Pharmacodynamics of L-Asparaginase in Childhood Acute Leukemia

Pharmacodynamiek van L-Asparaginase
in acute leukemie bij kinderen

ISBN: 978-90-8559-346-1

The Foundation Sophia Children's Hospital Fund (SSWO), the Pediatric Oncology Foundation Rotterdam (KOCR), Medac (GmbH, Hamburg, Germany), and Lamepro (B.V., The Netherlands) are gratefully acknowledged for their financial support of the work presented in this thesis.

The print and the reproduction of this thesis were kindly supported by Novartis B.V., and CSL-Behring B.V.

Cover: Asparagus compactus T.M. Salter, by E.G. Rice. Picture printed with permission of Compton Herbarium (South Africa).
Lay-out: Margo Terlouw-Willebrand, Nieuwerkerk aan den IJssel
Printed by: Optima Grafische Communicatie, Rotterdam (optima@ogc.nl)

Pharmacodynamics of L-Asparaginase in Childhood Acute Leukemia

Pharmacodynamiek van L-Asparaginase
in acute leukemie bij kinderen

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus
Prof.dr. S.W.J. Lamberts
en volgens besluit van het College voor Promoties

De openbare verdediging zal plaats vinden op
vrijdag 1 februari 2008 om 13.30 uur

door

Inge Maria Appel

geboren te Groningen

ERASMUS UNIVERSITEIT ROTTERDAM

PROMOTIECOMMISSIE

Promotor:	Prof.dr. R. Pieters
Overige leden:	Prof.dr. P. Sonneveld Prof.dr. H.A.P. Pols Dr. F.W.G. Leebeek
Copromotor:	Dr. M.L. den Boer

The motive that will conquer cancer will not be pity nor horror;
it will be curiosity to know how and why...
Pity never made a good doctor, love never made a good poet.
Desire for service never made a discovery.

H.G. Wells 1927

CONTENTS

1 chapter

General introduction

HISTORICAL PERSPECTIVES

The 'Three Princes of Serendip' is an old Persian fairy tale about three men who were on a mission and encountered things that looked irrelevant at first sight but which turned out to be important later on. They discovered things by serendipity and sagacity. Serendip is the Persian name for Sri Lanka. Later, in 1754 Horace Walpole coined the word *serendipity.* One of the founders of pediatric oncology Giulio D'Angio[1] pointed to this fable when he analyzed the discovery of treatment tools in pediatric oncology. It has often happened that *serendipitous* observations lead to a break-through in quite another field. This story includes the discovery of L-Asparaginase:

The enzymatic deamination of asparagine was already studied by Clementi[2] in 1922. In 1952 Kidd[3] discovered that the injection of guinea pig serum inhibited the growth of murine lymphomas. His experiments indicated that a protein was responsible for the antilymphoma activity. In 1956, Neuman and McCoy[4] showed that Walker carcinoma tissue cultures had an absolute requirement for asparagine. It took until 1961 before an explanation was found for these observations: Broome,[5] working in Kidd's laboratory, presented evidence that the enzyme L-Asparaginase was responsible for the antitumor activity of guinea pig serum. An effective drug was discovered by serendipity.

The next big step in the development of L-Asparaginase as an effective antitumor agent took place in 1964. Mashburn *et al.* demonstrated that L-Asparaginase produced by the microorganism *Escherichia coli* (*E. coli)* had the same antitumor activity as that gained from guinea pig serum.[6] L-Asparaginase could be extracted from two bacterial sources: *Escherichia coli*[6] and *Erwinia chrysanthemi.*[7] The bacterial source made it possible to produce and utilize larger quantities of the enzyme and a series of preclinical and clinical studies was initiated.[8,9] A polyethylene glycol modified version of the enzyme (PEG-Asparaginase) was developed in the 1970s and 1980s and was first used in clinical trials in the 1980s.

The importance of L-Asparaginase in the treatment of acute lymphoblastic leukemia (ALL) was demonstrated.[10,11] ALL is the most common cancer in childhood with 110 - 120 newly diagnosed children in the Netherlands each year. The 5-year disease free survival for children with ALL could be improved with more intensive combination chemotherapy from 4% in the early 1960s to more than 80% in the 1990s.[12] The continuously improving treatment results through the years are an example of accurate registration, medical development and the handling of therapeutic schedules according to often international treatment protocols. L-Asparaginase was used as a single agent or in combination with other drugs to treat ALL. This drug appeared to be highly effective especially in children with newly diagnosed ALL.[13] Prolonged L-Asparaginase intensification improved the outcome significantly as was demonstrated in the Dana-Farber Cancer Institute ALL Consortium Protocol 91-01.[14] Nowadays, L-Asparaginase is an essential drug that is used to treat children with ALL all over the world.

DRUG PROPERTIES AND PHARMACOLOGY

Chemistry

The chemical properties of L-Asparaginase are extensively described in several biochemical reviews.[15-17] The enzymatic hydrolysis of the non-essential amino acids asparagine and glutamine to aspartic acid, glutamic acid and ammonia causes a depletion of the corresponding serum amino acids. The glutaminase activity amounts about 3 - 4 % of the L-Asparaginase activity.[18] Therefore, high doses of L-Asparaginase will also reduce the level of glutamine in serum. Complete asparagine depletion is already achieved within a few minutes after administration of L-Asparaginase whereas complete glutamine depletion is not achieved because of an excess of glutamine in the serum.

Pharmacokinetics

Different L-Asparaginase preparations have different half-lives.[19-21] Asselin *et al*. found that native *E. coli* preparations had a half-life of 1.24 ± 0.17 days (i.e. 29.8 ± 4.1 hours) in blood, whereas the half-life of identically applied Erwinia chrysanthemi Asparaginase, Erwinase® (Ipsen, Maidenhead, UK) was much shorter. Different half-lives of the L-Asparaginase preparations lead to different durations of asparagine depletion (Table 1.1).

Pegylation, i.e. the covalent binding of monomethoxy-polyethylene glycol (PEG) molecules to proteins, is a common method to reduce the immunogenic potential of therapeutically applicable proteins. Pegylation of L-Asparaginase extended the half-life of the enzyme activity to more than 5 days.[19] The longer half-life of PEG-Asparaginase is caused by the higher molecular weight, the slower degradation of the enzyme by proteases and a reduced absorption in the reticular endothelial system.[22]

Table 1.1 Half-lives of different L-Asparaginase preparations

	Asselin *et al.*[19] **Elimination half-life (hours)** **Dose: 25,000 IU/m² i.m.**	**Werber *et al*.[21]** **Elimination half-life (hours)** **Dose: 10,000 IU/m² i.v.**
PEG-Asparaginase (Oncaspar®)	137.5 ± 77.8	
E. coli L-Asparaginase (Medac® / Paronal®)		23 ± 2.5
E. coli L-Asparaginase (Crasnitin® / Elspar®)	29.8 ± 4.1	17.7 ± 2.5
E. chrysanthemi L-Asparaginase (Erwinase®)	15.6 ± 3.1	7.2 ± 4.1

Plasma L-Asparaginase activity levels of ≥ 100 IU/L guaranteed complete plasma asparagine depletion, i.e. < 0.2 µmol/L.[23] The established dosage scheme should therefore be adapted to the different half-lives of the different products. The German BFM studies on the native *E. coli* L-Asparaginase preparation L-Asparaginase medac® and on Erwinase®, originally both dosed as 10.000 IU/m^2 intravenously, led to the recommendation of reducing the dose of L-Asparaginase medac® to 5000 IU/m^2 and increasing the Erwinase® dose to 20.000 IU/m^2 in order to achieve complete asparagine depletion in serum. 1000 IU/m^2 PEG-Asparaginase resulted in L-Asparaginase activities > 100 IU/L and thus complete asparagine depletion in serum.[24]

It has been suggested that L-Asparaginase hardly penetrates the central nervous system (CNS).[25] The activity of native *E. coli* L-Asparaginase in the cerebro-spinal fluid (CSF) was less than 1% of the corresponding serum activity. Yet, L-Asparaginase is believed to play a role in the prevention of meningeal leukemia.[25]

Intravenous versus intramuscular application of both L-Asparaginase medac® or Erwinase® application revealed no difference in pharamacokinetics.[26,27]

Pharmacodynamics

Asparagine depletion

The effectiveness of L-Asparaginase therapy in ALL is said to be a result of depletion of asparagine within leukemic cells. L-Asparaginase hydrolyzes asparagine to aspartic acid and ammonia and as a consequence asparagine in serum will be depleted promptly (Figure 1.1). In normal cells asparagine is a non-essential amino acid because cells use the enzyme asparagine synthetase to synthesize asparagine. Asparagine synthetase, encoded by a single gene located on chromosome 7q21.3,[28] is the only enzyme available for the synthesis of asparagine. This enzyme adds an amine from glutamine to aspartic acid, thereby forming the characteristic amide group of asparagine. In contrast to normal cells, asparagine is supposed to be an essential amino acid for leukemic cells because of their relative lack of asparagine synthetase.[29] Asparagine depletion inhibits protein and RNA synthesis[30] and induces cell cycle arrest and apoptosis in murine leukemia cell lines.[31] Complete asparagine depletion in the human circulation will be reached as long as the L-Asparaginase activity level in serum is > 100 IU/L.[23]

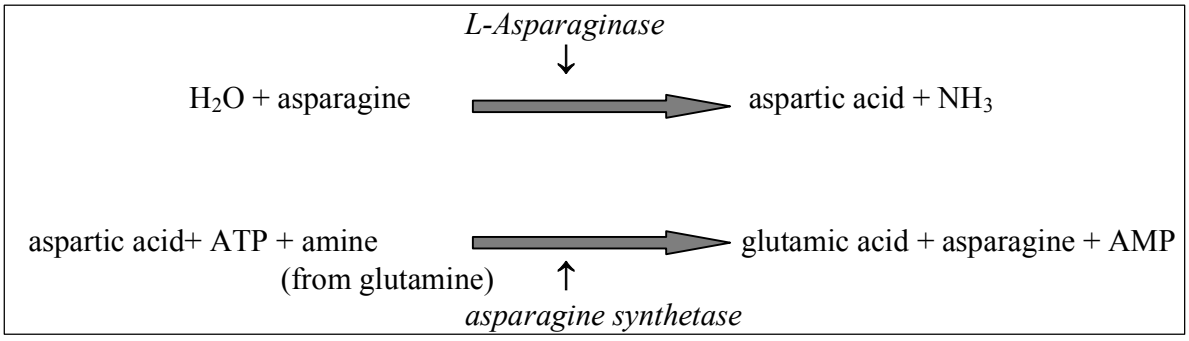


Figure 1.1 Mechanism of action of L-Asparaginase

Glutamine depletion

Glutamine levels will also drop upon L-Asparaginase exposure, an effect explained by the low glutaminase activity of L-Asparaginase[32] (Figure 1.2). A reduction in glutamine levels also contributes to the antileukemic effect of L-Asparaginase, since glutamine plays a role in cellular metabolism and protein synthesis. Serum levels of glutamine (650 μmol/L) are about tenfold higher than the amount of asparagine. Glutamine hydrolysis leads to an increase in glutamic acid. However, glutamine concentrations rapidly return to normal levels and remain normal during prolonged L-Asparaginase activity.[18] This is due to the fact that the body can increase its rate of glutamine synthesis by glutamine synthetase.[33]

Asparagine synthetase and resistance to L-Asparaginase

Cell line studies showed that L-Asparaginase sensitive leukemic cells had low intracellular asparagine synthetase activity and were dependent on the availability of extracellular asparagine.[34] Complete *in vitro* depletion of asparagine resulted in an amino-acid dependent upregulation of both mRNA, protein and activity of asparagine synthetase.[28] Resistance to L-Asparaginase in leukemic cell lines has been associated with upregulation of asparagine synthetase mRNA.[28,35] However, Fine *et al.* showed that L-Asparaginase results differ between leukemic cell lines and primary samples from leukemic patients and hence, cell line data can not be easily extrapolated to primary patient's cells.[36]

The relevance of *in vitro* resistance to antileukemic drugs in pediatric ALL was demonstrated by the fact that clinical outcome of patients resistant to L-Asparaginase, vincristine and prednisone was significantly worse than of sensitive patients.[37-39] In contrast, the prognostic favourable subtype of *TEL-AML1* positive ALL is associated with *in vitro* L-Asparaginase sensitivity.[40,41] However, a high baseline intracellular asparagine synthetase level was not related to *in vitro* L-Asparaginase resistance in children with *TEL-AML1* positive ALL,[40] whereas the two factors were correlated in other genetic subclasses of precursor B-ALL.[42] These data suggest that mechanisms other than high asparagine synthetase activity are important to L-Asparaginase resistance in *TEL-AML1* rearranged ALL. These factors may probably also contribute to L-Asparaginase resistance in other types of ALL. Knowledge of resistance mechanisms to L-Asparaginase is therefore important for improving outcome of childhood ALL.

