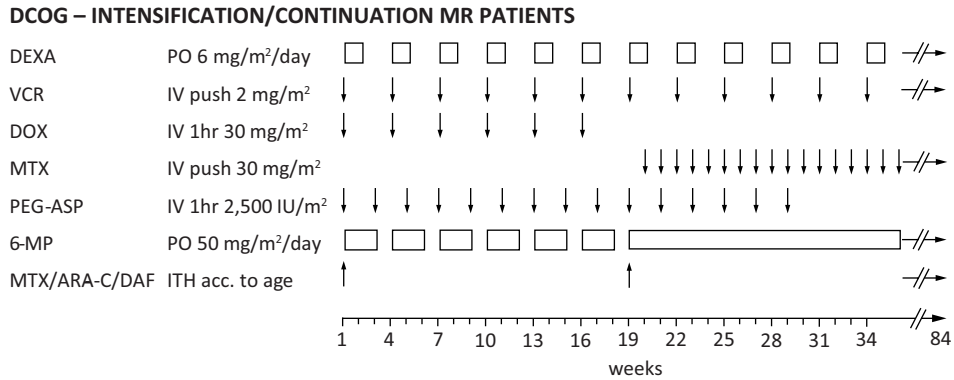
Lipoprotein lipase and hepatic lipase activities

Supplemental Table 1 shows the lipoprotein lipase (LPL) and hepatic lipase (HL) activities. Four patients had normal LPL activities during asparaginase courses, one of these patients had strong decreased HL activity. Four patients had measurements at start of intensification of which two patients had decreased LPL activities. Finally, we obtained in four patients LPL and HL activities at week 37 of continuation. Only one patient showed decreased LPL and HL activities. No associations with increased triglyceride levels were found.

Supplemental Table 1: Lipoprotein lipase (LPL) and hepatic lipase (HL) activities during asparaginase courses.

Patient	Week	LPL	HL	Interpretation of LPL and HL	TG
P1	19	160	241	normal	7.9
P1	37	89	377	normal	1.1
P2	19	213	257	normal	9.5
P3	37	123	346	both lower range	2.2
P4	37	74	352	LPL is low	0.7
P5	37	92	289	both lower range	0.5
P6	19	218	275	normal	6.9
P7	1	150	529	normal	0.4
P7	19	91	153	HL is very low	1.4
P8	1	159	554	normal	0.9
P9	1	88	298	LPL is low	1
P10	1	76	221	both low	0.9

Reference values[1]:
Girls; LPL (100 mU/ml-230 mU/ml) and HL (245 mU/ml-445 mU/ml)
Boys; LPL (70 mU/ml-180 mU/ml) and HL (225 mU/ml-515 mU/ml)
TG; triglyceride. Upper limit of normal: 1 mmol/L.
Week; treatment week of intensification/continuation.



Supplemental Figure 1: DCOG ALL-10 medium risk intensification protocol.
The intensification/continuation phase of the Dutch Childhood Oncology Group ALL-10 protocol (medium risk group, MRG).

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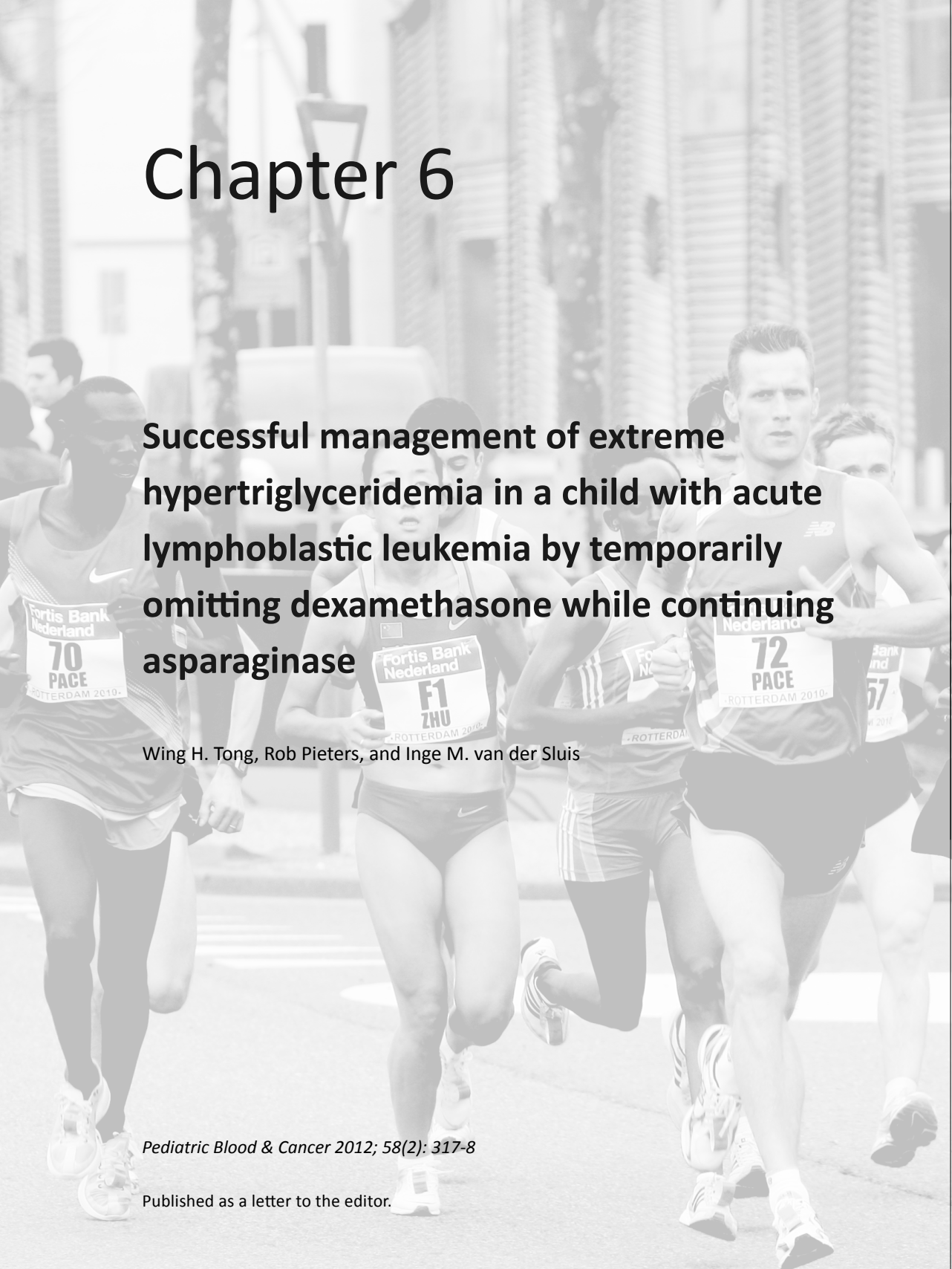
Chapter 6



Successful management of extreme hypertriglyceridemia in a child with acute lymphoblastic leukemia by temporarily omitting dexamethasone while continuing asparaginase

Wing H. Tong, Rob Pieters, and Inge M. van der Sluis

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ABSTRACT

Childhood acute lymphoblastic leukemia (ALL) patients treated with asparaginase and corticosteroids can suffer from disturbed lipid metabolism. Asparaginase is assumed to be responsible for this side effect. We report a child with ALL with extreme hypertriglyceridemia and hypercholesterolemia during intensification phase. These lipid abnormalities tended to normalize by temporarily omitting corticosteroids while continuing asparaginase. This report shows that not asparaginase itself, but the combination of corticosteroids and asparaginase is responsible for severe disturbed lipid metabolism.

INTRODUCTION

Asparaginase is an essential compound in the treatment of childhood acute lymphoblastic leukemia (ALL). This is usually administered in treatment phases that also include corticosteroids (prednisone or dexamethasone), i.e. induction and intensification. One of the side effects of asparaginase are lipid disorders, like hypercholesterolemia and hypertriglyceridemia. It is not clear whether interruption of asparaginase is indicated. Some clinicians do not treat these lipid abnormalities whereas others advise temporary fasting or low fat diet.[1-3] Oral fibrates can be used to reduce triglyceride levels, but have modest effects on LDL-cholesterol, HDL-cholesterol and total cholesterol levels.[4] Furthermore, the use of plasmapheresis has been reported.[2] Earlier reports described lipid disorders especially when corticosteroids and asparaginase were combined.[5,6] We report a patient with extreme hypertriglyceridemia during the intensification phase of the medium risk group managed successfully by omitting corticosteroids temporarily, while asparaginase infusions were still continued.

CASE REPORT

A twelve-year-old female, height 1.61 meter and weight 51 kilograms, was diagnosed with ALL. There was no family history of lipid disorders. She was treated according to Dutch Childhood Oncology Group (DCOG) ALL-10 protocol[7] with a good prednisone response after one week of therapy and complete remission at day 33. Based on minimal residual disease analysis at days 33 and 79 of the induction phase, she was stratified to the medium risk group (MRG). During induction insulin therapy was needed temporarily for hyperglycemia. After protocol I-b and protocol M, the intensification of the MRG started with oral dexamethasone (6 mg/m^2 divided in three doses per day) for 5 days, every three weeks. Vincristine (VCR) (2 mg/m^2/dose, maximum of 2 mg per dose) was intravenously given every three weeks. Doxorubicine (DOX) (30 mg/m^2/dose) was administered every three weeks for six times. Methotrexate (MTX) (30 mg/m^2/dose) was given intravenously once a week at the first day of week 20 of MRG intensification until week 84. PEGasparaginase was given biweekly intravenously (2,500 IU/m^2/dose), in total 15 times. 6-Mercaptopurine (6-MP) (50 mg/m^2/day) was given orally in 6 courses of 2 weeks with 1 week interval between the courses and from week 19 to 84 continuously. At week 1, 19, 37, 55 and 73 intrathecal therapy with methotrexate/ cytosine arabinoside/ diadreson F aquosum was given (Figure 1).

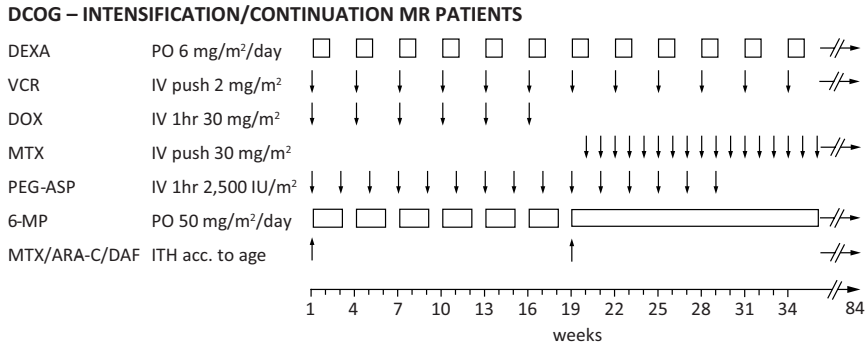

Figure 1: The intensification/continuation phase of the Dutch Childhood Oncology Group ALL-10 protocol (medium risk group, MRG).

Before start of intensification phase (week 1), serum levels of lipids, glucose and amylase were normal. These parameters were measured prospectively during intensive PEGasparaginase administration. For practical reasons, the measured values were non-fasting, except at week 1 and week 19. Triglyceride and total cholesterol levels increased after the first dexamethasone course and PEGasparaginase infusion (Figure 2).

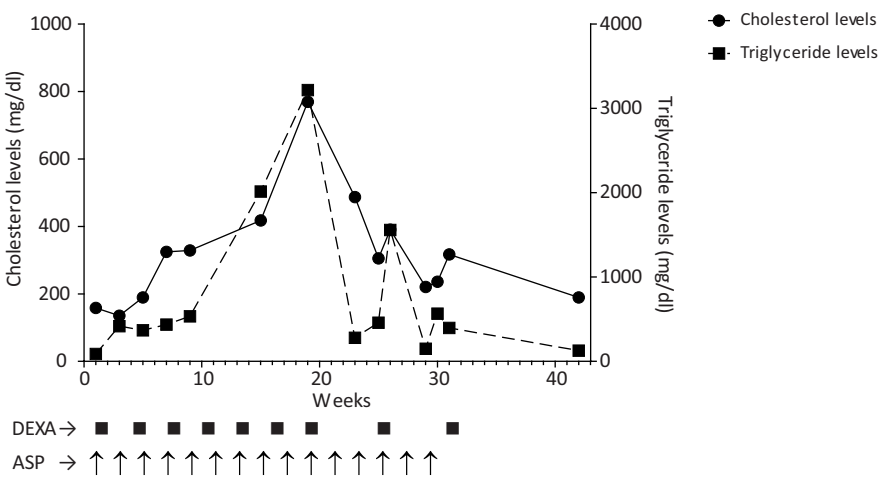

Figure 2: The effect of PEGasparaginase and dexamethasone pulses on serum triglyceride and cholesterol levels.

A small block indicates oral dexamethasone (DEXA), 5 days per 3 weeks and an arrow means one PEGasparaginase infusion (ASP) per 2 weeks. The reference values for children aged 12-18 years are: cholesterol: 116-213 mg/dl and triglyceride: 35-142 mg/dl.

The maximum peak values of triglyceride (3,218 mg/dL, normal upper level: 142 mg/dL) and total cholesterol (770 mg/dL, normal upper level: 213 mg/dL) were noticed at week 19 of the intensification phase. In an attempt to lower the lipid levels, the dexamethasone course of week 22 was omitted. Administration of PEGasparaginase was continued every 2 weeks according to protocol. The lipid values decreased dramatically to 279 mg/dL for triglyceride and 487 mg/dL for total cholesterol, both measured after omittance of dexamethasone at week 23. Within one week after restarting dexamethasone at week 25, the triglyceride level increased and total cholesterol level was slightly reduced, 1,559 mg/dL and 391 mg/dL, respectively. The dexamethasone course at week 28 was therefore not administered and consequently the levels of triglyceride and total cholesterol decreased to almost normal levels (triglyceride 151 mg/dL and total cholesterol 220 mg/dL, both measured at week 29). At all time points, the HDL-cholesterol levels were within the normal range of 35-74 mg/dL. The LDL-cholesterol levels at week 25 (177 mg/dL) and week 31 (186 mg/dL) were slightly increased (normal upper level: 147 mg/dL).