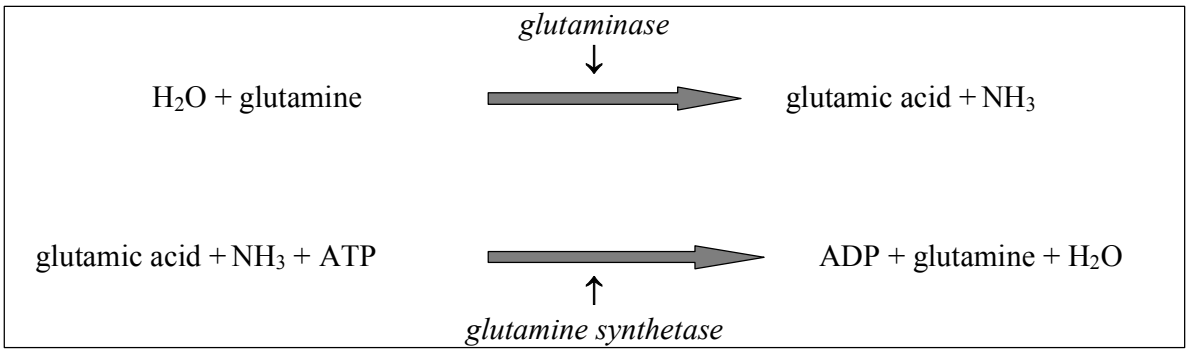


Figure 1.2 Mechanism of action of glutaminase

Interaction of L-Asparaginase with amino acids other than asparagine and glutamine

In 1969 Miller *et al.* already determined the serum levels of other amino acids during treatment with *E. coli* L-Asparaginase in two patients.[18] Besides the well-known effects on the level of asparagine and glutamine no significant changes were observed in the concentrations of other amino acids. With more modern techniques the group of Avramis monitored a decrease of 19% - 83% in serine, threonine, histidine, proline and arginine serum levels of 73 pediatric ALL patients.[43,44] It has been suggested that this effect on other amino acids is involved in the antileukemic activity of L-Asparaginase through interference with protein synthesis. In addition, acute leukemic cells had consistently lower taurine levels compared to normal cells and required L-cysteine probably due to a tenfold decrease in the activity of γ-cystathionase[45] (Figure 1.3).

Depletion of asparagine by L-Asparaginase resulted in decreased glycine concentrations in an animal model presented by Ryan.[46] In this model intraperitoneally injected glycine or asparagine effectively eliminated the antitumor effect of L-Asparaginase. However, Chakrabarti *et al.* could not confirm these results.[47] They found that the transamination of glyoxylate to glycine was significantly decreased in sensitive cells compared to cells resistant to L-Asparaginase.[48] This made them suggest that an increase in glyoxylate content in sensitive cells might attribute to the antileukemic effect.

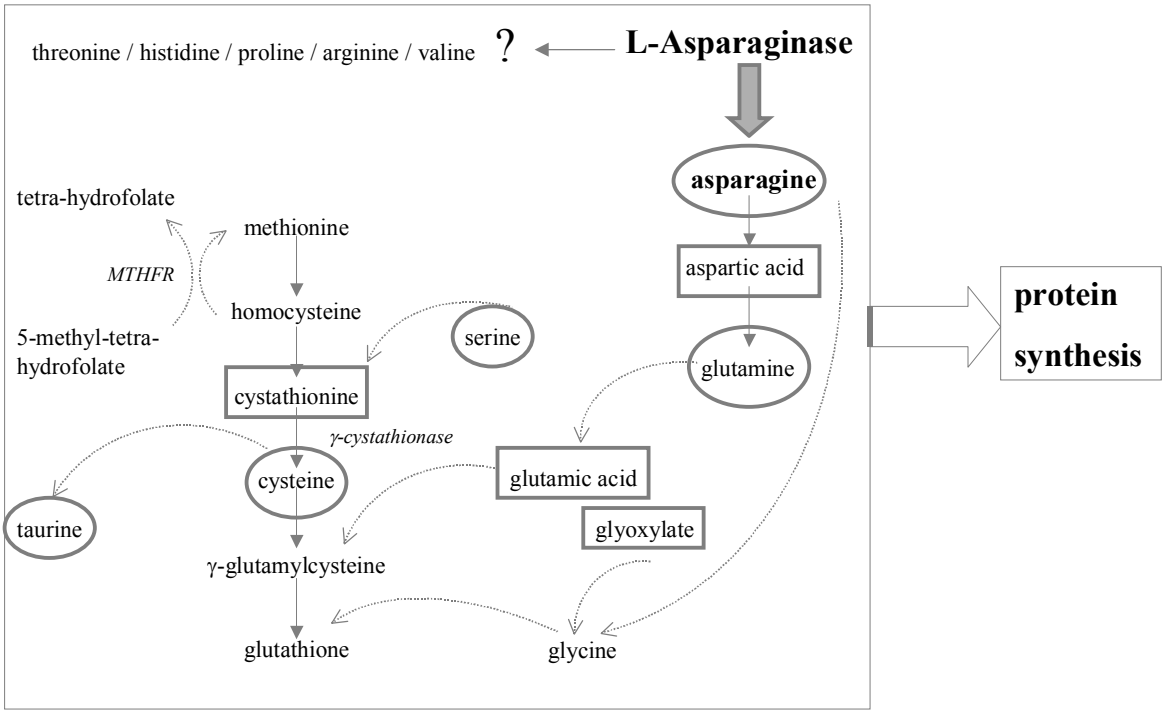


Figure 1.3 Interference of L-Asparaginase with the metabolism of amino acids
The interference of L-Asparaginase with serum amino acid levels: squares point to increased levels upon L-Asparaginase exposure, circles to decreased levels upon L-Asparaginase exposure.

L-ASPARAGINASE AND HEMOSTASIS

The depletion of asparagine and glutamine is followed by metabolic and cellular events leading to clinical side effects of therapy such as neurotoxicity and hepatotoxicity,[32] hyperglycemia, pancreatitis and alterations in hemostasis.[49,50] The duration of asparagine depletion in serum of patients correlated with the incidence of coagulation disorders.[51] Changes in laboratory parameters of coagulation were related to the L-Asparaginase activity in plasma.[52] Because the administration of L-Asparaginase takes place in conjunction with other drugs, especially corticosteroids, it is difficult to relate adverse reactions in hemostasis to L-Asparaginase only.

Most of the thrombotic events occur during induction therapy. The rate of clinical thrombosis was 5.2% in a meta-analysis of 1752 children.[53] Studies have been performed to investigate the role of L-Asparaginase therapy alone[54-56] or in combination with other drugs, especially corticosteroids.[57,58] The decrease in coagulation proteins upon L-Asparaginase exposure is in part counterbalanced by corticosteroids which increase procoagulant factors.[59] There are no data showing that different kinds of corticosteroids e.g. prednisone or dexamethasone interfere in different ways with L-Asparaginase.

The degree of disturbance of the coagulation system is dependent on the dose and on the type of L-Asparaginase preparation[51,60] and is mainly attributed to a decrease in coagulation protein synthesis. This will generate a disturbance in the hemostatic balance between bleeding and thrombosis. Increased thrombin generation and a decreased antithrombotic potential of the plasma are thought to be of critical importance in generating thrombo-embolic complications in children with ALL treated with chemotherapy. There are no data on changes in the coagulation profile after the administration of PEG-Asparaginase only.

The results of a randomized study on the use of antithrombin supplementations aiming to reduce the incidence of thrombo-embolic events were not conclusive (PARKAA study).[61] The incidence of thrombosis in patients treated with antithrombin was 28%, compared to 37% in the non-treated arm. However, no significant differences were seen in levels of markers of endogenous thrombin generation indicating that the suppression of antithrombin by L-Asparaginase was not the critical event in the development of thrombo-embolism. Replacement therapy with fresh frozen plasma[62,63] was also ineffective to correct for L-Asparaginase-induced coagulation factor deficiency.

The prophylactic use of Enoxaparin®, a low molecular weight heparin, during L-Asparaginase treatment seemed safe and might be effective in preventing thrombotic events.[64] Thrombotic events were not found in 41 children with ALL analyzed. However, also in a historical control group of 50 children without prophylactic low molecular weight heparin treatment only two events were observed. Therefore, there is currently no solid data indicating which strategy may prevent thrombo-embolic events caused by L-Asparaginase.

OUTLINE OF THE THESIS

In **Part I** we studied pharmacokinetic and pharmacodynamic aspects of a monotherapy with PEG-Asparaginase.

In **Part II** the main toxic side effect of L-Asparaginase treatment related to changes in hemostasis was studied.

Part I

Resistance to L-Asparaginase has *in vitro* been attributed to high levels of intracellular asparagine synthetase (AS). However, it was unknown whether baseline and/or L-Asparaginase induced AS mRNA levels were linked to the clinical response to this drug. We investigated in **chapter two** whether *in vivo* baseline and/or L-Asparaginase induced AS mRNA levels were related to the clinical response to a single dose of 1000 IU/m^2 PEG-Asparaginase in children (1 - 18 years) with newly diagnosed ALL before starting combined chemotherapy. Changes in AS mRNA expression were analyzed in time during this investigational window and related to the clinical response after 5 days. Because proB ALL is not frequently found in children older than 1 year, we additionally measured the baseline AS mRNA expression in 23 infants with proB ALL to compare data of this type of leukemia with those of other subtypes of ALL patients enrolled in the window study.

In **chapter three** we investigated the clinical and biological effects of this single dose of PEG-Asparaginase in children with newly diagnosed ALL. We compared the clinical response to PEG-Asparaginase with the *in vitro* sensitivity to L-Asparaginase at diagnosis and with clinical long-term outcome. The relationship between immunophenotype, genotype and clinical response was studied. *In vivo* response to PEG-Asparaginase was studied in relationship with baseline as well as L-Asparaginase-induced changes in serum and intracellular amino acid levels, and with parameters of apoptosis. In addition, clinical toxicity and changes in coagulation profiles were analyzed both after one single infusion with PEG-Asparaginase and after the combination chemotherapy given thereafter.

Pharmacokinetics and pharmacodynamics of L-Asparaginase in the cerebro spinal fluid (CSF) had not been characterized well for PEG-Asparaginase. In **chapter four** we monitored L-Asparaginase activity levels and asparagine concentrations in plasma and CSF after the administration of 1000 IU/m^2 PEG-Asparaginase on day -5. Regular chemotherapy according to the DCOG-ALL-9 schedule was started at day 0. From day -5 till day 0 blood samples were collected daily, and later on twice a week. A lumbar puncture was performed at diagnosis (day -5), at day 0 and at day 15 when intrathecal therapy was administered and asparagine levels in the CSF could be monitored.

Part II

A main side effect of L-Asparaginase is the increased risk of thrombo-embolic complications. In **part II** of this thesis we focused on the effects of L-Asparaginase

on coagulation parameters. We performed a randomized study between native *E. coli* L-Asparaginase and *Erwinia* L-Asparaginase **(chapter five)** to evaluate differences in the risk on thrombo-embolic complications. The study was performed during induction therapy of the DCOG-ALL-7 protocol. L-Asparaginase was administered 8 times as 10.000 IU/m^2 intravenously every three days starting at day 19. Thrombin generation and fibrinolysis was studied in blood samples taken before each L-Asparaginase infusion.

The concept of developmental hemostasis points to age-related changes in the coagulation system. Infants and adolescents are at risk for thrombo-embolic events due to physiological changes in their coagulation profile. Most serious thrombo-embolic complications during L-Asparaginase treatment are seen in adolescence. We hypothesized that these physiological changes in the coagulation system might influence the risk on thrombosis during L-Asparaginase therapy. In **chapter six** we describe the differences in changes in coagulation between patients in different age groups: 1 - 5, 6 - 10 and 11 - 16 years old. The children were treated according to the DCOG-ALL-9 protocol with four doses of *E. coli* L-Asparaginase Paronal® during induction. Procoagulant and anticoagulant factors and parameters of thrombin generation and fibrinolysis were monitored.