The asparaginase activity and asparagine levels in serum were measured according to validated procedures (AHA assay and RP-HPLC, respectively)[8,9] before each new PEGasparaginase infusion and several times one week after the infusion. The asparaginase levels of our patient ranged from 552 U/L to 3,200 U/L, much higher than 100 U/L required for complete asparagine depletion and were independent from discontinuing the dexamethasone administration.

From week 34 until end of treatment, the dexamethasone courses were definitely discontinued because of toxicity, among others, severe mood alteration (depression). The last measurement of triglyceride and cholesterol levels at week 42 revealed normal levels. Our patient had normal amylase levels and no signs of pancreatitis during asparaginase therapy. Also, she did not develop hyperglycemia in the intensification phase.

DISCUSSION

This case report demonstrates that extreme hypertriglyceridemia can be managed by omitting dexamethasone courses while continuing asparaginase infusions, without other therapy. Although lipid abnormalities associated with asparaginase are transient, it may be important to monitor triglyceride (TG) levels to prevent possible (future) complications.[2] For instance, triglyceride levels above 1,000 mg/dL are suggested to cause triglyceride-induced pancreatitis.[10] Ridola *et al.* and Kfoury-Baz *et al.* used plasmapheresis to treat extreme asparaginase-associated lipid disorders.[2,11]

To our knowledge, our case report is the first describing temporarily omittance of dexamethasone courses while continuing asparaginase infusions to treat hypertriglyceridemia.

This was done at two different time points with the same successful result on triglyceride and cholesterol levels that reduced dramatically. At all time points, PEGasparaginase was continued according to protocol. We preferred to omit dexamethasone and not asparaginase to assure

complete and continuous asparagine depletion during the first 30 weeks of the intensification phase. Dexamethasone courses in this protocol are given 3-weekly during 84 weeks, hence omitting a few courses was assumed a less significant reduction of therapy.

Corticosteroids are known to induce production of TG-rich particles.[12,13] Asparaginase might decrease the activity of lipoprotein lipase (LPL) activity,[6] an enzyme that removes triglyceride from plasma.[14,15] However, normal LPL values were observed in ALL patients with hypertriglyceridemia treated with asparaginase and steroids.[16] Omitting dexamethasone courses resulted in decreased lipid levels in our patient. This suggests that the primary cause of hypertriglyceridemia in this patient is an increased production by corticosteroids.

In conclusion, this report shows that not asparaginase itself, but the combination of corticosteroids and asparaginase is responsible for severe disturbed lipid metabolism. Temporarily omitting corticosteroids is a good and safe strategy to try to normalize lipid levels when asparaginase infusions are combined with corticosteroids. However, omitting corticosteroids should be balanced with the risk of leukemia recurrence and may therefore be riskful in case the treatment protocol does not include further use of corticosteroids. Also, it is unclear at present what the consequences of a transient hyperlipidemia are.

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Chapter 7

Cost-analysis of treatment of childhood acute lymphoblastic leukemia with asparaginase preparations: the impact of expensive chemotherapy

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ABSTRACT

Asparaginase is an expensive drug, but important in childhood acute lymphoblastic leukemia. In order to compare costs of PEGasparaginase, *Erwinia* asparaginase, and native *E.coli* asparaginase, we performed a cost-analysis in the Dutch Childhood Oncology Group ALL-10 medium-risk group intensification protocol. Treatment costs were calculated based on patient level data of 84 subjects, and were related to the occurrence of allergy to PEGasparaginase. Simultaneously, decision tree and sensitivity analyses were conducted. The total costs of the intensification course of 30 weeks were $57,893 in patients without PEGasparaginase allergy (n=64). The costs were significantly higher ($113,558) in case of allergy (n=20) necessitating a switch to *Erwinia* asparaginase. Simulated scenarios (decision tree analysis) using native *E.coli* asparaginase in intensification showed that the costs of PEGasparaginase were equal to those of native *E.coli* asparaginase. Also after sensitivity analyses, the costs for PEGasparaginase were equal to those of native *E.coli* asparaginase. Intensification treatment with native *E.coli* asparaginase, followed by a switch to PEGasparaginase, and subsequently to *Erwinia* asparaginase in case of allergy had similar overall costs compared to the treatment with PEGasparaginase as the first-line drug (followed by *Erwinia* asparaginase in case of allergy). PEGasparaginase is preferred over native *E.coli* asparaginase, because it is administered less frequently, with less day care visits. PEGasparaginase is less immunogenic than native *E.coli* asparaginase and is not more expensive. Asparaginase costs are mainly determined by the percentage of patients who are allergic and require a switch to *Erwinia* asparaginase.

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most common type of childhood cancer.[1] Annually, approximately 120 new cases of childhood ALL are diagnosed in the Netherlands. The treatment of childhood ALL has improved dramatically and survival increased from 0-5% in the 1960's to 80-85% nowadays.[1] Treatment consists of induction, consolidation, intensification and continuation phases. Asparaginase is one of the key drugs in this treatment.[2-4] Asparaginase is a non-human enzyme which hydrolyses asparagine into aspartic acid and ammonia. Given that leukemic blasts depend heavily on asparagine, deprived of this amino acid, they undergo apoptosis.[5]

Currently, several asparaginase preparations are available on the market: these are derived from *Escherichia coli* in its native form (Paronal® or Asparaginase medac®) or as a pegylated enzyme (PEGasparaginase, Oncaspar®) or extracted from *Erwinia* chrysanthemi (*Erwinia* asparaginase, Erwinase®). Many studies have shown that intensification by asparaginase is essential to improve the event-free survival of children with ALL.[2-4,6-8]

Unfortunately, asparaginase can cause an allergic reaction leading to inactivation of the drug or silent inactivation. Silent inactivation is the formation of anti-asparaginase antibodies which neutralize asparaginase without their being clinical symptoms of an allergy.[9] In the case of allergic reactions to PEGasparaginase, *Erwinia* asparaginase is given instead. *Erwinia* asparaginase is given three times per week. The different dose schedules for native *E.coli* asparaginase, PEGasparaginase and *Erwinia* asparaginase are based on differences in the pharmacokinetics of the three products.

Compared to native *E.coli* asparaginase, PEGasparaginase is expensive,[10] and *Erwinia* asparaginase is even more expensive. Little information is available on the exact costs of asparaginase in the treatment of ALL.[11-12] Recently, Litsenburg *et al.*[13] concluded that medication and diagnostics were the major contributors to the increased costs of the ALL-10 protocol compared to the previous ALL-9 protocol. However, in this study the costs of asparaginase were not analyzed separately and the costs were not related to the occurrence of allergies. Since native *E.coli* asparaginase was not administered during intensification in the ALL-10 protocol, we used hypothetical scenarios to study this strategy. The trial data of the ALL-10 protocol was used to compare PEGasparaginase to native *E.coli* asparaginase. Because of hospital budget restrictions and increasing costs of treatment of childhood ALL more insight into costs of asparaginase preparations is desired.

In the present study, we studied the costs of asparaginase in childhood ALL patients treated with PEGasparaginase or *Erwinia* asparaginase during the first 30 weeks of the intensification phase of the ALL-10 medium-risk (MR) protocol. The aim was to assess whether there are savings from using PEGasparaginase as the first-line drug rather than the native *E.coli* asparaginase.

DESIGN AND METHODS

Overall study design

For this cost-analysis, we compared the costs of asparaginase related to allergy in three treatment scenarios. Scenario 1 is based on trial data from the ALL-10 MR protocol. Scenario 2 & 3 are based on assumptions. A decision tree model was also used to relate costs for each scenario to different allergy rates.

Patients and the ALL treatment protocol

From November 2004 to April 2012, children with ALL were enrolled on the Dutch Childhood Oncology Group (DCOG) ALL-10 protocol[14] approved by the Institutional Review Board. Patients were stratified into three risk groups after induction treatment: standard risk (SR), MR and high risk (HR).[15] The intensification/ continuation scheme for the ALL-10 MR patients including asparaginase (administered intravenously) is shown in Supplemental Figure 1 and described in the Supplemental Design and Methods section. For this cost-analysis between April 2005 and October 2009, only MR patients from two pediatric oncology centers were included. Allergic reactions were graded according to the National Cancer Institute (NCI) Common Terminology Criteria for Adverse Events (CTCAE) version 3.0.

Description of three scenarios

The ALL-10 MR protocol was used as scenario 1 (Figure 1). Due to the fact that native *E.coli* asparaginase was not administered during intensification in this protocol, we used two hypothetical scenarios. In scenarios 2 and 3, patients were hypothetically treated with native *E.coli* asparaginase (5,000 IU/m^2, twice weekly) for a duration of 30 weeks (Figure 1).

In scenario 2, in case of an allergic reaction to native *E.coli* asparaginase, *Erwinia* asparaginase was given. In scenario 3, patients were switched to PEGasparaginase in case of an allergic reaction to native *E.coli* asparaginase. Scenario 3 was based, among others, on the ALL-10 induction and the ALL-BFM 2000[16] protocols which prescribed PEGasparaginase as second-line and *Erwinia* asparaginase as third-line therapy. In this scenario, it was assumed that an allergy to PEGasparaginase after an allergic reaction to native *E.coli* asparaginase will occur at the second dose which is the case in practically all allergic reactions according to the interim results of the ALL-10 protocol.

Costs data

Data on volumes were adapted from hospital electronic databases and medical files. For the unit prices, we applied the microcosting method[17] and Dutch tariffs. More details are given in the Supplemental Design and Methods section.

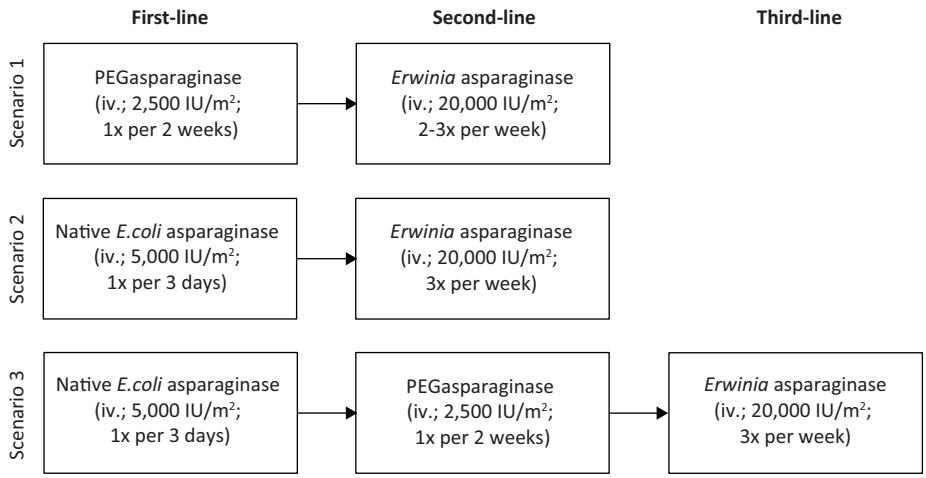

Figure 1: The flowchart of three distinct scenarios of asparaginase treatment in children with acute lymphoblastic leukemia.

Statistical, decision tree, sensitivity analyses

The data were analyzed with the software package SPSS for Windows version 17.0.2 (SPSS, Chicago, IL, USA). The mean total costs were not normally distributed (as shown by the Shapiro-Wilk test). The non-parametric Mann-Whitney U-test was used to compare the subgroups with or without an allergy to asparaginase. A two-sided p-value <0.05 was considered statistically significant. Data are presented as mean ± standard deviation and median (range) where appropriate.

We developed a decision tree model to compare costs of PEGasparaginase or *Erwinia* asparaginase to those of native *E.coli* asparaginase, while taking into account the incidence of allergy to asparaginase and the different associated costs (Figure 2). To account for uncertainty in the used prices and calculated costs, sensitivity analyses were performed. More details are given in the Supplemental Design and Methods section.

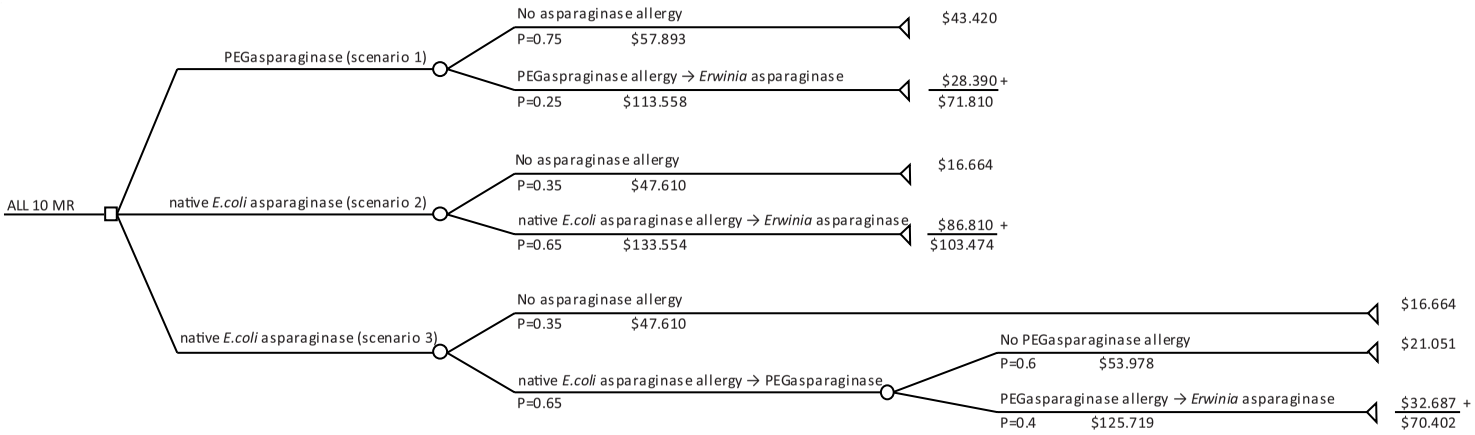

Figure 2: Decision tree of asparaginase treatment in children with acute lymphoblastic leukemia in the intensification according to three scenarios.

The mean costs after each probability of allergy (P) are the calculated costs per patient in scenario 1. The costs at the end of each branch represent the multiplied costs with each probability of allergy. The costs in scenarios 2 and 3 are simulated costs. The costs after each probability of allergy are the costs per patient. The costs at the end of each branch represent the multiplied costs with each probability of allergy in scenarios 2 and 3. In the last branch of scenario 3, the costs are calculated by multiplying the probability of allergy of 0.65 with the allergy probabilities of 0.6 and 0.4, respectively.

RESULTS

Characteristics

In total 84 children with ALL (33 girls) were included in this study. The median age was 5.2 years (range 1.8-18.6 years) at the start of the intensification. The baseline characteristics of each subgroup are presented in Table 1.

Table 1: Characteristics of patients with and without an allergic reaction to PEGasparaginase.

Characteristic	No PEG-asp allergy	PEG-asp allergy
Patients (%)	64 (76)	20 (24)
Boys / girls	37 / 27	14 / 6
Age (years), median (range)	5.4 (1.8-18.6)	4.3 (2-14.4)
Body surface area (m^2), median (range)	0.8 (0.5-2)	0.8 (0.5-1.6)
Patients aged <6 years (%)	35 (42)	12 (14)
Patients aged 6-12 years (%)	18 (22)	6 (7)
Patients aged >12 years (%)	11 (13)	2 (2)
Day care visits, median (range)	28 (19-42)	61 (6-84)
Inpatient days, median (range)	7 (0-40)	3 (0-77)
PEG-asp infusions, median (range)	15 (14-16)	2 (2)
Time point of PEG-asp allergy in weeks, median (range)	na	2 (2-4)
Erw-asp infusions, median (range)	na	68 (10-84)

ALL-10 MRG= ALL-10 protocol medium-risk group, PEG-asp= PEGasparaginase, Erw-asp= *Erwinia* asparaginase, na= not applicable.

In total 20 patients (24%) were switched to *Erwinia* asparaginase because of a proven allergic reaction to PEGasparaginase (grade 2 or higher according to the CTCAE criteria). No grade 1 allergies were seen. Two patients had an allergy to PEGasparaginase in a period (June 2005)

that no *Erwinia* asparaginase was commercially available in the Netherlands. Five patients were switched to twice weekly *Erwinia* asparaginase based on sufficient trough asparaginase levels.

Calculated costs (scenario 1)

Table 2 presents the costs per subgroup for all patients. The total mean treatment costs of all patients treated according to the first 30 weeks of the ALL-10 MR protocol were $ 71,147 ± 35,763 per patient.

Table 2: Treatment costs with PEGasparaginase (scenario 1) for patients with and without an allergic reaction to PEGasparaginase. Scenario 1: PEGasparaginase used as first-line treatment in intensification; and switch to *Erwinia* asparaginase as second-line after PEGasparaginase allergy.

Cost category	No PEG-asp allergy median (mean; SD) ($)	PEG-asp allergy median (mean; SD) ($)	p-value
Chemotherapy without ASP	1,026 (1,163; 450)	949 (1,143; 422)	0.6
PEG-asp per used vials	24,465 (27,324; 7,785)	3,262 (5,384; 4,642)	<0.001
Erw-asp per used vials	na	81,900 (66,424; 34,580)	-
Additional medication	1,467 (2,589; 3,750)	2,110 (4,027; 5,341)	0.03
Daycare treatment	8,627 (8,931; 2,2062)	21,667 (20,202; 8,854)	<0.001
Inpatient care	5,181 (8,879; 9,741)	2,827 (9,138; 16,792)	0.4
Blood products	143 (609; 1,412)	0 (43; 105)	0.05
Laboratory activities	2,498 (3,089; 1,573)	2,055 (2,505; 1,911)	0.04
Other hospital activities	4,812 (5,310; 2,184)	3,981 (4,692; 2,718)	0.2
Total costs	54,587 (57,893; 16,247)	126,613 (113,558; 47,187)	<0.001

PEG= poly-ethylene glycol, ASP= asparaginase, PEG-asp= PEGasparaginase, Erw-asp= *Erwinia* asparaginase, na= not applicable.

The distribution of these costs was mainly accounted for by asparaginase use (47%) calculated per used vial. Day care treatment and inpatient care accounted for 15% of the total costs. The mean treatment costs for patients with no allergy to PEGasparaginase were $ 57,893 ± 16,247 per patient, which was significantly lower than the costs of the subgroup with an allergy to PEGasparaginase ($ 113,558 ± 47,187 per patient). The total costs were calculated per two weeks of asparaginase exposure; for PEGasparaginase ($ 3,860 ± 1,083 per patient), and for *Erwinia* asparaginase ($ 7,571 ± 3,146 per patient).

Simulated costs (scenarios 2 and 3)

Table 3 shows the mean costs of the two hypothetical scenarios with native *E.coli* asparaginase administered.

Table 3: Treatment costs according to two different hypothetical treatments (scenarios 2 and 3). Scenario 2: hypothetical treatment scenario with native *E.coli* asparaginase used as first-line treatment; and switch to *Erwinia* asparaginase as second-line treatment after allergy to native *E.coli* asparaginase. Scenario 3: hypothetical treatment scenario with native *E.coli* asparaginase used as first-line treatment; PEGasparaginase used as second-line after native *E.coli* asparaginase allergy; and switch to *Erwinia* asparaginase as third-line treatment after PEGasparaginase allergy.

	Native *E.coli* ASP as first-line, *Erwinia* ASP as second-line		**Native *E.coli* ASP as first-line, PEG-asp as second-line and *Erwinia* ASP as third-line**		
	No PEG-asp allergy	**PEG-asp allergy**	**No PEG-asp allergy**	**Only *E.coli* ASP allergy**	***E.coli* ASP allergy and PEG-asp allergy**
Cost category	**median (mean; SD) ($)**	**median (mean; SD) ($)**	**median (mean; SD) ($)**	**median (mean; SD) ($)**	**median (mean; SD) ($)**
Chemotherapy without ASP	1,026 (1,163; 450)	949 (1,143; 422)	1,026 (1,163; 450)	949 (1,143; 422)	949 (1,143; 422)
E.coli ASP per used vials	5,337 (5,503; 936)	178 (222; 140)	5,337 (5,503; 936)	178 (222; 140)	178 (222; 140)
PEG-asp per used vials	na	na	na	22,834 (23,731; 5,278)	3,262 (3,425; 729)
Erw-asp per used vials	na	76,650 (87,439; 21,900)	na	na	68,250 (77,936; 19,412)
Additional medication	1,846 (2,981; 3,757)	2,148 (4,129; 5,388)	1,846 (2,981; 3,757)	2,046 (3,985; 5,345)	2,100 (4,042; 5,350)
Outpatient care	19,843 (20,076; 449)	24,341 (24,244; 213)	19,843 (20,076; 449)	8,438 (8,519; 363)	22,718 (22,572; 307)
Inpatient care	5,181 (8,879; 9,741)	2,827 (9,138; 16,792)	5,181 (8,879; 9,741)	2,827 (9,138; 16,792)	2,827 (9,138; 16,792)
Blood products	143 (609; 1,412)	0 (43; 105)	143 (609; 1,412)	0 (43; 105)	0 (43; 105)
Laboratory activities	2,498 (3,089; 1,573)	2,055 (2,505; 1,911)	2,498 (3,089; 1,573)	2,055 (2,505; 1,911)	2,055 (2,505; 1,911)
Other hospital activities	4,812 (5,310; 2,184)	3,981 (4,692; 2,718)	4,812 (5,310; 2,184)	3,981 (4,692; 2,718)	3,981 (4,692; 2,718)
Total costs	42,947 (47,610; 13,317)	118,784 (133,554; 31,252)	42,947 (47,610; 13,317)	47,852 (53,978; 24,538)	111,951 (125,719; 30,623)

ASP= asparaginase, *E.coli*-asp= native *E.coli* asparaginase, PEG-asp= PEGasparaginase, Erw-asp= *Erwinia* asparaginase, na = not applicable.

The mean costs would be $ 47,610 ± 13,317 for the subgroup with no allergy to native *E.coli* asparaginase. These costs were significantly lower than for patients who developed an allergy to native *E.coli* asparaginase and switched to *Erwinia* asparaginase with mean costs of $ 133,554 ± 31,252 ($p<0.001$, scenario 2). A switch from native *E.coli* asparaginase to PEGasparaginase was accompanied by mean costs of $ 53,978 ± 24,538, which are higher but not significantly so, than the costs in the group without an allergy to native *E.coli* asparaginase. A second switch in the intensification to *Erwinia* asparaginase after an allergy to PEGasparaginase had a cost of $ 125,719 ± 30,623 ($p<0.001$, scenario 3).

Decision tree and sensitivity analyses

The decision tree analysis was used to relate costs for each scenario to different probabilities of allergy. Figure 2 shows that the treatment costs using either native *E.coli* asparaginase as the first-line preparation (scenario 3: $ 70,402) or PEGasparaginase as the first-line preparation (scenario 1: $ 71,810) were the lowest. The costs of using native *E.coli* asparaginase in the first-line followed by *Erwinia* asparaginase second line (scenario 2) would be higher ($ 103,474).

One-way sensitivity analysis (Figure 3) showed that the largest range in treatment costs was in the subgroup with allergy to native *E.coli* asparaginase (scenario 2). Furthermore, Figure 3 illustrates that when treatment costs were calculated with the new price of *Erwinia* asparaginase, treatment with PEGasparaginase as the first-line preparation (scenario 1) would be less expensive ($ 100,199) compared to the native *E.coli* asparaginase scenario 2 ($ 190,284) and scenario 3 ($ 103,089).

Two-way sensitivity analysis (Figure 4) revealed that treatment with PEGasparaginase (scenario 1) is less expensive than treatment with native *E.coli* asparaginase (scenario 2) for allergy probabilities of PEGasparaginase ranging from zero to 0.8 with a fixed allergy rate of 0.65 for native *E.coli* asparaginase (Figure 4A). This also holds true if a fixed allergy rate to native *E.coli* asparaginase of 0.4 is used, which is frequently found in studies using less native *E.coli* asparaginase in intensification after native *E.coli* asparaginase in induction.[16,18] The treatment with PEGasparaginase (scenario 1) carries equal costs compared to the treatment with native *E.coli* asparaginase (scenario 3) at a fixed rate of allergy to PEGasparaginase of 0.25 (Figure 4B,C). This holds true if the probabilities for a second allergy to PEGasparaginase allergy are lower than the base case value of 0.4 (Figure 4B) or if the allergy probabilities for native *E.coli* asparaginase are higher than the base case value of 0.65 (Figure 4C).

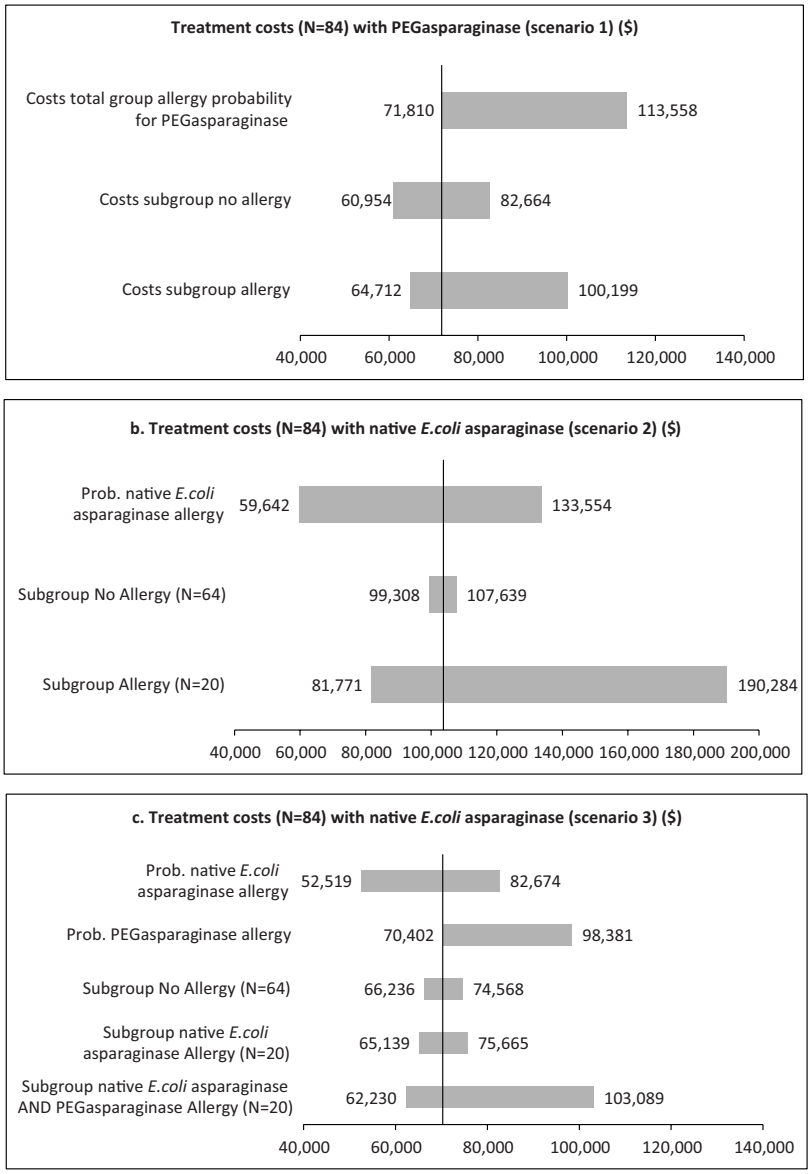

Figure 3-A,B,C: One-way sensitivity analyses of three asparaginase treatment scenarios.
Each bar indicates treatment costs per patient when the allergy probability or the costs were varied from the lowest to the highest value. The vertical line indicates base case value. (**A**) Treatment costs with PEGasparaginase (scenario 1). The first bar represents the allergy probability ranging from 25% to 100%. In the second and third bars the costs were varied in non-allergic (minus 25% to 25%) and allergic patients (minus 25% to 200%), respectively. (**B**) Treatment costs with native *E.coli* asparaginase (scenario 2). The first bar represents the allergy probability ranging from 14% to 100%. In the second and third bars the costs were varied in non-allergic (minus 25% to 25%) and allergic patients (minus 25% to 200%), respectively: 200% indicates the new price of *Erwinia* asparaginase (price level as of March 14, 2011). (**C**) Treatment costs with native *E.coli* asparaginase (scenario 3). The first bar represents the allergy probability ranging from 14% to 100%. The second bar shows the allergy probability ranging from 40% to 100%. In the third bar the costs were varied in non-allergic patients (minus 25% to 25%). The fourth bar represents the costs in patients allergic to native *E.coli* asparaginase (minus 25% to 25%). The last bar shows the costs of patients allergic to native *E.coli* asparaginase and subsequently to PEGasparaginase (minus 25% to 200%).