There is an interaction between the effects of corticosteroids and L-Asparaginase on the coagulation system. Different schedules are used during anti-leukemic treatment including differences in dosages of corticosteroids and L-Asparaginase, but also different timing and duration of administering these drugs. We analyzed two different regimens of concomitant use of these drugs in **chapter seven** in a group of high-risk ALL patients receiving dexamethasone and L-Asparaginase during induction and during intensification treatment of ALL according to the DCOG-ALL-9 protocol. We monitored procoagulant and anticoagulant factors and parameters of thrombin generation and fibrinolysis.

Finally, all results are discussed in **Part III**, **chapter eight** and summarized in **chapter nine** in English and Dutch.

REFERENCES

1. D'Angio GJ, Adamson PC. A tale of three princes and two physicists--the importance of "why?" Med Pediatr Oncol. 2003;41:132-135.
2. Clementi A. Désamidation enzymatique de l'asparaginase. Arch Internat Physiol. 1922;19:369.
3. Kidd JG. Regression of transplanted lymphomas induced in vivo by means of normal guinea pig serum. I. Course of transplanted cancers of various kinds in mice and rats given guinea pig serum, horse serum, or rabbit serum. J Exp Med. 1953;98:565-582.
4. Neuman RE, McCoy TA. Dual requirement of Walker carcinosarcoma 256 in vitro for asparagine and glutamine. Science. 1956;124:124-125.
5. Broome JD. Evidence that L-Asparaginase activity of guinea pig serum is responsible for its anti-lymphoma effects. Nature. 1961;191:1114-1115.
6. Mashburn LT, Wriston JC, Jr. Tumor Inhibitory Effect of L-Asparaginase from Escherichia Coli. Arch Biochem Biophys. 1964;105:450-452.
7. Wade HE, Robinson HK, Phillips BW. Asparaginase and glutaminase activities of bacteria. J Gen Microbiol 1971;69:299-312.
8. Campbell HA, Mashburn LT, Boyse EA, Old LJ. Two L-asparaginases from Escherichia coli B. Their separation, purification, and antitumor activity. Biochemistry. 1967;6:721-730.
9. Whelan HA, Wriston JC, Jr. Purification and properties of asparaginase from escherichia coli B. Biochemistry. 1969;8:2386-2393.
10. Hill JM, Roberts J, Loeb E, Khan A, MacLellan A, Hill RW. L-asparaginase therapy for leukemia and other malignant neoplasms. Remission in human leukemia. Jama. 1967;202:882-888.
11. Oettgen HF, Old LJ, Boyse EA, Campbell HA, Philips FS, Clarkson BD, et al. Inhibition of leukemias in man by L-asparaginase. Cancer Res. 1967;27:2619-2631.
12. Pui CH, Evans WE. Acute lymphoblastic leukemia. N Engl J Med. 1998;339:605-615.
13. Capizzi RL, Bertino JR, Skeel RT, Creasey WA, Zanes R, Olayon C, et al. L-asparaginase: clinical, biochemical, pharmacological, and immunological studies. Ann Intern Med. 1971;74:893-901.
14. Silverman LB, Gelber RD, Dalton VK, Asselin BL, Barr RD, Clavell LA, et al. Improved outcome for children with acute lymphoblastic leukemia: results of Dana-Farber Consortium Protocol 91-01. Blood. 2001;97:1211-1218.
15. Wriston JC, Jr., Yellin TO. L-asparaginase: a review. Adv Enzymol Relat Areas Mol Biol. 1973;39:185-248.
16. Maita T, Matsuda G. The primary structure of L-asparaginase from Escherichia coli. Hoppe Seylers Z Physiol Chem. 1980;361:105-17.
17. Jackson RC, Handschumacher RE. Escherichia coli L-asparaginase. Catalytic activity and subunit nature. Biochemistry. 1970;9:3585-3590.
18. Miller HK, Salser JS, Balis ME. Amino acid levels following L-asparagine amidohydrolase (EC.3.5.1.1) therapy. Cancer Res. 1969;29:183-187.
19. Asselin BL, Whitin JC, Coppola DJ, Rupp IP, Sallan SE, Cohen HJ. Comparative pharmacokinetic studies of three asparaginase preparations. J Clin Oncol. 1993;11:1780-1786.
20. Albertsen BK, Jakobsen P, Schroder H, Schmiegelow K, Carlsen NT. Pharmacokinetics of Erwinia asparaginase after intravenous and intramuscular administration. Cancer Chemother Pharmacol. 2001;48:77-82.
21. Werber G. Drug monitoring von Asparaginase im Rahmen des pädiatrischen Therapieprotokolls der ALL/NHL-BFM 90 Studie. Inaugural-Dissertation, Münster. 1995.
22. Ho DH, Brown NS, Yen A, Holmes R, Keating M, Abuchowski A, et al. Clinical pharmacology of polyethylene glycol-L-asparaginase. Drug Metab Dispos. 1986;14:349-352.
23. Boos J, Werber G, Ahlke E, Schulze-Westhoff P, Nowak-Gottl U, Wurthwein G, et al. Monitoring of asparaginase activity and asparagine levels in children on different asparaginase preparations. Eur J Cancer. 1996;32A:1544-1550.

24. Muller HJ, Loning L, Horn A, Schwabe D, Gunkel M, Schrappe M, et al. Pegylated asparaginase (Oncaspar) in children with ALL: drug monitoring in reinduction according to the ALL/NHL-BFM 95 protocols. Br J Haematol. 2000;110:379-384.

25. Riccardi R, Holcenberg JS, Glaubiger DL, Wood JH, Poplack DG. L-asparaginase pharmacokinetics and asparagine levels in cerebrospinal fluid of rhesus monkeys and humans. Cancer Res. 1981;41:4554-4558.

26. Nesbit M, Chard R, Evans A, Karon M, Hammond GD. Evaluation of intramuscular versus intravenous administration of L-asparaginase in childhood leukemia. Am J Pediatr Hematol Oncol. 1979;1:9-13.

27. Rizzari C, Zucchetti M, Conter V, Diomede L, Bruno A, Gavazzi L, et al. L-asparagine depletion and L-asparaginase activity in children with acute lymphoblastic leukemia receiving i.m. or i.v. Erwinia C. or E. coli L-asparaginase as first exposure. Ann Oncol. 2000;11:189-193.

28. Andrulis IL, Argonza R, Cairney AE. Molecular and genetic characterization of human cell lines resistant to L-asparaginase and albizziin. Somat Cell Mol Genet. 1990;16:59-65.

29. Kiriyama Y, Kubota M, Takimoto T, Kitoh T, Tanizawa A, Akiyama Y, et al. Biochemical characterization of U937 cells resistant to L-asparaginase: the role of asparagine synthetase. Leukemia. 1989;3:294-297.

30. Goody HE, Ellem KA. Nutritional effects on precursor uptake and compartmentalization of intracellular pools in relation to RNA synthesis. Biochim Biophys Acta. 1975;383:30-39.

31. Ueno T, Ohtawa K, Mitsui K, Kodera Y, Hiroto M, Matsushima A, et al. Cell cycle arrest and apoptosis of leukemia cells induced by L-asparaginase. Leukemia. 1997;11:1858-1861.

32. Ollenschlager G, Roth E, Linkesch W, Jansen S, Simmel A, Modder B. Asparaginase-induced derangements of glutamine metabolism: the pathogenetic basis for some drug-related side-effects. Eur J Clin Invest. 1988;18:512-516.

33. Tapiero H, Mathe G, Couvreur P, Tew KD. II. Glutamine and glutamate. Biomed Pharmacother. 2002;56:446-457.

34. Broome JD. L-Asparaginase: discovery and development as a tumor-inhibitory agent. Cancer Treat Rep. 1981;65 Suppl 4:111-114.

35. Avramis VI, Sencer S, Periclou AP, Sather H, Bostrom BC, Cohen LJ, et al. A randomized comparison of native Escherichia coli asparaginase and polyethylene glycol conjugated asparaginase for treatment of children with newly diagnosed standard-risk acute lymphoblastic leukemia: a Children's Cancer Group study. Blood. 2002;99:1986-1994.

36. Fine BM, Kaspers GJ, Ho M, Loonen AH, Boxer LM. A genome-wide view of the in vitro response to l-asparaginase in acute lymphoblastic leukemia. Cancer Res. 2005;65:291-299.

37. Den Boer ML, Harms DO, Pieters R, Kazemier KM, Gobel U, Korholz D, et al. Patient stratification based on prednisolone-vincristine-asparaginase resistance profiles in children with acute lymphoblastic leukemia. J Clin Oncol. 2003;21:3262-3268.

38. Pieters R, Huismans DR, Loonen AH, Hahlen K, van der Does-van den Berg A, van Wering ER, et al. Relation of cellular drug resistance to long-term clinical outcome in childhood acute lymphoblastic leukaemia. Lancet. 1991;338:399-403.

39. Kaspers GJ, Veerman AJ, Pieters R, Van Zantwijk CH, Smets LA, Van Wering ER, et al. In vitro cellular drug resistance and prognosis in newly diagnosed childhood acute lymphoblastic leukemia. Blood. 1997;90:2723-2729.

40. Stams WA, den Boer ML, Beverloo HB, Meijerink JP, Stigter RL, van Wering ER, et al. Sensitivity to L-asparaginase is not associated with expression levels of asparagine synthetase in t(12;21)+ pediatric ALL. Blood. 2003;101:2743-2747.

41. Ramakers-van Woerden NL, Pieters R, Loonen AH, Hubeek I, Drunen van E, Beverloo HB, et al. TEL-AML1 gene fusion is related to in vitro drug sensitivity for L-Asparaginase in childhood acute lymphoblastic leukemia. Blood. 2000;96:1094-1099.

42. Stams WA, den Boer ML, Holleman A, Appel IM, Beverloo HB, van Wering ER, et al. Asparagine synthetase expression is linked with L-asparaginase resistance in TEL-AML1-negative but not TEL-AML1-positive pediatric acute lymphoblastic leukemia. Blood. 2005;105:4223-4225.

43. Panosyan EH, Grigoryan RS, Avramis IA, Seibel NL, Gaynon PS, Siegel SE, et al. Deamination of glutamine is a prerequisite for optimal asparagine deamination by asparaginases in vivo (CCG-1961). Anticancer Res. 2004;24:1121-1125.

44. Grigoryan RS, Panosyan EH, Seibel NL, Gaynon PS, Avramis IA, Avramis VI. Changes of amino acid serum levels in pediatric patients with higher-risk acute lymphoblastic leukemia (CCG-1961). In Vivo. 2004;18:107-112.

45. Wakayama K, Besa EC, Baskin SI. Changes in intracellular taurine content of human leukemic cells. Nagoya J Med Sci. 1983;45:89-96.

46. Ryan WL, Sornson HC. Glycine inhibition of asparaginase. Science. 1970;167:1512-1513.

47. Chakrabarti R, Schuster SM. L-Asparaginase: Perspectives on the mechanism of action and resistance. Int J Ped Hem/Oncol. 1997;4:597-611.

48. Keefer JF, Moraga DA, Schuster SM. Comparison of glycine metabolism in mouse lymphoma cells either sensitive or resistant to L-asparaginase. Biochem Pharmacol. 1985;34:559-565.

49. Athale UH, Chan AK. Thrombosis in children with acute lymphoblastic leukemia. Part II. Pathogenesis of thrombosis in children with acute lymphoblastic leukemia: effects of the disease and therapy. Thromb Res. 2003;111:199-212.

50. Holle LM. Pegaspargase: an alternative? Ann Pharmacother. 1997;31:616-624.

51. Nowak-Gottl U, Werber G, Ziemann D, Ahlke E, Boos J. Influence of two different Escherichia coli asparaginase preparations on fibrinolytic proteins in childhood ALL. Haematologica. 1996;81:127-131.

52. Ahlke E, Nowak-Gottl U, Schulze-Westhoff P, Werber G, Borste H, Wurthwein G, et al. Dose reduction of asparaginase under pharmacokinetic and pharmacodynamic control during induction therapy in children with acute lymphoblastic leukaemia. Br J Haematol. 1997;96:675-681.

53. Caruso V, Iacoviello L, Di Castelnuovo A, Storti S, Mariani G, de Gaetano G, et al. Thrombotic complications in childhood acute lymphoblastic leukemia: a meta-analysis of 17 prospective studies comprising 1752 pediatric patients. Blood. 2006;108:2216-2222.

54. Mitchell L, Hoogendoorn H, Giles AR, Vegh P, Andrew M. Increased endogenous thrombin generation in children with acute lymphoblastic leukemia: risk of thrombotic complications in L'Asparaginase-induced antithrombin III deficiency. Blood. 1994;83:386-391.

55. Miniero R, Pastore G, Saracco P, Messina M, Lange MM, Fiandino G, et al. Hemostatic changes in children with acute lymphoblastic leukemia treated according to two different L-asparaginase schedules. Am J Pediatr Hematol Oncol. 1986;8:116-120.

56. Homans AC, Rybak ME, Baglini RL, Tiarks C, Steiner ME, Forman EN. Effect of L-asparaginase administration on coagulation and platelet function in children with leukemia. J Clin Oncol. 1987;5:811-817.

57. Nowak-Gottl U, Ahlke E, Fleischhack G, Schwabe D, Schobess R, Schumann C, et al. Thromboembolic events in children with acute lymphoblastic leukemia (BFM protocols): prednisone versus dexamethasone administration. Blood. 2003;101:2529-5233.

58. Gaynon PS, Lustig RH. The use of glucocorticoids in acute lymphoblastic leukemia of childhood. Molecular, cellular, and clinical considerations. J Pediatr Hematol Oncol. 1995;17:1-12.

59. van Giezen JJ, Brakkee JG, Dreteler GH, Bouma BN, Jansen JW. Dexamethasone affects platelet aggregation and fibrinolytic activity in rats at different doses which is reflected by their effect on arterial thrombosis. Blood Coagulation and Fibrinolysis. 1994;5:249-255.

60. Nowak-Gottl U, Ahlke E, Klosel K, Jurgens H, Boos J. Changes in coagulation and fibrinolysis in childhood acute lymphoblastic leukaemia re-induction therapy using three different asparaginase preparations. Eur J Pediatr. 1997;156:848-50.

61. Mitchell L, Andrew M, Hanna K, Abshire T, Halton J, Wu J, et al. Trend to efficacy and safety using antithrombin concentrate in prevention of thrombosis in children receiving l-asparaginase for acute lymphoblastic leukemia. Results of the PAARKA study. Thromb Haemost. 2003;90:235-244.

62. Nowak-Gottl U, Rath B, Binder M, Hassel JU, Wolff J, Husemann S, et al. Inefficacy of fresh frozen plasma in the treatment of L-asparaginase- induced coagulation factor deficiencies during ALL induction therapy [see comments]. Haematologica. 1995;80:451-453.

63. Halton JM, Mitchell LG, Vegh P, Eves M, Andrew ME. Fresh frozen plasma has no beneficial effect on the hemostatic system in children receiving L-asparaginase. Am J Hematol. 1994;47:157-61.

64. Elhasid R, Lanir N, Sharon R, Weyl Ben Arush M, Levin C, Postovsky S, et al. Prophylactic therapy with enoxaparin during L-asparaginase treatment in children with acute lymphoblastic leukemia. Blood Coagul Fibrinolysis. 2001;12:367-370.

I PART

PHARMACOKINETICS AND PHARMACODYNAMICS OF L-ASPARAGINASE

2 chapter

Up-regulation of asparagine synthetase expression is not linked to the clinical response to L-Asparaginase in pediatric acute lymphoblastic leukemia

Inge M. Appel
Monique L. den Boer
Jules P.P. Meijerink
Anjo J.P. Veerman
Nathalie C.M. Reniers
Rob Pieters

Blood 2006;107:4244-4249

ABSTRACT

L-Asparaginase (L-Asp) is an effective drug for treatment of children with acute lymphoblastic leukemia (ALL). The effectiveness is generally thought to result from a rapid depletion of asparagine in serum and cells. Asparagine synthetase (AS) opposes the action of L-Asp by resynthesis of asparagine. *In vitro,* resistance to L-Asp has been associated with up-regulation of AS mRNA expression. We monitored AS mRNA levels in leukemic cells before and during 5 days after intravenous administration of 1000 IU/m² of pegylated L-Asparaginase (PEG-Asp) in a therapeutic window in children with ALL at initial diagnosis. Within 24 hours, AS mRNA levels increased by 3.5-fold and remained stable in the following 4 days. Baseline and L-Asp-induced expression levels of AS did not differ between clinically good, intermediate, and poor responders to PEG-Asp. No significant difference of AS mRNA up-regulation was found between precursor B- and T-ALL or between hyperdiploids, *TEL-AML1* rearranged ALL or absence of genetic abnormalities. In 3 of 12 patients with T-ALL even a slight down-regulation of AS mRNA expression upon L-Asp exposure was found. In conclusion, although L-Asp exposure induces the expression of AS mRNA, the up-regulated gene expression does not correlate with an early clinical poor response to this drug in children with ALL.

INTRODUCTION

L-Asparaginase (L-Asp) is an effective drug for the treatment of children with acute lymphoblastic leukemia (ALL).[1,2] In newly diagnosed patients with ALL, 25% to 60% will reach a complete remission after monotherapy with L-Asp.[1] The efficacy of this drug is generally thought to result from a rapid and complete depletion of asparagine in plasma by hydrolyzing this amino acid to aspartic acid.

L-Asp resistance has been attributed to high levels of intracellular asparagine synthetase (AS).[3] Cell line studies showed that L-Asp sensitive leukemic cells have low intracellular AS activity and are dependent on the availability of extracellular asparagine.[4] Andrulis *et al.* demonstrate that complete asparagine depletion *in vitro* results in an amino-acid dependent up-regulation of mRNA, protein and activity of AS.[5] Resistance to L-Asp in cell lines is *in vitro* mediated by an up-regulation of AS expression in response to asparagine depletion of culture medium.[6,7] Whereas these cell line studies suggest that up-regulation of AS expression is an important mechanism of L-Asp resistance, clinical evidence is lacking for this assumption. In recent studies we found evidence that a high baseline intracellular AS gene expression is related to *in vitro* L-Asp resistance in children with *TEL-AML1* negative ALL,[8] but not in *TEL-AML1* positive children.[9] This suggests that the genotype plays an important role in the cause of L-Asp resistance. However, it is yet unknown whether baseline and/or L-Asp induced AS mRNA levels are linked to the clinical response to this drug given as a therapeutic window upfront of combination chemotherapy.

In the present *in vivo* study we investigate whether baseline and/or L-Asp-induced AS mRNA levels are related to the clinical response to a therapeutic window with L-Asp in children with newly diagnosed ALL.

PATIENTS, MATERIALS, AND METHODS

Patients and therapeutic window with PEG-Asp

In close collaboration between our institution and the Dutch Childhood Oncology Group (DCOG; the former Dutch Childhood Leukemia Study Group) a window study with pegylated *Escherichia coli* L-Asp (PEG-Asp) upfront to the ALL-9 treatment schedule was initiated in July 2000. The DCOG ALL-9 study was implemented in the Netherlands to confirm the good results of the ALL-6 study,[10] which was originally based on the German ALL-BFM strategy. The aim of our study is to determine the clinical response as well as molecular determinants of L-Asp response in ALL. Children with ALL at initial diagnosis and presenting with white blood count (WBC) greater than 10 x 10^9/L were eligible. Similar to a study from the Dana Farber Cancer Institute,[11] we assessed a 5-day investigational window. A complete and persistent depletion of asparagine is considered to be the mechanism of action of L-Asp treatment. Boos *et al.*[12] showed that a plasma *E coli* Asp activity of more than 100 IU/L leads to an asparagine depletion of less than 0.2 µM in plasma. Muller demonstrated that one dose of 1000 IU/m^2 PEG-Asp

resulted into more than 100 U/L serum enzyme activity of L-Asp for 3 weeks.[13] In a previous study we confirmed that 1000 IU/m^2 PEG-Asp given as a therapeutic window at day -5 (*ie*, 5 days before starting combined chemotherapy) results in more than 100 U/L L-Asp activity for at least 10 days in children with ALL at initial diagnosis.[14] In the present study patients received a single dose of 1000 IU/m^2 PEG-Asp in a 1-hour infusion 5 days before starting the DCOG-ALL-9 combination chemotherapy treatment schedule. PEG-Asp, kindly provided by Medac (Hamburg, Germany), was used mainly because of its lower immunogenicity than native (unpegylated) L-Asp.[15] This lower immunogenicity is important since these patients will be treated with unpegylated L-Asp as part of their regular combination chemotherapy hereafter.

We decided to use the same definition for clinical response that is used for response to prednisone:[16] more than 1 x 10^9 leukemic blasts per liter (1000/μL) of peripheral blood has been shown to be highly predictive for an inferior outcome. So, the clinical response on day 0 (5 days after the PEG-Asp infusion) was defined as good when the number of leukemic cells had declined to less than 1 x 10^9/L of peripheral blood, as intermediate when leukemic cells were 1 x 10^9/L to 10 x 10^9/L, and as poor when leukemic cells were greater than 10 x 10^9/L.

Between July 2000 and October 2002, 31 patients with ALL were enrolled in the study. Of these, 25 children were diagnosed in the Erasmus MC-Sophia Children's Hospital, Rotterdam, and 6 children in 3 other university hospitals in the Netherlands. Patients' characteristics are shown in Table 2.1.

Table 2.1 Characteristics of 31 patients treated with one dose of PEG-Asp before the DCOG-ALL-9 study

Characteristic	Value
Male / Female	17/14
Age, median, y (range)	4.2 (1.2 - 13.1)
Median WBC count at diagnosis, x 10^9/L (range)	47 (11.4 - 417)
Immunophenotype	
Pro-B-ALL	1
common ALL	9
Pre-B-ALL	9
T-ALL	12
Cytogenetic characteristics	
Hyperdiploid	9*
TEL-AML 1 fusion	5
BCR-ABL fusion	1*
MLL gene rearranged	0
others	17
CNS involvement**	
Yes	0
No	31

Patients were administered one intravenous dose of 1000 IU/m^2 pegylated L-Asparaginase before undergoing combination chemotherapy as part of the DCOG (Dutch Childhood Oncology Group) ALL-9 study.
** one patient had both a hyperdiploidy and a BCR-ABL fusion,*
*** CNS involvement defined as more than 5 cells/μL with blasts in the cerebrospinal fluid*

Because pro-B-ALL is not frequently found in children older than 1 year, we additionally measured the baseline AS mRNA expression in 23 infants with pro-B-ALL (Interfant-99) to compare data of this type of leukemia with those of other subtypes of ALL patients enrolled in the window study.

The immunophenotyping was performed at reference laboratories of the participating groups. The B-lineage ALL cells ($CD19^+$, $HLA\text{-}DR^+$) were classified into the following differentiation stages: pro-B-ALL cells were $CD10^-$, cytoplasmic μ chain-negative ($c\mu^-$), and surface immunoglobulin-negative (sIg^-); cALL cells were $CD10^+/c\mu^-/sIg^-$; pre-B-ALL cells were $CD10^{+/-}/c\mu^+/sIg^-$. B-ALL cells characterized by $CD10^-/c\mu^-/sIg^+$ were excluded from the study.

The window study on PEG-Asp and the Interfant study on infants with pro-B-ALL were approved by the local ethical committee and by the institutional research board of the DCOG. The patient and/or the parents and guardians have given informed consent for these studies in accordance with the Declaration of Helsinki.

Patient samples

Bone marrow and peripheral blood samples were obtained at initial diagnosis of ALL (day-5) before the administration of PEG-Asp. To perform daily analyses of asparagine synthetase (AS) expression in leukemic cells we decided for ethical reasons that daily collection of bone marrow was unacceptable. Stams *et al.*[9] have shown that purified leukemic cells out of peripheral blood revealed comparable AS mRNA levels compared with leukemic cells isolated out of bone marrow. Therefore, blood samples were collected during 5 consecutive days until the start of combination chemotherapy at day 0. Within 24 hours after sampling, mononuclear cells were isolated by density gradient centrifugation using Lymphoprep (density 1.077 g/mL; Nycomed Pharma, Oslo, Norway) and centrifuged at 480g for 15 minutes at room temperature. The collected mononuclear cells were washed twice and kept in culture medium consisting of RPMI 1640 medium (Dutch modification without L-glutamine; Gibco BRL, Life Technologies, Breda, MD), 20% fetal calf serum (FCS; Integro, Zaandam, The Netherlands), 2 mM L-glutamine (Gibco BRL, Life Technologies), 5 μg/mL insulin, 5 μg/mL transferrin, 5 ng/ml sodium selenite (ITS media supplement; Sigma, St Louis, MO), 100 IU/mL penicillin, 100 μg/mL streptomycin, 0.125 μg/mL amphotericin B (Life Technologies), and 0.2 mg/mL gentamycin (Life Technologies). Contaminating nonleukemic cells in the ALL samples were removed by immunomagnetic beads as described by Kaspers *et al*.[17] All samples contained more than 90% leukemic cells, as determined morphologically on May-Grünwald-Giemsa-stained (Merck, Darmstadt, Germany) cytospins.