DISCUSSION

The aim of this study was to assess whether there could be savings from using PEGasparaginase as the first-line drug compared to native *E.coli* asparaginase during the first 30 weeks of the intensification phase of the ALL-10 MR protocol in the Netherlands. We showed that overall costs of treatment with native *E.coli* asparaginase, followed by a switch to PEGasparaginase, and subsequently to *Erwinia* asparaginase in case of allergy were $ 70,402. This sum was equivalent to that of treatment with PEGasparaginase as the first-line drug (followed by *Erwinia* asparaginase in case of allergy) which had overall costs of $ 71,810 (scenario 1), as applied in the ALL-10 MR protocol. Because of the comparable costs of these two scenarios, the latter one is preferable, because PEGasparaginase is administered less frequently (once every 2 weeks versus 4 times every 2 weeks), resulting in reduced burden for the patient and family. Treatment with native *E.coli* asparaginase, followed by a switch to *Erwinia* asparaginase is the most expensive alternative with overall costs of $ 103,474 (scenario 2).

We have shown that the distribution of the calculated and simulated costs was mainly accounted for by asparaginase use (47%) and that these asparaginase costs were mainly determined by allergy percentages necessitating a switch to *Erwinia* asparaginase. So, reducing the number of allergies by using PEGasparaginase would also reduce costs. Furthermore, PEGasparaginase is administered less frequently than native *E.coli* asparaginase or *Erwinia* asparaginase and is less immunogenic.[19-20] Scenarios 1 and 3 were less expensive than scenario 2; scenario 1 with PEGasparaginase as first-line treatment is the most patient-friendly option. For these reasons, PEGasparaginase is being used as the first-line drug in induction and intensification in the new DCOG ALL-11 protocol (opened in April 2012).

Two earlier studies investigated the costs of PEGasparaginase. Both found that treatment costs with PEGasparaginase were similar or slightly less than those with native *E.coli* asparaginase.[11-12] This is in line with our observations. However these studies did not study the costs related to asparaginase allergy and in these studies asparaginase was given less intensively. Litsenburg *et al.* also studied costs in the ALL-10,[13] but they calculated costs for the total period of treatment and did not study the exact role of asparaginase-related costs.

The costs in the different scenarios were based on actual resource utilization in childhood ALL patients. We also accounted for the costs of discarding unused asparaginase vials by calculating the costs per used vial. Despite of the sensitivity analysis to account for different percentages of asparaginase-related allergy, it is important to note that these percentages depend on several factors such as dose schedule and the type of asparaginase, earlier exposure to asparaginase in induction and the route of asparaginase administration (intravenous or intramuscular).[21] PEGasparaginase has been shown to result in less antibody formation than native *E.coli* asparaginase.[22] The percentage of patients switching to another asparaginase preparation can also depend on silent inactivation of asparaginase.[9] With monitoring of asparaginase activity levels, more cases of inactivation will be detected, necessitating a switch in asparaginase preparations more often.

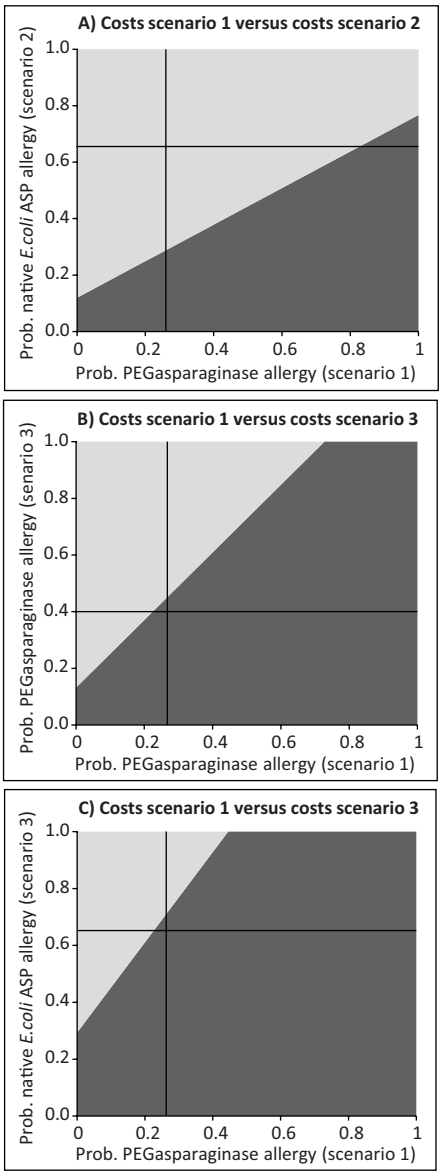

Figure 4-A,B,C: Two-way sensitivity analyses of three asparaginase treatment scenarios.

Each graph indicates the comparison between two hypothetical scenarios 2 and 3 with scenario 1 as baseline. For each allergy probability the values are varied, represented as the vertical line or the horizontal line. The intercept between these lines indicates the base case value. In all graphs, the horizontal axis is the same.

The light colored area indicates for which allergy probability combinations the treatment costs with scenario 1 are the lowest. The dark colored area indicates for which allergy probability combinations the treatment costs with scenario 2 in (A) or scenario 3 in (B) and (C) are the lowest.

The line between the light and dark colored area represents equal costs in each comparison.

(A) For the vertical axis the probability of native *E.coli* asparaginase allergy (scenario 2) is used.

(B) For the vertical axis the probability of PEGasparaginase allergy (scenario 3) is used. For the costs of scenario 3, the probability of native *E.coli* asparaginase is assumed to be 0.65.

(C) For the vertical axis the probability of native *E.coli* asparaginase allergy (scenario 3) is used. For the costs of scenario 3, the probability of PEGasparaginase is assumed to be 0.4.

Prob.= probability, *E.coli*-asp= native *E.coli* asparaginase, PEG-asp= PEGasparaginase

This study had some limitations. First, we had to simulate part of the treatment in order to calculate costs. To achieve reliable simulations for treatment with native *E.coli* asparaginase, simulations were based on the allergy rates in the ALL-9 HR and ALL-10 MR protocols. Furthermore, some satellite hospitals were not visited to collect data which were retrieved from academic hospital files. A mean cost based on data collected from satellite hospitals was imputed for the missing data. We did not evaluate silent inactivation of asparaginase; this is now being done in a prospective setting. The generalizability of this study might be limited, because there is tremendous heterogeneity across the world in dose, frequency and type of asparaginase used in the treatment of childhood ALL. Nowadays, the new childhood ALL treatment protocols also include PEGasparaginase for instance in Germany[23] and the United Kingdom,[24] while native *E.coli* asparaginase and *Erwinia* asparaginase are used in different countries. We studied the costs of asparaginase preparations which were related to different allergy probabilities. Additionally, the costs were presented for two weeks of exposure to asparaginase. Taken together, our results could be generalized for these countries using different asparaginase preparations.

To conclude, we have shown that the costs of PEGasparaginase and native *E.coli* asparaginase in intensification therapy are comparable. However, PEGasparaginase is preferred, because it is administered less frequently, requiring fewer day care visits and is, therefore, more patient-friendly. PEGasparaginase is less immunogenic than native *E.coli* asparaginase and is not more expensive. Since the price of *Erwinia* asparaginase has been doubled, the saving of costs will be clearly in favor of PEGasparaginase. Finally, asparaginase costs are mainly determined by allergy rates, necessitating a switch to *Erwinia* asparaginase.

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Supplementals accompanying Chapter 7

SUPPLEMENTAL METHODS

Patients and acute lymphoblastic leukemia treatment protocol

Medium-risk (MR) group patients from two pediatric oncology centers, Erasmus MC – Sophia Children's Hospital (Rotterdam) and the VU University Medical Center (Amsterdam), were included in this study. The ALL-10 protocol was approved by the Institutional Review Board and informed consent was obtained from parents or children's guardians in accordance with the Declaration of Helsinki. All patients received native *E.coli* asparaginase (5,000 IU/ m^2 per dose) eight times every three days in the induction phase. A patient stratified to the MR group was given PEGasparaginase (2,500 IU/ m^2 per dose) every two weeks during the first 30 weeks of the intensification. In the case of an allergy to PEGasparaginase, the patient was switched to *Erwinia* asparaginase (20,000 IU/ m^2 per dose) three times per week for 30 weeks. In the case of high serum levels of *Erwinia* asparaginase, the frequency was reduced to twice a week. All asparaginase preparations were administered intravenously.

Costs of scenario 1

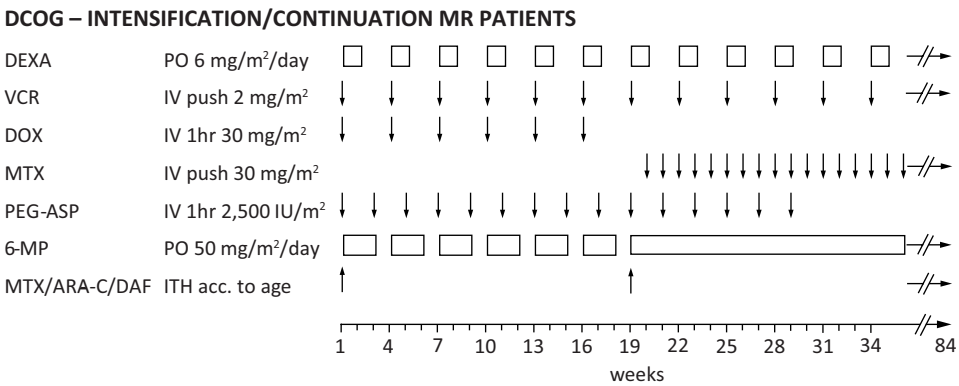
Treatment costs in the first 30 weeks of intensification were based on patient level data and according to intention-to-treat analysis. Direct medical costs were calculated from a hospital perspective. Each component of resource use was collected for every patient and linked to a unit price. Data on volumes were adapted from hospital electronic databases and medical files. Some patients were partly treated in satellite hospitals: in these cases volume data were retrieved from chart review.

For the unit prices, we applied the microcosting method[1] and Dutch tariffs. All costs were converted to US dollars (€ 1 = $ 1.40) according to the price level in 2010.

Different cost categories were used: (1) inpatient care including room and board, nursing and physician fees. Inpatient care estimates were for a pediatric ICU day in an academic hospital ($ 2,213), inpatient day in an academic hospital ($ 942) and an inpatient day in a satellite hospital ($ 589); (2) day care treatment, this estimate was $ 325 for academic hospitals and $ 290 for satellite hospitals; (3) chemotherapy other than asparaginase; (4) PEGasparaginase per used vial, one vial contains 3,750 IU ($ 1,729); (5) *Erwinia* asparaginase per used vial, one vial contains 10,000 IU ($ 560); (6) Native *E.coli* asparaginase per used vial, one vial contains 10,000 IU ($ 85); (7) additional medication such as antibiotics; (8) laboratory activities; (9) other hospital activities (e.g. imaging, placement of Port-a-Cath® device, bone marrow puncture), and (10) blood products.

Costs of scenarios 2 and 3

In the two hypothetical scenarios, it was assumed that all other treatment besides asparaginase was according to the ALL-10 MRG protocol. Only the costs of asparaginase, number of day care visits and anti-emetic drugs administered together with asparaginase were changed. For all parameters, each item was multiplied by the number needed by the patient to calculate the costs. The number of day care visits was adjusted for the type of asparaginase, according to the dose schedule in the protocol. The costs of anti-emetic drugs (always combined with asparaginase) were calculated according to changes in the dose schedule and the body surface area of the patients.



Supplemental Figure 1: The intensification/continuation phase of the ALL-10 MR protocol.

Decision tree analysis

We developed a decision tree model with TreeAge for Health Care (TreeAge Pro 2009, TreeAge Software, Williamstown, MA, USA) to compare costs of PEGasparaginase or *Erwinia* asparaginase to native *E.coli* asparaginase, while taking into account the incidence of allergy to asparaginase and the different associated costs (Figure 2).

For scenario 1, the allergy rate to PEGasparaginase of the interim results of the ALL-10 MRG protocol was 25%, and this served as the base case value. In scenario 2, the historical allergy rate to native *E.coli* asparaginase in the intensification phase of the ALL-9 protocol after exposure of native *E.coli* asparaginase in induction was 65%.[2] This served as the base case value in this scenario. In scenario 3, the base case value for allergy was similar as that in scenario 2: 65%. If patients subsequently also had an allergic reaction to PEGasparaginase, they were switched

to *Erwinia* asparaginase. The base case value of PEGasparaginase allergy after native *E.coli* asparaginase allergy in the intensification was estimated at 40%.

In every branch of this tree, either the calculated or the simulated costs and the base case probabilities were included. We used the mean values of costs in this decision tree model.

Sensitivity analysis

To account for uncertainty in the used prices and calculated costs, a sensitivity analysis was performed. With one-way sensitivity analysis, costs and allergy probabilities were changed one by one to assess the impact of each change. The costs of subgroups with or without allergy who did not receive *Erwinia* asparaginase were varied 25% each way.[1] Because the price of *Erwinia* asparaginase was doubled in March 2011 when analyzing all cost data, the costs were varied from minus 25% up to 200%. The allergy probabilities were varied according to the lowest probability found in clinical trials 14%[3] to 100%.

Two-way sensitivity analysis was used to assess the effect of different allergy percentages on treatment costs. These percentages were varied from zero to 100%. For every allergy probability the treatment costs of PEGasparaginase or *Erwinia* asparaginase (scenario 1) were compared to those of native *E.coli* asparaginase (either scenario 2 or 3).

Missing data

Missing information from satellite hospitals was retrieved from medical files of the academic hospitals. To account for missing data, estimation based on duration of treatment in the satellite hospitals and the costs were also imputed for each patient.

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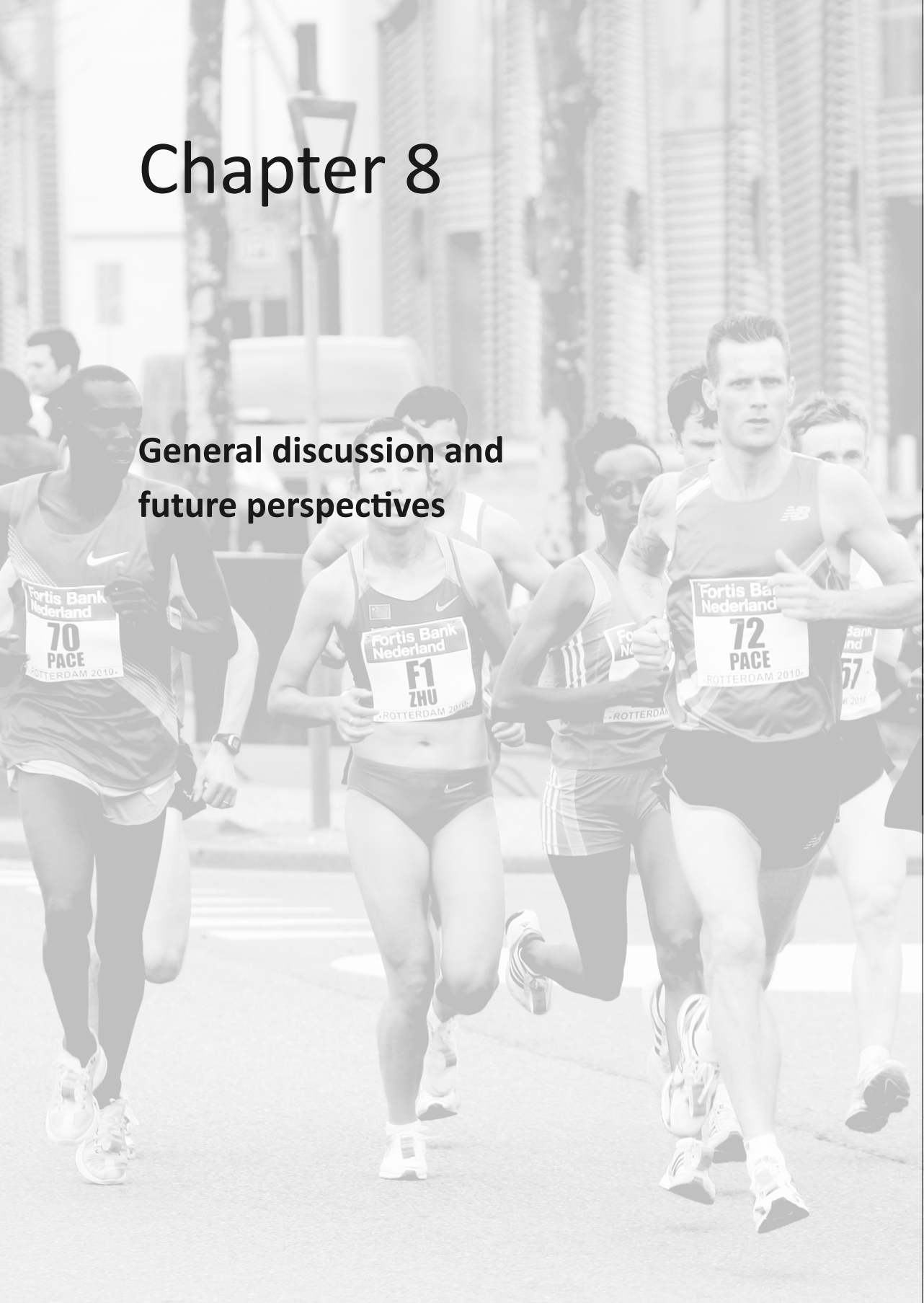
Chapter 8

General discussion and future perspectives



GENERAL DISCUSSION

The aim of this thesis was to study efficacy, toxicity, and putative mechanisms of asparaginase resistance during intensive PEGasparaginase and *Erwinia* asparaginase therapy in the Dutch Childhood Oncology Group (DCOG) ALL-10 medium risk intensification protocol in order to improve the use of these asparaginase preparations. Also we studied whether the use of intensive PEGasparaginase is more expensive compared to the use of native *E.coli* asparaginase in the intensification phase.

Efficacy of asparaginases

In the current childhood acute lymphoblastic leukemia (ALL) treatment, the non-human enzyme asparaginase is used which is derived from bacteria sources.[1-2] Three main types of asparaginase have been used; either these are derived from *Escherichia coli* in its native form (native *E.coli* asparaginase) or as a pegylated enzyme (PEGasparaginase), or is extracted from *Erwinia chrysanthemi* (*Erwinia* asparaginase). The different dose schedules of native *E.coli* asparaginase, PEGasparaginase and *Erwinia* asparaginase are based upon differences in pharmacokinetics. Equivalent doses for optimal efficacy have been suggested for different asparaginase preparations, indicating that one dose of 1,000-2,500 IU/m^2 PEGasparaginase in two weeks is as effective as four doses of 5,000-10,000 IU/m^2 native *E.coli* asparaginase or six doses of 10,000-25,000 IU/m^2 *Erwinia* asparaginase in two weeks.[3-8] However, a lot of variation in asparaginase doses and dose frequency is observed. Therefore, it is important to study what the optimal dosing schedule is for very prolonged use of PEGasparaginase and *Erwinia* asparaginase in childhood ALL.

Chapter 2 showed the results of our prospective drug monitoring study. We assessed the asparaginase activity levels of patients who were treated with 2,500 IU/m^2 PEGasparaginase every two weeks in the intensification. All patients received prior native *E.coli* asparaginase in induction. The mean serum trough level of 899 U/L was found in patients without clinical allergy to or silent inactivation of PEGasparaginase. Recently, the Dana-Farber Cancer Institute (DFCI 05-001 protocol) consortium found similar trough levels of 758 U/l using PEGasparaginase in the same dose schedule.[9] So, the use of 2,500 IU/m^2 PEGasparaginase leads to unnecessary high asparaginase activity levels. Therefore, a dose reduction of PEGasparaginase (1,500 IU/m^2) is applied in the current ALL-11 protocol and the doses are individualized. This dose reduction seems feasible as different re-induction protocols used 1,000 IU/m^2 PEGasparaginase with adequate trough levels of ≥100 U/L in approximately 80% of the patients.[10-11] Appel *et al*. showed earlier that 1,000 IU/m^2 PEGasparaginase in induction resulted in asparaginase levels above 100 U/L for at least two weeks with complete asparagine depletion in all patients.[12] However, a dose reduction of PEGasparaginase should be guided by careful monitoring of asparaginase activity levels.

We found that patients with an allergy to PEGasparaginase (22%) showed asparaginase activity levels of zero. Even very mild allergies (grade 1) were accompanied by undetectable

activity levels and continuing PEGasparaginase was useless. Eight percent of the patients showed silent inactivation of PEGasparaginase with asparaginase activities of zero. Patients can be treated effectively with *Erwinia* asparaginase in case of an allergy to or silent inactivation of PEGasparaginase. The far majority of the patients that switched to *Erwinia* asparaginase showed effective asparaginase activity levels during the first two weeks of *Erwinia* asparaginase, namely median trough activity level of 183 U/L (48-hour) and 93 U/L (72-hour) and asparaginase activity levels of ≥100 U/L in 100% (48-hour) and 33% (72-hour) of the patients. Similar rates were found by Vrooman *et al*. (83% (48-hour) and 45% (72-hour) using a dose of 25,000 IU/m^2 three times per week intravenously.[13] These activity levels were lower compared to that of Salzer *et al*., who reported median trough levels of 645 U/L (48-hour) and 248 U/L (72-hour) and asparaginase activity levels of ≥100 U/L in 93% (48-hour) and 88% (72-hour) of the patients using intramuscular *Erwinia* asparaginase in a dose of 25,000 IU/m^2 three times per week.[14] The route of *Erwinia* asparaginase administration may explain the higher median asparaginase activity levels which were found by Salzer *et al*.[14] However, previous studies have shown no differences in mean asparaginase activity levels, asparagine depletion and asparaginase antibodies after intravenous or intramuscular administration of *Erwinia* asparaginase.[15-17]

In our study, only 3% of the patients treated with *Erwinia* asparaginase had an allergy to *Erwinia* asparaginase which was accompanied by asparaginase activity levels of zero. This is explained by the fact that *Erwinia* asparaginase was not administered earlier in induction and these patients only received continuous *Erwinia* asparaginase courses in intensification. A dosing schedule of three times per week *Erwinia* asparaginase is preferred over a twice weekly dosing schedule in the majority of patients. Close drug monitoring remains necessary to ensure adequate drug levels at the longest time interval (72-hour) during a three times per week dosing schedule as some patients will need an alternate-day dosing schedule. Important conclusion is that the use of *Erwinia* asparaginase as second-line agent is justified in patients who develop a clinical allergy to or silent inactivation of PEGasparaginase.

The above mentioned data make clear that the production of asparaginase antibodies hamper the efficacy of asparaginase.[6,18-20] Detection of silent inactivation is crucial to avoid useless continuation of an inactive asparaginase product which may lead to a worse event-free survival in two independent studies.[19,21]

We found a high incidence of hypersensitivity reactions to PEGasparaginase in the intensification phase due to antibody development against native *E.coli* asparaginase which was used in induction. This implicates that PEGasparaginase should be used upfront already in induction instead of native *E.coli* asparaginase. This is supported by a randomized study showing that 26% of patients treated with native *E.coli* asparaginase developed asparaginase antibodies *versus* only 2% of patients treated with PEGasparaginase in induction phase.[22] We and others show that the presence of asparaginase antibodies is related to allergy to and silent inactivation of asparaginase.[23-24] Chapter 2 revealed that approximately half of the children developed native *E.coli* asparaginase antibodies before start of consolidation. Not all children who had

asparaginase antibodies showed an allergy to or silent inactivation of PEGasparaginase in the intensification phase after the asparaginase break in consolidation. Table 1 shows the sensitivity and specificity of asparaginase antibodies during consolidation and during the first two weeks of the intensification phase.

Table 1: Sensitivity and specificity of asparaginase antibodies to predict an allergy to or silent inactivation of PEGasparaginase.

	day 79 to day 140 consolidation % (CI)	**day 140 to day 154 first two weeks of intensification % (CI)**
sensitivity of Coli-AAA	87% (60%-98%)	100% (95%-100%)
specificity of Coli-AAA	64% (43%-82%)	73% (59%-84%)
sensitivity of PEG-AAA	nd	70% (46%-88%)
specificity of PEG-AAA	nd	100% (95%-100%)

Coli-AAA; native *E.coli* asparaginase antibodies, PEG-AAA; PEGasparaginase antibodies, CI; confidence interval, nd; not determined.

Willer *et al.* found a good sensitivity (97%) and high specificity of 96% using the same antibody test.[23] However, we found low specificity using asparaginase antibodies in both phases to predict an allergy or asparaginase inactivation. Possible explanations of this difference could be the differences in dosing schedule and the cut-off points used. Most important is that we showed that asparaginase antibodies are not perfect to predict asparaginase allergy or silent inactivation of asparaginase. Therefore, the clinical use of antibody monitoring is limited and monitoring drug levels has to be preferred.

Finally, in this part of the thesis, we questioned the use of a desensitization protocol in childhood ALL patients with silent inactivation of PEGasparaginase. Unintended, a desensitization program was applied in our five patients with silent inactivation of PEGasparaginase. In this situation, continuing PEGasparaginase leads to desensitization in which antibodies declined coinciding with rise of PEGasparaginase activity levels over time in all 5 patients. However this desensitization program took an unpredictable and sometimes long time period (several months). Most importantly, the risk of a desensitization course with an uncertain outcome must be outweighed with the switch to another effective asparaginase preparation. As intensive asparaginase has proven to be of relevance for the survival rate, we do not recommend such desensitization approaches, but switching to *Erwinia* asparaginase.

Pharmacodynamics (asparagine/glutamine levels)

This prospective drug monitoring study showed that measuring asparaginase activity levels is preferred over asparagine levels to monitor asparaginase efficacy. If the samples for asparagine measurements are not properly handled, this amino acid is very rapidly degraded *ex vivo* in the tube by asparaginase. This leads to false low asparagine levels. To minimise the problem

of ongoing asparaginase activity in the tube, we rapidly deproteinized the blood samples for amino acid analysis.[8] Therefore, the absence of asparagine is not due to processing errors.[25-27] Chapter 2 showed that serum asparagine was strongly depleted, but not always completely depleted when using *Erwinia* asparaginase compared to PEGasparaginase. With our very low level of quantification of 0.2 μM,[28] 33% of the patients showed no complete asparagine depletion receiving *Erwinia* asparaginase. If the threshold asparagine level of 0.5 μM from literature[29-32] was used, 5% of the *Erwinia* asparaginase treated patients with activity levels of ≥100 U/L showed incomplete asparagine depletion. Therefore, asparagine measurements during *Erwinia* asparaginase may be clinically more relevant than during PEGasparaginase therapy. If lower asparaginase activity levels are accepted, asparagine levels could play an important role to guide the efficacy of asparaginase preparations. In general, the role of asparagine measurements seems to be limited because of the technical limitations.