RNA extraction and cDNA synthesis

Total cellular RNA was extracted from a minimum of 5×10^6 cells using Trizol reagent (Life Technologies) according to the manufacturer's protocol, with minor modifications as reported previously.[9] The concentration of RNA was quantified spectrophotometrically and the quality was checked on agarose gels. Following a denaturation step of 5 minutes at 70 ºC, 1 μg of RNA was reverse-transcribed into

single-stranded cDNA. The reverse transcription (RT) was performed in a total volume of 25 µL, containing 2.5 nM random hexamers and 20 nM oligo dT primers (Amersham Pharmacia Biotech, Piscataway, NJ), 200 U Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI), and 25 U RNAsin (Promega) and was incubated at 37 ºC for 30 minutes, 42 ºC for 15 minutes and 94 ºC for 5 minutes. The obtained cDNA was diluted to a final concentration of 8 ng/µl and stored at -80 ºC.

Quantitative real-time PCR

The mRNA expression levels of AS and the endogenous housekeeping gene encoding for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as a reference, were quantified using real-time polymerase chain reaction (RTQ-PCR) analysis (TAQMAN chemistry) on an ABI Prism 7700 sequence detection system (PE Applied Biosystems, Foster City, CA).

Amplification of specific PCR products was detected using dual-fluorescent nonextendable probes labeled with 6-carboxyfluorescein (FAM) at the 5' end and 6-carboxytetramethylrhodamine (TAMRA) at the 3' end. The primers and probe combinations were designed using OLIGO 6.22 software (Molecular Biology Insights, Cascade, CO) and have been published elsewhere.[9] Because all PCRs were performed with equal efficiencies (> 95%), relative mRNA expression levels of AS for each patient could directly be normalized for input RNA using the GAPDH expression of the patient. The relative mRNA expression level of the target gene in each patient was calculated using the comparative cycle time (C_t) method.[18]

Briefly, the target PCR C_t values (*ie,* the cycle number at which emitted fluorescence exceeds 10 x the standard deviation [SD] of baseline emissions, as measured from cycles 3 to 12) are normalized by subtracting the GAPDH C_t value from the target PCR C_t value, which gives the ΔC_t value. From the ΔC_t value, the relative expression level to GAPDH for AS is calculated using the following equation: relative mRNA expression = $2^{-\Delta Ct}$ x 100%.

Statistics

Differences in mRNA expression levels measured at different days were analyzed using the Wilcoxon matched-pairs signed rank test. The relationship between AS mRNA expression and *in vivo* PEG-Asp response and between AS expression and immunophenotype or cytogenetic subtype was analyzed with the Mann-Whitney *U* (MWU) test.

RESULTS

Children with newly diagnosed ALL and WBC greater than 10 x 10^9/L were consecutively enrolled into the study. As is shown in Table 2.1, 31 children were eligible at the moment of analysis: 1 with pro-B-ALL, 9 with common ALL, 9 with pre-B-ALL, and 12 with T-ALL.

Similar to day 7 for a prednisone window response, we evaluated the *in vivo* response to PEG-Asp by counting the number of leukemic blasts in the peripheral blood at day 0, 5 days after PEG-Asp was given. As can be seen in Figure 2.1, the leukemic cells in the peripheral blood dropped continuously over 5 days. The number of leukemic cells reduced 224-fold from median 44.7 x 10^9/L at day-5 to median of 0.2 x 10^9/L at day 0. This was more than a 2-log decrease in leukemic cell burden. There were 21 (68%) children who were PEG-Asp good responders (blast number < 1 x 10^9/L at day 0), 6 (19%) who were intermediate responders (blasts 1 x 10^9/L to 10 x 10^9/L at day 0) and 4 (12%) children who were poor responders (blasts > 10 x 10^9/L at day 0) (Figure 2.1).

The baseline expression level of AS mRNA relative to GAPDH was median 0.26% (range 0.05% - 2.5%) in leukemic cells (> 90% purity). This was in the range of healthy controls as described in our previous study.[9] The expression levels of AS in leukemic cells relative to GAPDH increased significantly median 3-fold, from 0.26% (basal expression) to 0.75% 24 hours later (P < 0.001; Figure 2.2). During the following 4 days the expression of AS mRNA remained stable at the level of 24 hours (Figure 2.2; Table 2.2).

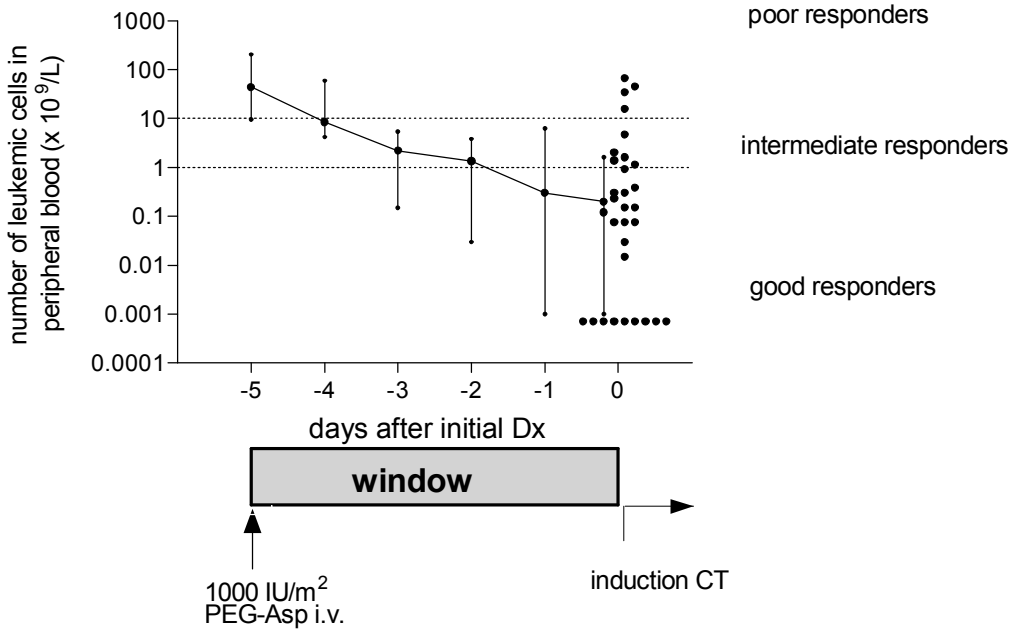


Figure 2.1 Clinical response to pegylated L-asparaginase (PEG-Asp) in pediatric acute lymphoblastic leukemia (ALL)

Clinical response to 1000 IU/m^2 intravenous PEG-Asp, measured as the decrease in the absolute number of leukemic cells in peripheral blood of 31 children with ALL. The final values at day 0 are shown for each individual by dots. The clinical response line shows the median and 25th and 75th percentiles. A good clinical response is defined by < 1 x 10^9 blasts/L at day 0, an intermediate response by 1 x 10^9 blasts/L to 10 x 10^9 blasts/L on day 0, and poor response by more than 10 x 10^9 blasts/L at day 0. Dotted lines indicate the cut-off values for these clinical responses.

Table 2.2 Asparagine synthetase (AS) mRNA expression values in time

	day					
	-5	**-4**	**-3**	**-2**	**-1**	**0**
AS Expression compared to GAPDH (%)						
Median	0.26	0.75	0.75	0.87	1.05	0.99
25^{th} – 75^{th} percentile	0.16-0.46	0.45-1.4	0.52-1.18	0.47-1.17	0.48-1.05	0.53-1.8
Wilcoxon signed rank test, *P*						
compared between successive days	NA	< 0.001	0.4	0.4	0.06	0.4
Day -5 compared with days -4, -3, -2, -1, and 0	NA	< 0.001	0.001	0.001	0.001	0.002

Median expression values (and 25^{th} - 75^{th} percentiles) of AS mRNA compared with GAPDH in time in leukemic cells of 31 children induced by pegylated L-asparaginase (PEG-Asp). Wilcoxon signed rank test compared between successive days, and day -5 values compared to levels of day -4, day -3, day -2, day -1, and day 0, NA indicates not applicable.

The baseline expression level of AS mRNA did not differ between good and intermediate (P = 0.614), good and poor (P = 0.852) responders, and intermediate and poor (P = 1.0) responders; nor did the up-regulated AS levels after 24 hours of PEG-Asp differ between good and intermediate (P = 0.614) responders, good and poor (P = 0.737) responders, and intermediate and poor (P = 0.914) responders (Figure 2.3A-B). The fold-change in AS mRNA expression levels was also not related to the relative (P = 0.997) or absolute (P = 0.804) decrease in leukemic cells in all 31 patients.

The AS expression for the different immunophenotypic ALL subgroups at diagnosis is shown in Figure 2.4. The median levels of AS mRNA relative to GAPDH mRNA for c/pre-B-ALL patients (0.21%) and for the T-ALL patients (0.28%) did not significantly differ (P = 0.376). One window patient had a pro-B-ALL for which the baseline expression of AS mRNA was 3-fold higher than the other c/pre-B-ALL patients. To explore whether pro-B-ALL is associated with a high AS mRNA expression we analyzed the AS expression of 23 infant pro-B-ALL cases. Infants with pro-B-ALL had a median 0.15% (range, 0.07% - 1.43%) AS mRNA expression level, which was not significantly different from the baseline AS expression values in non-infants with c/pre-B- or T-ALL (Figure 2.4A). The c/pre-B-ALL group had a median baseline AS expression level of 0.21% that rose significantly to a median of 0.72% 1 day later (median 3.99-fold individual up-regulation, P = 0.001). Patients with T-ALL demonstrated a significant increase from baseline 0.28% to 0.68% 1 day later (median 1.94-fold individual up-regulation, P = 0.012). Patients with T-ALL tended to have a lower individual up-regulation of AS mRNA compared with the children with c/pre-B-ALL, but this was not statistically different (P = 0.107; Figure 2.4B). Only 3 cases had a slight down-regulation of the AS mRNA expression (-1.6-, -1.2-, and -1.05-fold). These 3 cases were all patients with T-ALL, of whom 2 were clinically good responders.

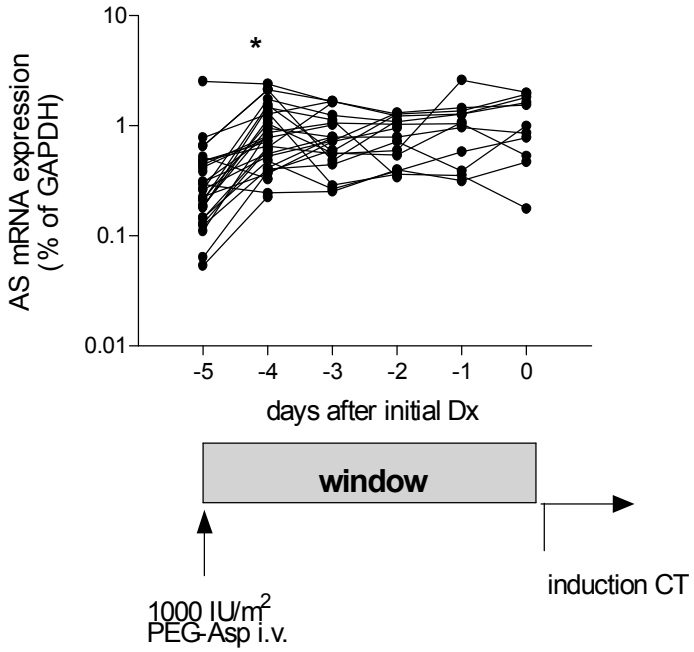


Figure 2.2 Asparagine synthetase (AS) mRNA expression induced by pegylated L-Asparaginase (PEG-Asp) in time

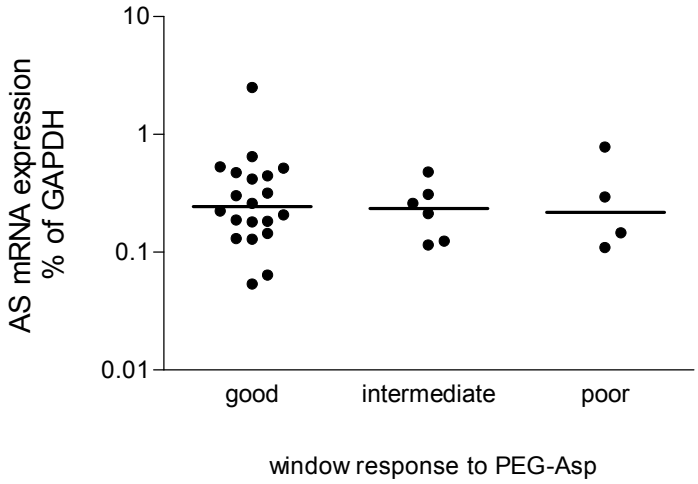
Time-response curves of AS mRNA expression in leukemic cells of 31 children after one single dose of PEG-Asp (1000 IU/m^2 given intravenously at day -5).
** 3-fold increase in AS mRNA from day-5 to day-4 (P < 0.001).*

The baseline and PEG-Asp induced expression levels of AS mRNA did not differ between hyperdiploid (n = 9), *TEL-AML1* positive (n = 5) and other B-lineage ALL (n = 5). For infants with MLL gene-rearranged ALL, no data were available for the effect of L-Asp on AS mRNA levels, because these patients were not eligible for the PEG-Asp window study.

DISCUSSION

Studies on putative causes of L-Asp resistance have been performed most extensively in mouse cell lines.[5,6] L-Asp sensitive tumor cells that did not contain detectable levels of AS developed resistance to L-Asp through exposure of cells to sublethal concentrations of this drug.[19] Resistant cells up-regulated AS expression and activity by 60-fold. It is well known that AS plays a crucial role in maintaining amino acid homeostasis in cells.[20] A rapid transcriptional control of the AS gene occurs following deprivation of any single essential amino acid.[6,21] In 1997, Hutson *et al.*[6] demonstrated that depletion of the intracellular asparagine pool by L-Asp was sufficient to activate *in vitro* AS expression in human leukemic cell lines. The increase in AS mRNA expression also resulted in a simultaneous up-regulation of AS protein levels and AS enzyme activity.[6,7] The direct correlation among mRNA, protein, and activity levels was confirmed by Irino *et al*.[22] The activity of AS was inversely related to the sensitivity to L-Asp in human leukemia cell lines.[6,23] These studies suggest that the expression of the AS gene is linked to resistance to L-Asp.

A



B

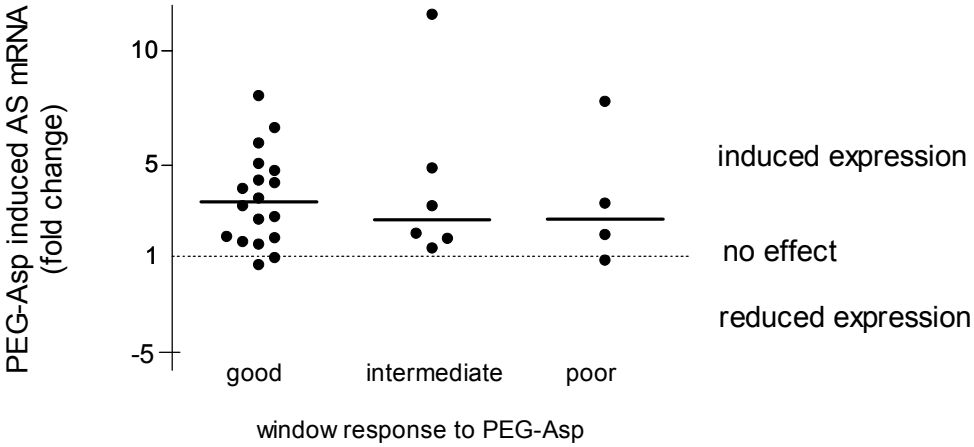


Figure 2.3 Relationship between clinical response and asparagine synthetase (AS) mRNA expression

(A) Baseline AS mRNA expression levels. (B) PEG-Asp-induced changes in AS mRNA expression levels measured after 24 hours of in vivo exposure to PEG-Asp compared to baseline expression levels. Dots represent individual expression values; solid lines, represent the median expression value per group. For definition of clinical response: see Figure 2.1.

In addition to the fact that these studies dealt not with primary patient samples but with cell lines, Wagner and Boos[24] argued that the test conditions in Hutson's experiments were not comparable with *in vivo* situations, where various products and substrates (such as aspartate, glutamate, glutamine, and ammonia, among others) are all part of metabolic pathways and equilibrium conditions.[5]

In 1969 Haskell et al. studied in vivo AS activity in 18 patients with leukemia.[3] Prior to therapy, AS activity was nearly undetectable in leukemic cells. Patients were treated with 200 IU/kg E coli L-Asp daily for 3 days to 3 weeks. A 7-fold increase in AS activity was found in 5 L-Asp-resistant compared to 4 L-Asp sensitive patients (mixed cohort of ALL, acute myeloid leukemia [AML], chronic

myelogenous leukemia [CML], and chronic lymphocytic leukemia [CLL]). Haskell et al.[3] suggested that L-Asp resistance was related to the capacity of leukemic cells to up-regulate AS expression for asparagine biosynthesis. However, besides the limited number of patients in a very heterogeneous group, the criteria used to determine whether the patient was resistant or sensitive to L-Asp were not described by Haskell.

In order to study the effect of monotherapy with L-Asp on leukemic blasts we used PEG-Asp. The effectivity of different L-Asp products like Erwinase, *E coli*, or PEG-Asp is the same if the serum enzyme activity of L-Asp is higher than 100 IU/L.[13] We studied whether baseline levels or up-regulated levels of AS mRNA expression in leukemic cells after an *in vivo* treatment with PEG-Asp monotherapy were associated with short-term clinical response to this drug in children with ALL. The baseline AS expression levels were in the same range as healthy controls, as reported before.[9] Up-regulation of AS mRNA occurred already within 24 hours after PEG-Asp exposure and thereafter no further changes were found. Because the drop in leukemic cells was seen during the whole window period (Figures 2.1 and 2.2), it is unlikely that only leukemic cells resistant to PEG-Asp with intrinsic higher AS expression levels were left over on day -4. Baseline and L-Asp induced AS mRNA expression levels did not differ between patients with good, intermediate, or poor response (Figure 2.3). So, L-Asp-induced up-regulation of AS mRNA is not related to early *in vivo* blast reduction in childhood ALL and thus not predictive for the short-term clinical response to L-Asp. As mentioned earlier, cell line studies showed that mRNA, protein, and activity levels of AS are correlated,[6,7,23] but at present it is unknown whether this is also the case for clinical samples because only limited amounts of patients' samples can be obtained.

Immunophenotypic and genetic abnormalities are related to drug resistance and outcome in childhood ALL.[25-27] T-ALL cells from children are, *in vitro,* more resistant to L-Asp than cells from children with precursor B-lineage ALL.[28] The relative resistance to L-Asp of T-ALL cases can not be explained by altered expression of the AS gene, since both baseline and L-Asp induced changes in AS mRNA expression did not differ between T- and c/pre-B-ALL patients (Figure 2.4). Remarkable was the finding that 3 out of 12 children with T-ALL even demonstrated a slight AS mRNA down-regulation, which would, *in vitro,* even point to sensitivity for L-Asp. Hyperdiploidy and the *TEL-AML1* fusion are related to favorable outcome in childhood ALL,[26,29] and are both *in vitro* sensitive to L-Asp.[9,30,31] In a previous study in *TEL-AML1* positive ALL, Stams *et al.*[9] showed that *TEL-AML1* positive children expressed 5-fold more AS mRNA compared to *TEL-AML1* negative patients and healthy controls. In the present study, *TEL-AML1* and hyperdiploid cases do not show an impaired *in vivo* up-regulation of AS that might have explained their high sensitivity to L-Asp. Taken together, both studies suggest that sensitivity to L-Asp as found in *TEL-AML1* positive and hyperdiploid cells is not linked to decreased AS mRNA expression.

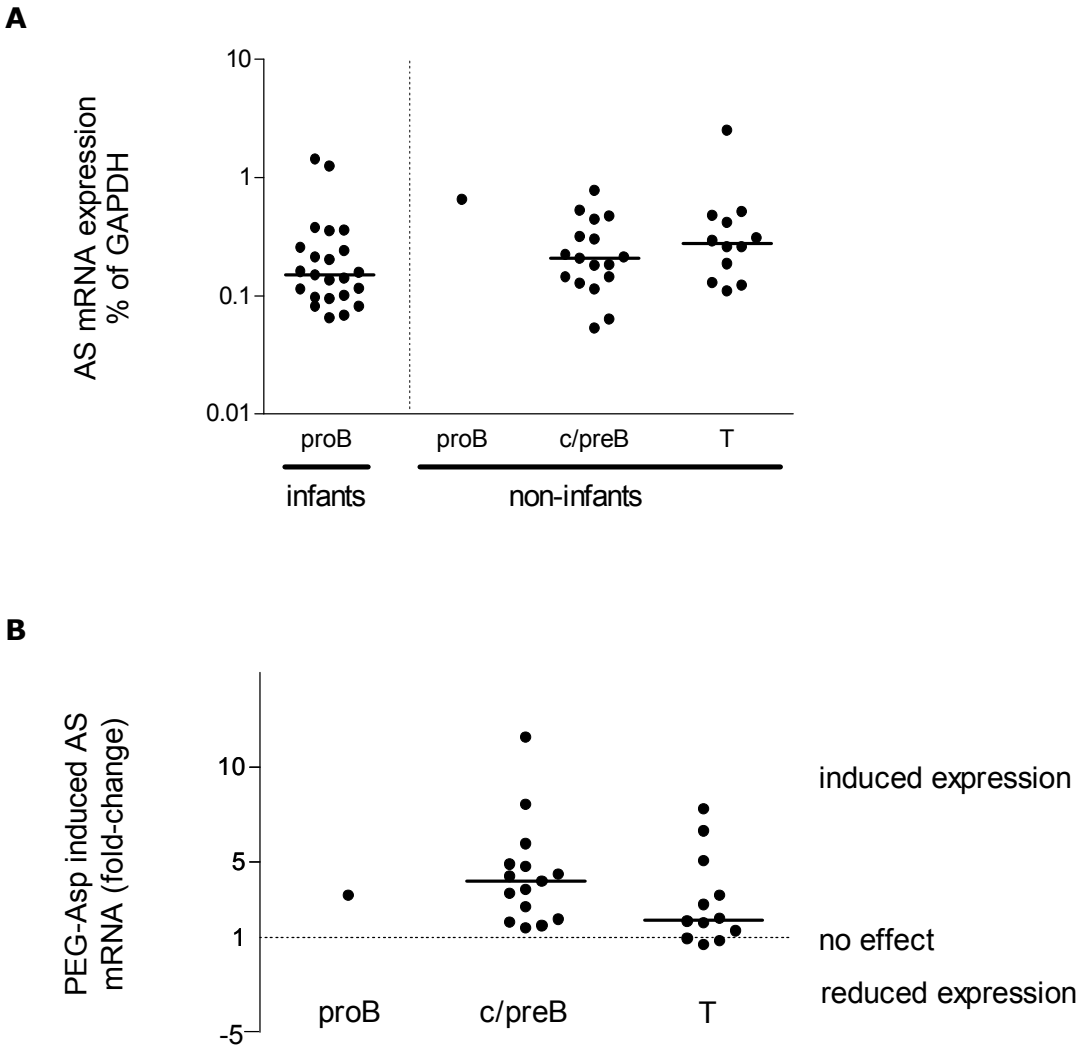


Figure 2.4 Relationship between immunophenotype and asparagine synthetase (AS) mRNA expression

(A) Baseline AS mRNA expression levels. (B) PEG-Asp induced changes in AS expression levels in ALL cells. Dots represent individual expression values; solid lines, represent the median expression value per group.