Striking differences in glutamine levels during asparaginase therapy have been reported. Glutamine "depletion" defined as a decline of glutamine level from 340 μM, before asparaginase to 88 μM, after asparaginase was observed by the Children's Cancer Group (CCG) studies.[33] Another study showed a decline of glutamine level after the first native *E.coli* asparaginase from 579 μM to 84 μM.[34] We demonstrated in chapter 3 that children who received native *E.coli* asparaginase (5,000 IU/m^2 every three days) showed no significant decline in glutamine levels. We observed lower glutamine levels in *Erwinia* asparaginase treated patients as compared to those in PEGasparaginase treated patients. This is remarkable as the serum PEGasparaginase activity levels were much higher than the *Erwinia* asparaginase activity levels. This can be explained by higher glutaminase activity of *Erwinia* asparaginase.[35] Although glutamine is broken down to glutamic acid by asparaginase, this does not lead to glutamine depletion *in vivo* as this is supplemented from other organ stocks. So, no glutamine depletion was seen during the induction phase using native *E.coli* asparaginase, nor during very prolonged PEGasparaginase or *Erwinia* asparaginase therapy in intensification. We conclude therefore that glutamine depletion does not play an important role in the clinical efficacy of asparaginase.

Mesenchymal cells

Iwamoto *et al.* showed that in contrast to leukemic cells, MSCs had a high activity of asparagine synthetase. *In vitro*, this led to high asparagine levels rescuing the ALL cells from the effect of asparaginase.[36] However, we could not confirm their results *in vivo*. Chapter 3 showed that asparagine levels were higher in the bone marrow compared to blood only at diagnosis.[28] This finding is consistent with increased asparagine synthesis within the bone marrow by the leukemic blasts. Asparagine was not detectable in the bone marrow nor in blood during asparaginase therapy. We conclude that the increased asparagine synthesis by MSCs is of relevance for asparaginase resistance of leukemic cells *in vitro*, but it is questionable whether this leads to asparaginase resistance *in vivo* in ALL patients.

Toxicity of asparaginase preparations

Allergy

Asparaginase preparations are associated with various (severe) side effects. Some of these imply a permanent discontinuation of asparaginase therapy. Allergy is a major side effect, and in case of this the *E.coli* derived asparaginase agents should be replaced by *Erwinia* asparaginase.

Most allergic reactions to PEGasparaginase occurred in intensification after the asparaginase-free interval. This is explained by the development of asparaginase antibodies following exposure to native *E.coli* asparaginase in the induction. Antibodies against *E.coli* asparaginase cross-react with the pegylated form of native *E.coli* asparaginase (PEGasparaginase). This is in line with our observation that the allergy almost always occurred at the first few drops of the second PEGasparaginase infusion of the intensification phase. This is due to a boost/rise in antibody titer after the first PEGasparaginase dose.

In two PEGasparaginase naive patients, an allergy to PEGasparaginase was seen at the first course. This can be caused by antibodies against *E.coli* asparaginase or by antibodies against the PEG-molecule. It has been suggested that previous exposure to PEG-containing products might lead to an allergy against the PEG-molecule.[37-38] Those two patients had no detectable anti-PEGasparaginase antibodies, however they had detectable native *E.coli* asparaginase antibodies. So, it is important to distinguish anti-PEG antibodies and anti-PEGasparaginase antibodies.

Interestingly, undetectable asparaginase activity levels were also found in patients with very mild (grade 1) allergic reactions. Consequently, patients with mild allergies need to be switched to *Erwinia* asparaginase as well. We conclude that real-time measurements of asparaginase activity levels are helpful to ensure that children receive the correct asparaginase preparation which leads to therapeutic activity levels.

Dyslipidemia/pancreatitis

Hypertriglyceridemia and hypercholesterolemia (dyslipidemia) are well-known side effects of asparaginase.[39-42] We demonstrated in chapter 5 that patients treated with PEGasparaginase had increased triglyceride levels in the first months of intensification, after week 19 of the intensification these levels decreased. Also *Erwinia* asparaginase treated patients showed slightly increased triglyceride levels, but these were significantly lower compared to the PEGasparaginase treated patients. Importantly, we found that temporary hypertriglyceridemia grade 3/4 was seen in approximately half of patients receiving PEGasparaginase which normalized completely after completion of 30 weeks of asparaginase therapy. As the dyslipidemia is self-limiting and is not associated with the occurrence of pancreatitis, thrombosis or other complications (see below) there is no need to discontinue asparaginase therapy in case of hypertriglyceridemia.

Triglyceride-induced pancreatitis has been suggested in the literature,[43] but we found no associations between the development of pancreatitis and hypertriglyceridemia nor with asparaginase activity levels. The occurrence of (severe) pancreatitis (6%) was comparable

with the literature.[21] Pancreatitis was in our study the only reason to discontinue asparaginase permanently.

Lipid disorders especially occur when corticosteroids and asparaginase are combined.[44-45] In almost all treatment protocols, asparaginase is administered in treatment phases that include corticosteroids. Chapter 6 showed that not asparaginase itself, but the combination of corticosteroids and asparaginase was responsible for severe disturbed lipid metabolism. We applied a simple, but effective strategy to normalize lipid levels in a pediatric ALL patient by temporarily omitting corticosteroids.[46] This strategy was applied successfully in another patient but did not lead to reduction of lipid levels in a third case. The omittance of corticosteroids should be balanced with the risk of leukemia relapse, so this strategy may be riskful.

Hyperammonemia/central neurotoxicity

Hyperammonemia is a logical consequence of asparagine breakdown by asparaginase therapy.

We observed that the ammonia levels after *Erwinia* asparaginase therapy were significantly higher compared to those after PEGasparaginase therapy, despite the lower *Erwinia* asparaginase activity levels. This is explained by higher glutaminase activity of *Erwinia* asparaginase.[47]

Hyperammonemia could have a direct neurotoxic effect.[48] However, we showed in chapter 5 that the level of hyperammonemia was not related to central neurotoxicity, defined as somnolence or depressed level of consciousness, ataxia, seizures, mood alteration or posterior reversible encephalopathy syndrome (PRES).

It is also hypothesized that an increased GABA-ergic tone plays a role in the neurotoxicity of asparaginase. Gamma-aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the brain. As glutamine and glutamate are precursors of GABA[49-50] changes in glutamine and glutamate levels due to asparaginase may contribute to encephalopathy. It has been reported that the severity of hepatic encephalopathy correlates better with the level of intracranial glutamate/glutamine than with ammonia levels. When hepatic encephalopathy occurs, the urea cycle does not work optimally, therefore glutamate and glutamine levels remain very high.[51] In contrast, glutamine levels will decrease during asparaginase therapy and the increase in glutamate levels is much less than observed in patients with hepatic encephalopathy.

In conclusion, we feel that nor hyperammonemia nor changes in glutamine or glutamate can easily explain the asparaginase-induced encephalopathy.

Thrombosis

Thrombosis is related to the inhibition of protein synthesis due to asparagine depletion. The UK ALL 2003 trial reported 3% of cases with venous thrombosis using intramuscular PEGasparaginase (1,000 IU/m^2).[52] However, this group did not use an intensified PEGasparaginase regimen. The DFCI 00-01 protocol[21] detected thrombosis in 8% of patients which is comparable to the 5% found by us using a comparable dose schedule of asparaginase. We found no relation between thrombosis and the asparaginase activity levels. This suggested that asparaginase activity level itself was not a risk factor for the development of thrombosis.

Our group published several studies on the risk of thrombosis and the combined use of corticosteroids and asparaginase. The occurrence of thrombosis is age-dependent with teenagers being at higher risk of thrombosis than children younger than 10 years. During asparaginase therapy anticoagulants proteins declined significantly and the fibrinolytic potential was decreased due to asparagine depletion.[53]

In this thesis, we observed that asparaginase courses could be safely continued after resolution of the vascular obstruction using low molecular weight heparin prophylaxis. No recurrence of thrombosis was seen thereafter.

The number of patients with pancreatitis, thrombosis or central neurotoxicity in our study was low. Therefore, the associations between side effects with asparaginase activity levels should be interpreted with caution.

Cost-analysis of asparaginase preparations

The more intensive use of asparaginase in the treatment of childhood ALL lead to higher drug costs. Chapter 7 showed that asparaginase use accounted for 47% of the costs of the intensification phase of the ALL-10 medium risk protocol. Not only drug costs, but for instance also costs of inpatient care and day care treatment should be taken into account. We found that the asparaginase costs are mainly determined by the percentage of allergic patients requiring a switch to the more expensive *Erwinia* asparaginase. PEGasparaginase is administered less frequently with fewer day care visits than native *E.coli* asparaginase or *Erwinia* asparaginase and PEGasparaginase is less immunogenic.[54-55] So, reducing the number of allergies will reduce the costs.

FUTURE PERSPECTIVES

This project resulted in significant changes in the use of PEGasparaginase in the Netherlands. Due to the high PEGasparaginase activity levels found in the ALL-10 protocol, a dose reduction is applied from 2,500 IU/m^2 to 1,500 IU/m^2 in the ALL-11 protocol. Also, we found that the use of native *E.coli* asparaginase in induction led to a high hypersensitivity and silent inactivation rate to PEGasparaginase in the ALL-10 medium risk intensification protocol. To decrease this, the ALL-11 protocol prescribes PEGasparaginase instead of native *E.coli* asparaginase in induction. A randomized study is performed to determine whether continuous dosing schedule compared to the non-continuous dosing schedule will result in less hypersensitivity reactions.

In the ALL-11 protocol, therapeutic drug monitoring of asparaginase has been implemented to effectively adjust asparaginase doses and to detect cases of silent inactivation in time. This nation-wide program has been initiated to individualize asparaginase treatment for all newly diagnosed ALL patients.

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Summary

Samenvatting

SUMMARY

This thesis presents new insights on efficacy and toxicity of very prolonged use of PEGasparaginase and *Erwinia* asparaginase in children with acute lymphoblastic leukemia (ALL). Also, the results of a cost-analysis of asparaginase preparations in the Dutch Childhood Oncology Group (DCOG) ALL-10 medium risk intensification protocol are shown.

Chapter 2 and Chapter 4 focus on the efficacy of asparaginases. Administration of asparaginase can be limited by the occurrence of hypersensitivity reactions by antibodies leading to inactivation of asparaginase. Asparaginase antibodies can also neutralize asparaginase without any clinical signs of hypersensitivity, so called silent inactivation. In a first prospective study, PEGasparaginase courses were monitored in eighty-nine newly diagnosed ALL patients. Thirty percent developed hypersensitivity to PEGasparaginase: 22% clinical allergy and 8% silent inactivation.

All patients with clinical allergies to or silent inactivation of PEGasparaginase showed PEGasparaginase activity levels of zero. Hypersensitivity to pegylated *E.coli* asparaginase was due to the antibody formation against native *E.coli* asparaginase which was used in the induction. Children without clinical allergy to and without silent inactivation of PEGasparaginase had asparaginase trough levels varying from 475 to 1786 U/L. These levels were much higher than the desired trough level of 100 U/L which is sufficient for complete asparagine depletion.

In a second nation-wide study, fifty-nine children who developed hypersensitivity to PEG-asparaginase were switched to *Erwinia* asparaginase 20,000 IU/m^2 three times per week. One-third of these patients could be switched to a twice weekly dosing schedule because of “high” asparaginase trough levels (72-hour levels ≥100 U/L) measured in the first two weeks of therapy. Most patients treated with *Erwinia* asparaginase showed effective asparaginase activity levels, namely 96% of the patients had at least one *Erwinia* asparaginase trough level of ≥100 U/L. Only 3% of the patients developed an allergy to *Erwinia* asparaginase and none silent inactivation. Serum asparagine was strongly depleted, but not always completely with *Erwinia* asparaginase compared to PEGasparaginase. We suggest that asparagine measurements during *Erwinia* asparaginase may be clinically more relevant than during PEGasparaginase therapy. A dosing schedule of three times per week *Erwinia* asparaginase is preferred over a twice weekly schedule in the majority of patients. Close drug monitoring remains necessary to ensure adequate drug levels at the longest time interval. The use of *Erwinia* asparaginase as second-line agent is justified in patients who develop a clinical allergy to or silent inactivation of PEGasparaginase.

Our study also showed that the presence of asparaginase antibodies is related to allergy and silent inactivation of asparaginase. The sensitivity of the (*E.coli*) antibody test to predict allergy or silent inactivation in the intensification was 87%. But predicting asparaginase allergy or silent inactivation based upon antibody formation is hampered by the low specificity (64%) of this test. Therefore, it is more useful to monitor the asparaginase activity levels.

Chapter 3 analysis whether the production of excess asparagine by mesenchymal cells in the bone marrow can lead to asparaginase resistance *in vivo*. Increased asparagine levels in the bone marrow compared to blood was not detected during asparaginase therapy. So, there is no evidence that resistance of leukemic cells to asparaginase is caused by increased asparagine levels in the bone marrow in the *in vivo* situation.

Chapter 5 and Chapter 6 describe the non-allergic toxicities during asparaginase therapy, such as dyslipidemia, pancreatitis, hyperammonemia, thrombosis and central neurotoxicity. Hypertriglyceridemia and hypercholesterolemia grade 3/4 were found in 47% and 25%, respectively, of the PEGasparaginase-treated patients. No grade 3/4 hypertriglyceridemia was found in patients receiving *Erwinia* asparaginase. However, hyperammonemia grade 3/4 was only found in *Erwinia* asparaginase-treated patients (10%). Triglyceride, cholesterol and ammonia levels increased rapidly and remained temporary elevated using each of both agents, but normalized after finishing the last asparaginase dose. High triglyceride and high cholesterol levels were associated with high asparaginase activities. Thrombosis occurred in 4.5%, pancreatitis in 6%, and central neurotoxicity in 10% of the patients; these toxicities were not related to asparaginase activity levels. No associations were found between hypertriglyceridemia and pancreatitis or thrombosis nor between ammonia and central neurotoxicity. In conclusion, severe dyslipidemia occurred frequently, but was temporary and not associated with other side effects and is no clinical reason to interrupt or discontinue asparaginase courses.