AS mRNA up-regulation in ALL cells occurs very rapidly (< 24 hours) after cellular asparagine depletion following PEG-Asp administration. Amino acids are required for protein synthesis, but they also play a role in the control of gene expression.[6,20] The promotor of AS contains a nutrient-sensing response unit (NSRU) that is responsible for the induction of AS gene transcription upon amino acid deprivation.[32] Iiboshi *et al.* showed that withdrawal of asparagine and glutamine by L-Asp resulted in a rapid inactivation of p70 S6 kinase.[33] P70 S6 kinase participates in the mammalian target of rapamycin (mTOR) protein synthesis by controlling translational initiation and elongation factors as well as protein kinases that affect ribosomal assembly. Recently, gene expression profiling revealed that L-Asp resistant ALL cells overexpressed several ribosomal protein-encoding genes as well as initiation factors.[34] Using gene expression profiling, Fine *et al.* showed that L-Asp resistant cell lines expressed more baseline AS mRNA

than sensitive leukemic cell lines, whereas no such association was found for primary pediatric ALL samples.[35] This study emphasizes the fact that leukemic cell lines and primary samples from leukemic patients are different from each other and cell line data can not be extrapolated to primary patient's cells that easily. Exposure to L-Asp altered in primary patient's samples the expression of a number of genes related to protein synthesis (*ie*, tRNA synthetases and amino acid transporters). However, no genes discriminative for L-Asp resistance in patient's samples were found. These data point to a consistent coordinated response to amino acid starvation, which occurs irrespective of the level of resistance to L-Asp in patient's cells. Therefore, AS up-regulation may be a consequence of amino acid deprivation by L-Asp but is not the limiting key-factor explaining resistance to L-Asp in pediatric ALL.

We conclude that up-regulation of AS mRNA in childhood ALL cells occurs within 24 hours after *in vivo* exposure to PEG-Asp, but these up-regulated levels are not associated with an early (poor) response to PEG-Asp in this small group of children.

REFERENCES

1. Capizzi RL, Bertino JR, Skeel RT. L-Asparaginase: clinical, biochemical, pharmacological and immunological status. Ann Int Medicine. 1971;74:893-901.
2. Sallan SE, Hitchcock-Bryan S, Gelber R, Cassady JR, Frei ED, Nathan DG. Influence of intensive asparaginase in the treatment of childhood non-T-cell acute lymphoblastic leukemia. Cancer Res. 1983;43:5601-5607.
3. Haskell CM, Canellos GP, Leventhal BG, Carbone PP, Block JB. L-Asparaginase resistance in human leukemia-Asparagine synthetase. Biochemical Pharmacology. 1969;18:2578-2580.
4. Broome JD. L-Asparaginase:discovery and development as a tumor-inhibitory agent. Cancer Treatment Reports. 1981;65:111-114.
5. Andrulis IL, Argonza R, Cairney AE. Molecular and genetic characterization of human cell lines resistant to L-Asparaginase and Albizziin. Somatic Cell Mol Genet. 1990;16:59-65.
6. Hutson RG, Kitoh T, Moraga Amador DA, Cosic S, Schuster SM, Kilberg MS. Amino acid control of asparagine synthetase: relation to resistance in human leukemia cells. Am J Physiol. 1997;272:1691-1699.
7. Aslanian AM, Kilberg MS. Multiple adaptive mechanisms affect asparagine synthetase substrate availability in asparaginase-resistant MOLT-4 leukaemia cells. Biochem J. 2001;358:59-67.
8. Stams WA, den Boer ML, Holleman A, Appel IM, Beverloo HB, van Wering ER, et al. Asparagine synthetase expression is linked with L-asparaginase resistance in TEL-AML1-negative but not TEL-AML1-positive pediatric acute lymphoblastic leukemia. Blood. 2005;105:4223-4225.
9. Stams WA, den Boer ML, Beverloo HB, et al. Sensitivity to L-asparaginase is not associated with expression levels of asparagine synthetase in t(12;21)+ pediatric ALL. Blood. 2003;101:2743-2747.
10. Veerman AJ, Hahlen K, Kamps WA, et al. High cure rate with a moderately intensive treatment regimen in non-high-risk childhood acute lymphoblastic leukemia. Results of protocol ALL VI from the Dutch Childhood Leukemia Study Group. J Clin Oncol. 1996;14:911-918.
11. Asselin BL, Kreissman S, Coppola DJ, et al. Prognostic significance of early response to a single dose of Asparaginase in childhood acute lymphoblastic leukemia. J Ped Hematol/Oncol. 1999;2:6-12.
12. Boos J, Weber G, Ahlke E, Schulze-Westhof P, Nowak-Gottl U, Wurthwein G. Monitoring on asparaginase activity and asparaginase levels in children on different asparaginase preparations. Eur J Cancer. 1996;32A:1544-1550.
13. Muller HJ, Loning L, Horn A, et al. Pegylated asparaginase (Oncaspar) in children with ALL: drug monitoring in reinduction according to the ALL/NHL-BFM 95 protocols. Br J Hematol. 2000;110:379-384.
14. Appel IM, Pinheiro JPV, Boer den ML, et al. Lack of asparagine depletion in the cerebrospinal fluid after one intravenous dose of PEG-asparaginase: a window study at initial diagnosis of childhood ALL. Leukemia. 2003;17:2254-2256.
15. Yoshimoto T, Nishimura H, Saito Y, et al. Characterization of polyethylene glycol-modified L-asparaginase from *Escherichia coli* and its application to therapy of leukemia. Jpn J Cancer Res. 1986;77:1264-1270.
16. Schrappe M, Reiter A, Zimmermann M, et al. Long-term results of four consecutive trials in childhood ALL performed by the ALL-BFM study group from 1981 to 1995. Berlin-Frankfurt-Munster. Leukemia. 2000;14:2205-2222.
17. Kaspers GJ, Veerman AJ, Pieters R, et al. Mononuclear cells contaminating acute lymphoblastic leukaemic samples tested for cellular drug resistance using the methyl-thiazol-tetrazolium assay. Br J Cancer. 1994;70:1047-1052.

18. Meijerink J, Mandigers C, Loch vd L, Tonissen E, Goodsaid F, Raemakers A. A novel method to compensate for different amplification efficiencies between patient DNA samples in quantitative real-time PCR. J Mol Diagn. 2001;3:55-61.

19. Horowitz B, Madras BK, Meister A, Old LJ, Boyse EA, Stockert E. Asparagine synthetase activity of mouse leukemias. Science. 1968;160:533-535.

20. Jousse C, Averous J, Bruhat A, Carraro V, Mordier S, Fafournoux P. Amino acids as regulators of gene expression: molecular mechanisms. Biochem Biophys Res Commun. 2004;313:447-452.

21. Chakrabarti R, Schuster SM. L-Asparaginase: Perspectives on the mechanisms of action and resistance. Int J Ped Hem/Oncol. 1997;4:597-611.

22. Irino T, Kitoh T, Koami K, et al. Establishment of real-time polymerase chain reaction method for quantitative analysis of asparagine synthetase expression. J Mol Diagn. 2004;6:217-224.

23. Aslanian AM, Fletcher BS, Kilberg MS. Asparagine synthetase expression alone is sufficient to induce L-asparaginase resistance in MOLT-4 human leukaemia cells. Biochem J. 2001;357:321-328.

24. Wagner A, Boos J. Unphysiological effects contributing to asparaginase toxicity in vitro. Am J Physiol. 1998;274:C1185-1186.

25. Pieters R, den Boer ML, Durian M, et al. Relation between age, immunophenotype and in vitro drug resistance in 395 childern with acute lymphoblastic leukemia - implications for treatment of infants. Leukemia. 1998;12:1344-1348.

26. Pui CH, Evans WE. Acute lymphoblastic leukemia. N Engl J Med. 1998;339:605-615.

27. Pieters R, den Boer ML. Molecular pharmacodynamics in childhood leukemia. Int J Hematol. 2003;78:402-413.

28. Pieters R, Loonen AH, Huismans DR, et al. In vitro drug sensitivity of cells from children with leukemia using the MTT assay with improved culture conditions. Blood. 1990;76:2327-2336.

29. McLean TW, Ringold S, Neuberg D, et al. TELAML-1 dimerizes and is associated with a favorable outcome in childhood acute lymphoblastic leukemia. Blood. 1996;88:4252-4258.

30. Ramakers-van Woerden NL, Pieters R, Loonen AH, et al. TEL-AML1 gene fusion is related to in vitro drug sensitivity for L-Asparaginase in childhood acute lymphoblastic leukemia. Blood. 2000;96:1094-1099.

31. Kaspers GJL, Veerman AJP, Pieters R, et al. In vitro cellular drug resistance and prognosis in newly diagnosed childhod acute lymphoblastic leukemia. Blood. 1997;90:2723-2729.

32. Barbosa-Tessmann IP, Chen C, Zhong C, et al. Activation of the human asparagine synthetase gene by the amino acid response and the endoplasmic reticulum stress response pathways occurs by common genomic elements. J Biol Chem. 2000;275:26976-26985.

33. Iiboshi Y, Papst PJ, Kawasome H, et al. Amino Acid-dependent Control of p70 s6k. J Biol Chem. 1999;274:1092-1099.

34. Holleman A, Cheok MH, den Boer ML, et al. Gene-expression patterns in drug-resistant acute lymphoblastic leukemia cells and response to treatment. N Engl J Med. 2004;351:533-542.

35. Fine BM, Kaspers GJL, Ho Minh, Loonen AH, Boxer LM. A Genome-Wide View of the in vitro Response to L-Asparaginase in Acute Lymphoblastic Leukemia. Cancer Res. 2005;65:291-299.

chapter 3

Pharmacokinetic, pharmacodynamic and intracellular effects of PEG-Asparaginase in newly diagnosed childhood acute lymphoblastic leukemia: results from a single agent window study

Inge M. Appel
Karin M. Kazemier
Joachim Boos
Claudia Lanvers
Jan Huijmans
Anjo J.P. Veerman
Elisabeth van Wering
Monique L. den Boer
Rob Pieters

Submitted

ABSTRACT

L-Asparaginase is an effective drug for treatment of children with acute lymphoblastic leukemia. The effectiveness is thought to result from depletion of asparagine in serum and cells. We investigated the clinical response *in vivo* of 1000 IU/m^2 PEG-Asparaginase and its pharmacokinetic, pharmacodynamic and intracellular effects in children with newly diagnosed ALL before start of combination chemotherapy. The *in vivo* window response was significantly related to immunophenotype and genotype: 26/38 common/pre B–ALL cases, especially those with hyperdiploidy and *TEL-AML1* rearrangement, demonstrated a good clinical response compared to 8/17 T-ALL (P = 0.01) and *BCR-ABL* positive ALL (P = 0.04). A poor *in vivo* clinical window response was related to *in vitro* resistance to L-Asparaginase (P = 0.02) and both were prognostic factors for long-term event-free survival (Hazard ratio 6.4; P = 0.004 and Hazard ratio 3.7; P = 0.01). After administration of one *in vivo* dose of PEG-Asparaginase no changes in apoptotic parameters or in intracellular levels of twenty amino acids in leukemic cells could be measured, in contradiction to the changes found after *in vitro* exposure. This may be explained by the rapid removal of apoptotic cells from the circulation *in vivo.* One additional dose of PEG-Asparaginase upfront ALL treatment did not lead to other severe toxicities.

INTRODUCTION

L-Asparaginase is an effective drug to treat newly diagnosed acute lymphoblastic leukemia (ALL).[1] Prolonged L-Asparaginase intensification significantly improved the outcome of ALL patients as was demonstrated in the Dana-Farber Cancer Institute ALL Consortium Protocol 91-01.[2] Several studies have shown that *in vitro* resistance to this drug is an independent prognostic factor in ALL.[3-8] Also a poor early *in vivo* response to L-Asparaginase as a single drug has been linked to an unfavourable outcome in pediatric ALL.[9]

The administration of L-Asparaginase results in the deamination of asparagine into aspartic acid leading to a rapid and complete depletion of serum asparagine, which ultimately affects the intracellular asparagine levels.[10,11] L-Asparaginase also has 3 - 4% glutaminase activity leading to serum glutamine depletion.[12] L-Asparaginase enzymatic activity should be > 100 IU/L in order to sufficiently diminish the asparagine serum levels required to induce leukemic cell kill.[13] Amino acid deficiency impairs protein synthesis and leads to apoptosis and cell death.[14]

In vitro studies demonstrated that cellular deprivation of asparagine and glutamine leads to increased levels of asparagine synthetase.[15] This enzyme opposes the action of L-Asparaginase and, thereby can rescue cells from the effect of L-Asparaginase.[16] Human leukemia cell line studies suggested that only L-Asparaginase resistant cells upregulate the activity of asparagine synthetase.[15] However, we recently demonstrated that up-regulation of asparagine synthetase mRNA occurs in both sensitive and in resistant cases within 24 hours of *in vivo* exposure to L-Asparaginase.[17] These data imply that mechanisms other than increased expression levels of asparagine synthetase contribute to cellular L-Asparaginase resistance.