In Chapter 7, the results of a cost-analysis of asparaginase preparations in the DCOG ALL-10 medium risk intensification protocol are shown. The total costs of using PEGasparaginase and native *E.coli* asparaginase in intensification therapy are comparable. The costs are mainly determined by the percentage of allergic patients requiring a switch to *Erwinia* asparaginase. Therefore, reducing the number of allergies by using PEGasparaginase will reduce the costs. Most importantly, PEGasparaginase is administered less frequently with fewer day care visits than native *E.coli* asparaginase or *Erwinia* asparaginase. Thus, the use of PEGasparaginase is the most patient friendly option as first line treatment.

In Chapter 8 the general discussion and the future perspectives are presented. Intensified and effective asparaginase therapy is very important in modern treatment of childhood ALL. The use of native *E.coli* asparaginase in induction leads to a high rate of hypersensitivity reactions to PEGasparaginase in the intensification phase of the DCOG ALL-10 medium risk protocol. Based on this research, the starting PEGasparaginase dose in the ongoing ALL-11 protocol has been lowered to 1,500 IU/m^2 because of the high asparaginase trough levels which were found in the ALL-10 protocol. This dose reduction of PEGasparaginase should be guided by careful monitoring of asparaginase activity levels. Currently, a nation-wide therapeutic drug monitoring (TDM) program is used to individualize the PEGasparaginase dose and to detect silent inactivation. In case of an allergy to or silent inactivation of PEGasparaginase, patients are switched to *Erwinia* asparaginase with TDM to allow individualized dosing of *Erwinia* asparaginase.

SAMENVATTING

Dit proefschrift biedt nieuwe inzichten over werkzaamheid en veiligheid van PEGasparaginase en *Erwinia* asparaginase bij kinderen met acute lymfatische leukemie (ALL). Het onderzoek is uitgevoerd bij kinderen die intensief asparaginase kregen toegediend volgens het ALL-10 protocol van Stichting Kinderoncologie Nederland (SKION). Ook worden de resultaten van een kostenanalyse van verschillende soorten asparaginase in de intensiveringsfase van het ALL-10 protocol gepresenteerd.

In de hoofdstukken 2 en 4 wordt de werkzaamheid van verschillende soorten asparaginase beschreven. Het toedienen van asparaginase kan worden belemmerd door het optreden van overgevoeligheidsreacties op asparaginase. Deze kunnen ontstaan door asparaginase antistoffen die asparaginase inactiveren. Asparaginase antistoffen kunnen ook asparaginase neutraliseren zonder klinische tekenen van overgevoeligheid, dit heet ook wel stille inactivatie. In een eerste prospectieve studie werden de asparaginasespiegels gemeten bij 89 kinderen met ALL. Dertig procent ontwikkelde een overgevoeligheidsreactie voor PEGasparaginase: 22% een klinische allergie en 8% stille inactivatie.

Alle patiënten met klinische allergieën of stille inactivatie van PEGasparaginase hadden PEG-asparaginase spiegels van nul. De overgevoeligheid voor PEGasparaginase ontstond, omdat er asparaginase antistoffen ontstaan na gebruik van native *E.coli* asparaginase in de inductiefase. Kinderen zonder klinische allergie of stille inactivatie van PEGasparaginase hadden bij een dosering van 2.500 eenheden/m^2 (één keer per twee weken) asparaginase dalspiegels variërend van 475 tot 1786 eenheden/L. Deze waren veel hoger dan de gewenste dalspiegel van 100 eenheden/L welke met complete asparagine depletie is geassocieerd.

In een tweede landelijke studie waren 59 kinderen overgevoelig voor PEGasparaginase en zij werden overgezet naar *Erwinia* asparaginase 20.000 eenheden/m^2 drie keer per week. Bij een derde van de patiënten kon de doseringsfrequentie worden verlaagd naar twee keer per week, omdat in de eerste twee weken hoge *Erwinia* asparaginase dalspiegels (72 uur spiegels ≥100 eenheden/L) werden gemeten. De meeste patiënten lieten effectieve asparaginasespiegels zien; zo had 96% van de patiënten tenminste één *Erwinia* asparaginase dalspiegel van ≥100 eenheden/L. Slechts 3% ontwikkelde een allergie op *Erwinia* asparaginase en er waren geen patiënten met stille inactivatie. Asparagine was sterk verlaagd, maar niet altijd compleet gedepleteerd bij patiënten die *Erwinia* asparaginase kregen toegediend. Niet allergische patiënten die met PEGasparaginase werden behandeld, hadden wel altijd volledige asparagine depletie. Asparagine metingen tijdens *Erwinia* asparaginase therapie zouden daarom meer van klinisch belang kunnen zijn dan tijdens PEGasparaginase therapie. Uit deze studie blijkt verder dat een doseringsschema van 3 keer per week *Erwinia* asparaginase de voorkeur verdient boven een schema van 2 keer per week voor de meeste kinderen. Het is aan te bevelen om regelmatig asparaginasespiegels te blijven meten. Dus het gebruik van *Erwinia* asparaginase als tweedelijnstherapie is gerechtvaardigd bij patiënten die een klinische allergie of stille inactivatie van PEGasparaginase ontwikkelen.

Het onderzoek toont ook aan dat de aanwezigheid van asparaginase antistoffen gerelateerd is aan een allergische reactie of stille inactivatie van asparaginase. De sensitiviteit van de antistoffentest om een allergie of stille inactivatie in de intensiveringsfase te voorspellen is 87%. De specificiteit van deze test was laag, namelijk 64%. Daarom is het voorspellen van een allergische reactie of een stille inactivatie gebaseerd op deze antistoffentest nog niet optimaal. Het meten van asparaginasespiegels verdient de voorkeur boven het meten van asparaginase antistoffen.

Hoofdstuk 3 beschrijft de productie van asparagine door stamcellen in het beenmerg als mogelijk mechanisme van asparaginase resistentie. Er werd geen verhoogde asparagine in het beenmerg in vergelijking met het bloed tijdens de asparaginase therapie gevonden. Daarom is er dus geen bewijs voor resistentie van de leukemiecellen voor asparaginase door verhoogde asparagine in het beenmerg, *in vivo*.

Hoofdstukken 5 en 6 zijn aan de niet-allergische bijwerkingen van asparaginase gewijd, zoals veranderingen in het vetmetabolisme, pancreatitis, hyperammonemie, trombose en centrale neurotoxiciteit. Hypertriglyceridemie en hypercholesterolemie graad 3/4 werden gezien in respectievelijk 47% en 25%, van de patiënten PEGasparaginase. Er werd geen hypertriglyceridemie graad 3/4 gevonden bij patiënten die *Erwinia* asparaginase kregen toegediend, maar hyperammonemie graad 3/4 werd alleen bij de patiënten (10%) gezien die *Erwinia* asparaginase kregen toegediend. Bij elke soort asparaginase stegen de triglyceride, cholesterol en ammoniak spiegels snel en bleven tijdelijk verhoogd. Na de laatste asparaginase infusie normaliseerden al deze spiegels. Hoge triglyceride en hoge cholesterol spiegels waren gecorreleerd met hoge asparaginasespiegels. Trombose, pancreatitis en centrale neurotoxiciteit kwamen voor in respectievelijk 4.5%, 6% en 10% van de patiënten. Deze bijwerkingen waren niet geassocieerd met asparaginasespiegels. Er werden geen associaties gevonden tussen hypertriglyceridemie met pancreatitis of trombose en ook niet tussen ammoniak spiegels met centrale neurotoxiciteit. Geconcludeerd, het verstoorde vetmetabolisme bij kinderen die behandeld werden met PEGasparaginase is tijdelijk van aard en niet geassocieerd met andere bijwerkingen. En er is geen klinische reden om de asparaginase therapie te onderbreken of permanent te staken.

In hoofdstuk 7 worden de resultaten van een kosten analyse van de verschillende soorten asparaginase in het ALL-10 protocol in de intensiveringsfase gepresenteerd. De totale kosten bij gebruik van PEGasparaginase en native *E.coli* asparaginase in de intensiveringsfase zijn vergelijkbaar. De kosten worden voornamelijk bepaald door het percentage van allergische patiënten die overgezet moeten worden naar *Erwinia* asparaginase. Het gebruik van PEGasparaginase zal het aantal allergieën verminderen en daardoor de kosten reduceren. Het is belangrijk om te benadrukken dat PEGasparaginase minder vaak toegediend hoeft te worden en daarom minder poliklinische bezoeken nodig zijn dan wanneer native *E.coli* asparaginase zou worden gebruikt. Dus het gebruik van PEGasparaginase is de meest patiëntvriendelijke optie als eerstelijnstherapie.

In hoofdstuk 8 worden de resultaten van alle uitgevoerde studies bediscussieerd en de toekomstplannen voor vervolgonderzoek besproken. Intensieve en effectieve asparaginase

therapie is heel belangrijk in de huidige behandeling van kinderen met ALL. Het gebruik van native *E.coli* asparaginase in de inductie leidt tot hoge percentages van overgevoeligheidsreacties, namelijk 22% allergie en 8% stille inactivatie van PEGasparaginase in de intensiveringsfase van het ALL-10 protocol, waarin 30 weken asparaginase wordt voorgeschreven. Gebaseerd op dit onderzoek is de startdosering van PEGasparaginase in het ALL-11 protocol verlaagd naar 1.500 eenheden/m^2, omdat er hoge asparaginase spiegels in het ALL-10 protocol werden gevonden. Deze reductie van de PEGasparaginase dosering zou met frequente metingen van asparaginase spiegels gepaard moeten gaan. Tegenwoordig wordt een landelijk therapeutic drug monitoring (TDM) programma gebruikt om de asparaginase dosering te individualiseren en om stille inactivatie te detecteren. In het geval van een allergische reactie of stille inactivatie van PEGasparaginase worden kinderen overgezet op *Erwinia* asparaginase met TDM, zodat ook deze dosering kan worden geïndividualiseerd.

List of publications

This thesis

Tong WH, Pieters R, De Groot-Kruseman HA, Hop WJC, Boos J, Tissing WJE, van der Sluis IM. Toxicity of very prolonged PEGasparaginase and *Erwinia* asparaginase courses in relation to asparaginase activity levels. **Submitted**.

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Plasma amino acids levels in nitric oxide responsive and non-responsive PPHN (Persistent Pulmonary Hypertension of the Newborn) newborns. **Submitted**.

WH Tong, MD, MSc, WCJ Hop, PhD, EAP Steegers, MD, PhD, JJ Duvekot, MD, PhD.
Subclinically increased Troponin T levels in preeclamptic patients are related to the development of pulmonary edema. **Submitted.**

LCJ van den Berk, A. van der Veer, ME Willemse, MJGA Theeuwes, MW Luijendijk, **WH Tong**, IM van der Sluis, R. Pieters, ML den Boer.
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TE Cohen-Overbeek, **WH Tong**, TR Hatzmann, JF Wilms, LCP Govaerts, EAP Steegers, WCJ Hop, JW Wladimiroff, D Tibboel. 2010.
Omphalocèle: comparison of outcome following prenatal or postnatal diagnosis.
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Increased adverse perinatal outcome of hospital delivery at night.
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Curriculum vitae

Wing Hung Tong was born on 5th March 1980 in Zeist. When he was eight years old, the family moved to Rotterdam. In 1999, Wing finished his pre-university education (Atheneum) at the "Christelijk College Henegouwerplein". After the four-year study "Health care, Policy and Management" he obtained his Master of Science degree at the Erasmus University Rotterdam. Then, still motivated to continue in medicine, Wing started a new academic study medicine in 2003, also in Rotterdam. Wing obtained his medical degree at the Erasmus Medical Center in 2009. Between May 2009 and May 2014, Wing undertook his PhD research at the department of Pediatric Oncology/Hematology of the Erasmus MC-Sophia Children's Hospital (promotor: prof. dr. Rob Pieters and co-promotor dr. Inge M. van der Sluis), where the current doctoral thesis was written. Wing previously had been a medical student assistant at the Obstetrics department in this hospital (2005-2009), and it was then when he gained the first experiences with scientific research, which led to two scientific articles in this field. Since 2009 he has been a volunteer for the track-and-field union ("PAC") Rotterdam as youth athletics trainer. His passion for marathon running inspired him to start raising money for charity: "Kinderen Kankervrij" (KiKa). To this aim, Wing ran the marathon of New York in 2011. Regrettably the 2012 edition of the New York marathon was blown off on account of hurricane "Sandy". Wing instead chose the marathon of Rotterdam 2013 as the race to collect money for the Princess Máxima Center for Pediatric Oncology (to be built in Utrecht) by selling "KiKa" stones. In June 2014 Wing will start as a resident at the department of Pediatrics at the Albert Schweitzer Hospital in Dordrecht.

PhD portfolio

Summary of PhD training and teaching activities

Name PhD candidate:	Wing Hung Tong
Erasmus MC department:	Pediatric Oncology/Hematology
Research school:	MolMed
PhD period:	May 2009 – May 2014
Promotor:	Prof.dr. R. Pieters
Supervisor:	Dr. I.M. van der Sluis

	Year	Workload
1. PhD TRAINING		
General courses:		
– Basiscursus Regelgeving & Organisatie voor Klinisch onderzoekers	2009	1.0 ECTS
– Minicursus methodologie van patiëntengebonden onderzoek en voorbereiding subsidieaanvragen	2010	0.3 ECTS
– Biomedical English Writing and Communication	2011	4.0 ECTS
– Principles of Clinical Pharmacology (NIH)	2011-2012	2.0 ECTS
– Course English Common European Framework of Reference	2012	4.0 ECTS
Research skills:		
– Endnote course	2009	0.1 ECTS
– NVK JOD proposal schrijven	2010	0.3 ECTS
– NVK JOD timemanagement	2012	0.3 ECTS
– NVK JOD intervisie	2013	0.3 ECTS
(Inter)National Conferences – participation and presentations:		
Oral presentations		
– Subclinically increased Troponin T levels in preeclamptic patients are correlated with the development of pulmonary edema. 16th ISCOMS. Groningen	2009	1.0 ECTS
– Plasma amino acids levels in NO responsive and non-responsive PPHN newborns. 8th Research day Pediatrics. Rotterdam	2009	1.0 ECTS
– Clinical studies on asparaginase in childhood acute lymphoblastic leukemia. Laboratory meeting. Muenster	2010	1.0 ECTS
– Asparaginase studies. Symposium: Landelijke bijscholingsdag voor kinderoncologieverpleegkundigen. Rotterdam	2012	1.0 ECTS
– Research: asparaginase. Meeting: marathon of New York. Den Bosch	2012	0.5 ECTS

	Year	Workload
– Asparaginase: a clinical study. Young Researchers Day. Veldhoven	2012	0.5 ECTS
– Het wetenschappelijke 'zorgpad' van asparaginase. 2nd National shared care symposium of the DCOG Netherlands. Amsterdam	2012	1.0 ECTS
– Efficacy, drug resistance and toxicity of extensive use of asparaginase in childhood acute lymphoblastic leukemia, project number: 44. KiKa site visit. Breukelen	2012	1.0 ECTS
– Therapeutic drug monitoring of asparaginase. KiKa PhD day: Tom Voûte Young Investigators Award. Utrecht	2013	1.0 ECTS
– A prospective study on drug monitoring of asparaginases in pediatric ALL. KiKa PhD day: Tom Voûte Young Investigators Award. Utrecht	2014	1.0 ECTS
– Toxicity of very prolonged PEGasparaginase and *Erwinia* asparaginase courses in relation to asparaginase activity levels. 9th CLS meeting. Prague	2014	1.0 ECTS
Poster presentations		
– Omphalocèle; comparison of the perinatal outcome following a prenatal diagnosis or a or a diagnosis at birth. 19th World Congress on Ultrasound in Obstetrics and Gynecology. Hamburg	2009	0.5 ECTS
– The bone marrow niche of patients with acute lymphoblastic leukemia produces no increased asparagine levels *in vivo* that may lead to clinical asparaginase resistance. 53th ASH. San Diego	2011	1.0 ECTS
– No evidence of increased asparagine levels in the bone marrow of patients with acute lymphoblastic leukemia during asparaginase therapy. 44th SIOP. London	2011	1.0 ECTS
– Cost-effectiveness of treatment of childhood acute lymphoblastic leukemia with PEGasparaginase and *Erwinia* asparaginase: the impact of expensive chemotherapy. 54th ASH. Atlanta	2012	1.0 ECTS
– A prospective study on drug monitoring of PEGasparaginase and *Erwinia* asparaginase and asparaginase antibodies in pediatric acute lymphoblastic leukemia. 55th ASH. New Orléans	2013	1.0 ECTS
– Should we use a desensitization protocol in acute lymphoblastic leukemia with silent inactivation of PEGasparaginase? 55th ASH. New Orléans	2013	1.0 ECTS
– A prospective study on drug monitoring of PEGasparaginase and *Erwinia* asparaginase and asparaginase antibodies in pediatric acute lymphoblastic leukemia. 9th CLS meeting. Prague	2014	1.0 ECTS
– Should we use a desensitization protocol in acute lymphoblastic leukemia with silent inactivation of PEGasparaginase? 9th CLS meeting. Prague	2014	1.0 ECTS

	Year	Workload
Meetings, seminars and workshops:		
– Weekly research meetings department of pediatric oncology/ hematology. Rotterdam	2009-2014	2.0 ECTS
– Weekly laboratory meetings department of pediatric oncology/ hematology. Rotterdam	2009-2014	2.0 ECTS
– Multi-center talks: asparaginase research projects (one talk per year per center, n=6)	2009-2014	4.0 ECTS
– Weekly QCAT research meetings during and after cancer treatment	2009-2014	2.0 ECTS
– Erasmus MC PhD day	2009	0.2 ECTS
– Guidelines and late effects research for childhood cancer survivors. 2nd National DCOG "LATER" Conference	2010	0.2 ECTS
– Presentatie in Groningen. De Silent Inactivation trial. (georganiseerd door EUSA Pharma/Genzyme)	2010	1.0 ECTS
– The Dutch Cancer Society. Symposium on Translational Oncology. Ede	2010	0.4 ECTS
– Zorg voor Kennis. Symposium georganiseerd door de alumnivereniging BMG. Rotterdam	2010	0.2 ECTS
– 1st National shared care symposium of the DCOG Netherlands. Utrecht	2011	0.4 ECTS
– SKION promovendi dag. Utrecht	2012	0.4 ECTS
– 2nd National shared care symposium of the DCOG Netherlands. Amsterdam	2012	0.4 ECTS
– SKION promovendi dag. Utrecht	2013	0.4 ECTS
– DCOG "SKION-dagen". Utrecht	2013	0.4 ECTS
– 2nd Daniel den Hoed Day. Rotterdam	2013	0.2 ECTS
– 1st Research retraite. Princess Máxima Center. De Bilt	2013	0.4 ECTS
– SKION promovendi dag. Utrecht	2014	0.4 ECTS
– DCOG "SKION-dagen". Utrecht	2014	0.4 ECTS
– Symposium Clinical Trial Center. Rotterdam	2014	0.4 ECTS

	Year	Workload
2. TEACHING ACTIVITIES		
Lecturing:		
– Curriculum primary school (KiKa, leukemie en onderzoek (Barendrecht). Voorlezen boekenweek)	2013	1.0 ECTS
– Curriculum secondary school (HAVO/VWO profielwerkstukken over een geneeskunde onderwerp)	2013	1.0 ECTS
– Curriculum secondary school (maatschappelijke stages bij hematologisch laboratorium specieel)	2013-2014	0.4 ECTS
Supervising Master thesis:		
– Supervising Ms. Cathelijne Alleman during her final year MSc Health Economics Policy and Law, EUR Rotterdam. Master thesis 12-month internship	2010-2011	10.0 ECTS
Other skills:		
– Training in statistics and SPSS for colleagues of the QCAT research group	2012-2013	1.0 ECTS
– Peer reviewing of articles for the following scientific journals: Pediatric Blood & Cancer, British Journal of Haematology, Cancer, Leukemia, Blood, Haematologica	2011-2014	2.0 ECTS

Dankwoord

Nu ik dit dankwoord aan het schrijven ben, zit ik midden in mijn training voor de marathon van Rotterdam 2014. Het uitvoeren van promotieonderzoek voelt voor mij net als het lopen van een marathon. Uren van noeste arbeid zitten hierin. De marathon en het promotieonderzoek hebben iets gemeenschappelijks en dat is samenwerking. Daarom wil ik beginnen met de kinderen en hun ouders/verzorgers te bedanken voor de samenwerking. Zonder jullie is er geen wetenschappelijk onderzoek mogelijk. Wat heb ik veel van jullie geleerd: respect voor de manier waarop jullie omgaan met de ziekte, hoe sommige patiënten al jong volwassen werden en de geïnteresseerdheid in de resultaten van "dokter Wing". Die resultaten zijn er zeker naar en daar ben ik jullie veel dank voor verschuldigd!

Geachte professor Pieters, beste Rob, ik kan nog heel goed ons eerste promotoroverleg herinneren. Ik wilde je graag de data van de eerste drie patiënten van de *Erwinia* asparaginase studie laten zien, maar altijd hield je mij de volgende vraag voor: "Wing, wat is de vraagstelling hierbij?" Het kritisch kijken naar de data en artikelen heb ik geleerd van je, maar ook jouw enorme motivatie en inspiratie hebben ervoor gezorgd dat ik met plezier naar het werk ging. Altijd kon ik snel de manuscripten retour krijgen voorzien van goede "comments", variërend van "nog niet goed" tot "Ok, Rob" of zelfs "goed stuk". Toen ik voor het eerst het woord "submission" zag staan op een artikel was ik blij hiermee. Je inspireerde mij om te gaan hardlopen voor het goede doel (eerst voor Kinderen Kankervrij, KiKa en later voor het Prinses Máxima Centrum voor Kinderoncologie, via de KiKa stenen). Zo mocht ik namens KiKa twee keer de New York marathon lopen. Graag wil ik je veel succes wensen met de volgende droom: de realisatie van het Prinses Máxima Centrum voor Kinderoncologie!

Geachte dr. Van der Sluis, beste Inge, eind 2008 toen ik midden in mijn co-schappen zat, heb ik voor het eerst van het woord "asparaginase" gehoord. Mijn eerste gedachte was: "heeft iets met een enzym te maken." Vele uren hebben wij gezeten op de diverse stukken, op een gegeven moment heb ik geen versienummers meer gezet op de stukken, maar alleen een datum. Maar het onderzoek is klaar en daarmee ook het proefschrift. Ik wil je bedanken voor het vertrouwen wat je altijd in mij hebt gehad. Soms was ik wel heel snel (en niet kritisch genoeg) met de stukken, maar mede door jouw overzicht, structuur en geduld ligt er nu een proefschrift dat staat als een huis. Je stond altijd klaar als "Wing weer eens een kort vraagje had" of als een "paper" met enige spoed (vanwege abstract deadline) doorgenomen moest worden. Graag wil ik je vooral ook plezier wensen met de vele nieuwe studies (fase 1/2) die gaan volgen en met het begeleiden van mijn opvolger van het asparaginase project.

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de grote commissie wil ik bedanken. Geachte professor Uyl-de Groot, beste Carin, geachte professor Hoogerbrugge, beste Peter en geachte professor Rings, beste Edmond, dank voor jullie bereidheid voor het zitting nemen in deze commissie. Extra dank gaat uit naar Carin, want jij zorgde voor het eerste contact met Rob toen wij elkaar spraken tijdens een BMG-feestje. Als ik nu niet op dat feestje was geweest…

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Dit onderzoek was niet mogelijk geweest zonder een uniek laboratorium. Het feit dat wij een diagnostisch laboratorium op de afdeling en polikliniek hebben, maakt de samenwerking tussen polikliniek, kliniek en laboratorium alleen maar sterker. Rolinda, ik vind het knap dat jij als hoofd

van het Haematologisch Laboratorium Specieel én het managen daarvan, analist kan zijn en ook goed onderwijs kan geven aan jonge artsen, maar ook aan middelbare scholieren. Het was fijn samenwerken met je, vooral door jou heb ik “leren pipetteren”! Dan Henk, bedankt voor alle hulp en interesse in mijn onderzoek, jij komt later aan bod. ;-) Bedankt Linda, Tineke, Carla, Fred en Miriam voor jullie interesse, maar ook de hulp op de “admi” als weer eens een asparaginase spiegel kwam en Wing weer eens in de VS zat voor een ASH congres.

Ook zou dit onderzoek niet mogelijk zijn geweest zonder de medewerking van de kinderoncologieverpleegkundigen. Zij hebben heel vaak briefjes van Wing Tong gezien in de behandelkamers, of mochten zij mij bellen dat het bloed afgenomen was of dat het kind naar de OK ging voor een beenmergpunctie. Graag wil ik alle verpleegkundigen van de unit kinderoncologie/ hematologie bedanken in willekeurige volgorde: Miranda, Ellen, Janna, Gea, Gerda, Rianne, Wanda, Melanie, Eline, Daniëlle van der Heijden, Jessica, Carina, Marianne, Christel, Mariska, Sanne, Sherriëne, Kees, Karin, Carine, Anja, Eveline, Romy, Magda, Stephany, Judith, Evelien, Ilse, Katinka, Bianca, Maud, Gemma, Ruth, Nel, Marja, Yvonne, Sarien, Astrid, Daniëlle Houwaard en Marjan. Naast kinderoncologieverpleegkundigen heb ik dankbaar gebruik mogen maken van alle hulp van de researchverpleegkundigen. Zij hadden mij ingewerkt in het “research” wereldje van asparaginase (toen liep er al de R-ASP 2 studie), zij namen mijn werk over als ik eens vakantie had, maar zij waren ook altijd in voor een gezellig praatje, bedankt: Inekee, Marian, Anke en Jolanda. Marian, jij zit (net als ik) midden in de marathonvoorbereiding. Hopelijk viel de marathon mee? Daarnaast hoop ik dat je het naar je zin hebt op de nieuwe afdeling? Natuurlijk wil ik ook alle kinderoncologieverpleegkundigen en researchverpleegkundigen van de andere kinderoncologie centra bedanken voor de interesse en alle hulp met de diverse asparaginase studies.

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Een onderzoek kan niet efficiënt lopen, zonder goede logistiek. Daarom wil ik het secretariaat kinderoncologie/hematologie hartelijk bedanken; Jeanine, Jacqueline en Anita (J&J&A). Jullie waren onafscheidelijk, 6 handen op 1 buik en vol passie en gedrevenheid spannen jullie je voor

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Elke donderdag was er de QCAT bespreking, waarin lange termijn effecten onderwerpen en onderzoeken werden besproken. Ik heb het als prettig ervaren om onderdeel uit te mogen maken van deze gezellige groep. Leuk is het om met jullie te hardlopen. Straks lopen wij weer voor de tweede keer de Run for KiKa Rotterdam, samen met Saskia Pluijm en Andrica. Daarnaast heeft de QCAT groep veelvuldig geborreld en elkaar op taart getrakteerd. Bedankt voor de gezellige tijd, Manita, Marjo, Lidewij, Karin, Wendy, Marjolein, Lizet, Saskia Pluijm, Saskia Gooskens, Annelies, Ivana, Marissa, Mark, Sebastian, Sebastiaan, Eva en Maite.

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Een promovendus kan niet zonder de hulp van zijn paranimfen. Daarom wil ik twee personen speciaal bedanken: in de eerste plaats Richard. Wij kennen elkaar sinds 1999 van de studie gezondheidswetenschappen, toen hadden wij nooit gedacht dat we promotieonderzoek zouden verrichten. Vervolgens promoveerde jij in 2009 en ik nu in 2014. Ik moet nog altijd denken aan de volgende opmerking: "die gangen met kamers zien er wel uit als een soort gevangenis." Wij spraken elkaar vaak op de universiteit, maar ook tijdens etentjes. Dit heb ik gewaardeerd. Jij bent nu vader geworden van prachtige Bess, ik kom haar eens opzoeken! Leuk dat ik uitgenodigd werd voor jouw trouwfeest met Mariska. Het was een eer om aan jouw zijde te staan toen jij promoveerde, het is nu een eer om je als mijn paranimf te hebben.

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Wing Hung Tong