Recently, gene expression profiling studies by microarray analysis revealed that leukemic cells of L-Asparaginase resistant ALL patients express higher levels of genes involved in protein synthesis than L-Asparaginase sensitive cells.[18] A deficiency in amino acids finally induces apoptosis in malignant cells. L-Asparaginase activates caspase 3 and inactivates poly-ADP-ribose-polymerase (PARP) in patients leukemic cells and resistance to L-Asparaginase is linked to an impaired capacity of cells to trigger the apoptotic pathway.[19] Hypothetically, an altered intracellular amino acid composition might rescue cells from the effects of L-Asparaginase. It is yet unknown whether the amino acid metabolism of L-Asparaginase differs between resistant and sensitive leukemic cells of patients.

In the present study we investigated pharmacokinetic and pharmacodynamic as well as intracellular effects of one *in vivo* dose of pegylated-L-Asparaginase (PEG-Asparaginase) in children with newly diagnosed ALL. *In vivo* response to PEG-Asparaginase was monitored by analyzing the decrease in leukemic cells during a therapeutic window of 5 days before start of combination chemotherapy. *In vivo* response to PEG-Asparaginase was compared with the baseline as well as PEG-Asparaginase-induced changes in serum and intracellular amino acid levels and with parameters of apoptosis. *In vitro* resistance to L-Asparaginase was determined and compared to the mentioned factors that might influence drug

resistance. In addition, the influence of PEG-Asparaginase given upfront regular antileukemic treament was evaluated on the incidence of allergic reactions during treatment and changes in hemostasis.

METHODS

Patients and treatment

In close collaboration between our institution and the Dutch Childhood Oncology Group (DCOG), a window study with PEG-Asparaginase upfront to the ALL-9 treatment schedule was initiated in July 2000. The ALL-9 protocol was based on the ALL-6 treatment strategy of the DCOG.[20] Non-high risk ALL was defined as white blood count (WBC) < 50 x 10^9 /L, no mediastinal mass, absence of t(9;22), t(4;11) or other MLL rearrangements, no T-cell phenotype and no central nervous system (CNS) or testicular involvement. All other leukemias were defined as high risk.

Eligible for the window study with PEG-Asparaginase were children with newly diagnosed ALL and presenting WBC > 10 x 10^9 /L. Patients received a single dose of 1000 IU/m^2 PEG-Asparaginase in an one hour infusion 5 days before starting combination chemotherapy. PEG-Asparaginase (Oncaspar®), kindly provided by Medac (GmbH, Hamburg, Germany), was used because of its lower immunogenicity than native (unpegylated) L-Asparaginase,[21] which was important since native *E. coli* L-Asparaginase was used as part of the regular combination chemotherapy given after the investigational window.

The window study with PEG-Asparaginase was approved by the local ethical committee and by the institutional research board of the DCOG. The patient and/or the parents/guardians gave informed consent for this study.

The immunophenotyping and cytogenetic characterizations were performed at the central reference laboratory of the DCOG and at laboratories of the participating centers. B-lineage ALL ($CD19^+$, $HLA\text{-}DR^+$) was classified into the following differentiation stages: proB-ALL cells were $CD10^-$, cytoplasmic μ chain $^-$ ($c\mu^-$) and surface immunoglobulin$^-$ (sIg^-); c-ALL cells were $CD10^+/c\mu^-/sIg^-$; pre-B cells were $CD10^{+or-}/c\mu^+/sIg^-$. B-ALL cells characterized by $CD10^-/c\mu^-/sIg^+$ were excluded from the study. Cytogenetic analyses were done by regular karyotyping and FISH.[22]

Patient samples

Bone marrow and peripheral blood samples were obtained at initial diagnosis of ALL before the administration of PEG-Asparaginase. Serum and leukemic cells from peripheral blood were collected one hour after the end of the infusion with PEG-Asparaginase and thereafter daily during 5 consecutive days till the start of combination chemotherapy at day 0. Mononuclear cells were isolated by density gradient centrifugation using Lymphoprep (density 1.077 g/ml; Lucron bioproducts, Gennep, The Netherlands) as described before.[17] Contaminating non-leukemic cells were removed by immunomagnetic beads as described earlier.[23] All samples contained over 90% of leukemic cells, as determined morphologically on May-Grünwald-Giemsa (Merck, Darmstadt, Germany) stained cytospins.

Clinical response

In correspondence with the definition of the window response to prednisone in ALL[24] the clinical window response to PEG-Asparaginase on day 0 (5 days after the PEG-Asparaginase infusion) was defined as good when the number of leukemic cells was < 1 x 10^9 /L of peripheral blood, as intermediate when leukemic cells were 1 - 10 x 10^9 /L, and as poor when leukemic cells were > 10 x 10^9 /L.

***In vitro* cytotoxicity**

In vitro L-Asparaginase cytotoxicity in leukemic cells taken at initial diagnosis (untreated) was determined using the MTT assay. Cells were exposed to six different concentrations of L-Asparaginase (Paronal®, Christiaens B.V., Breda, The Netherlands) ranging from 0.0032 - 10 IU/ml in duplicate. Control cells were cultured without L-Asparaginase. After four days of incubation at 37 °C in humidified air containing 5% CO_2, the MTT-assay was performed. Drug sensitivity was assessed by the LC_{50}, the drug concentration lethal to 50% of the cells. Evaluable assay results were obtained when a minimum of 70% of leukemic cells was present in the control wells after 4 days of incubation and when the control OD was ≥ 0.050.[8]

*In vitro s*ensitivity towards L-Asparaginase was defined as LC50 ≤ 0.033 IU/ml, *in vitro* resistance towards L-Asparaginase was defined as LC50 ≥ 0.912 IU/ml, and intermediate sensitive was defined as LC50 0.033 - 0.912 IU/ml.[8]

Since LC50 values were highly correlated between Paronal® and L-Asparaginase Medac® (Rs 0.93, *P* < 0.002) and between Paronal® and PEG-Asparaginase (Oncaspar®) (Rs 0.86, *P* < 0.002) all consecutive MTT-assays were performed using Paronal®.[3,8]

Apoptotic features

Determination of Annexin V, $DIOC_6$, cleaved-caspase-3 and cleaved-PARP were done by FACS analysis as described earlier.[19]

For *ex vivo* exposure to L-Asparaginase, cells were incubated with culture medium as control or incubated with 10 IU/ml of L-Asparaginase (Paronal®) in culture medium. 5 x 10^6 cells were harvested after 18, 24, 30 and 44 hours of incubation with L-Asparaginase and apoptotic markers were analyzed.

L-Asparaginase activity and amino acid levels in serum

Serum levels of L-Asparaginase and asparagine, glutamine, aspartic acid and glutamic acid were determined in the laboratory of Prof Dr J. Boos (University Children's Hospital Muenster, Department of Pediatric Hematology/Oncology, Germany). Blood samples were taken daily from day -5 to day 0, and twice a week from day 0 to 28. L-Asparaginase activity was analyzed as described before;[25] serum levels of asparagine, aspartic acid, glutamine and glutamic acid were done using HPLC.[26] The lower limit of detection was 0.2 µM for all amino acids; glutamine levels higher than 250 µM were not further analyzed and reported as > 250 µM. The serum levels of asparagine and the L-Asparaginase activity in a part of the patients included in the present study have been published before.[27]

Intracellular amino acid levels

Intracellular levels of all essential and non-essential amino acids were measured by liquid column chromatography on a Biochrome 20 amino acid analyzer with ninhydrin detection (Biochrome, Cambridge). Blood samples were taken daily from day -5 at diagnosis to day 0. At each time-point 5 x 10^6 leukemic cells were lysed in 100 μl of lysis buffer (150 ml NaCl, 30 mM Tris (pH 7.6), 10% glycerol, 1% Triton X-100, 1 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptine) for 15 minutes on ice. Cleared supernatants were stored at -80 ^{0}C until the time of analysis. The intracellular amounts of amino acids were expressed as μmol of amino acid per mg of protein. The lower limit of detection was 7 μmol (coefficient of variation 10%). Intracellular protein content was measured using the bicinchoninic acid (BCA) assay (Interchim Omnilabo[28]).

For *ex vivo* exposure to L-Asparaginase, cells were incubated with culture medium as control or incubated with 0.1 IU/ml or 10 IU/ml of L-Asparaginase (Paronal®) in culture medium. 5 x 10^6 cells were harvested after 1, 3, 6, 24, and 30 hours, and if possible after 48 and 72 hours of incubation with L-Asparaginase and amino acid levels were analyzed.

Clinical toxicity

Clinical manifestations of diabetes mellitus, pancreatitis, hyperlipidemia, neurotoxicity and stroke were monitored. Changes in coagulation and fibrinolysis were monitored throughout induction therapy. On day -5, day 0 and during induction on days 29, 33, 36, 40 and 43 peripheral blood samples were collected from the infusion line just before each Paronal® infusion. All coagulation assays were done with commercially available reagents and methods as described in detail previously.[29] Reference values were applied from the literature.[30-32]

Statistics

Differences between multiple groups were calculated using the Kruskal-Wallis test. The Mann-Whitney *U* test was used when 2 groups were compared. $P = 0.05$ (two sided) was used as level of significance.

Median follow-up time of patients at risk of an event was 4.1 years (range 1.9 to 5.5 years). Event-free survival defined as relapse-free survival was calculated according to Kaplan Meier (± SE). Multiple regression was conducted using Cox proportional hazards regression models to assess prognostic factors for event-free survival.

RESULTS

Patients

Between July 2000 and July 2004, 57 children with newly diagnosed ALL were enrolled in the PEG-Asparaginase-window study (Table 3.1). Since only patients with initial WBC > 10 x 10^9 /L were eligible for this study, more than half of these children were high-risk patients according to ALL-9 criteria.

Table 3.1 Patient characteristics of PEG-Asparaginase window study

Patients included	57
Male/female	36/21
Age (years) median (range)	4.9 (1.4 - 15.1)
WBC (10^9/L) median (range)	44.4 (11.3 - 417)
Non high risk/high risk	27/30
Immunophenotype (57):	
pro B ALL	2
common/pre B ALL	38
T ALL	17
Genotype of precursor B ALL (40):	
Hyperdiploid (> 50 chromosomes)	11
TEL-AML1/t(12;21)	8
BCRABL/t(9;22)	2
MLL rearranged (11q23)	0
Normal (46 XX/XY)	8
others	11

Clinical response

Administration of PEG-Asparaginase at day -5 resulted in a steadily drop in the number of leukemic cells in the peripheral blood over 5 consecutive days. The median leukemic cell count was reduced 192-fold from 34.5 x 10^9 /L at day -5 to 0.18 x 10^9 /L at day 0 (Figure 3.1). 35 (61%) out of 57 children were defined as good responders to PEG-Asparaginase, 16 (28 %) were intermediate responders and 6 (11 %) children were poor responders.

The good prognostic genotypes (hyperdiploid and *TEL-AML1* / t(12;21) positive ALL) were associated with a good clinical window response, whereas the poor prognostic genotype *BCR-ABL* / t(9;22) was associated with a poor clinical window response (P = 0.04) (Table 3.2). The 17 T-ALL patients had a significant poorer response to PEG-Asparaginase compared to the 36 common/pre B cases (P = 0.01).

Table 3.2 Correlation between clinical response to one dose of PEG-Asparaginase and immunophenotype and genotype in 57 children with newly diagnosed ALL

	Pro B		**Common/pre B**				**T**	**total**
	MLL germline	MLL rearranged	TEL-AML1	hyperdiploid	other	BCRABL	-	
Good responder	1		7	7	12		8	35
Intermediate responder	1		1	3	4		7	16
Poor responder					2	2	2	6
Total	**2**	**0**	**8**	**10**	**18**	**2**	**17**	**57**

Clinical response of 57 children with ALL to one dose of PEG-Asparaginase upfront ALL-induction treatment (day –5), related to immunophenotype and genotype. The clinical response to PEG-Asparaginase on day 0 was defined as good when the number of leukemic cells had declined to < 1 x 10^9/L of peripheral blood, as intermediate when leukemic cells were 1 - 10 x 10^9/L, and as poor when leukemic cells were > 10 x 10^9/L.

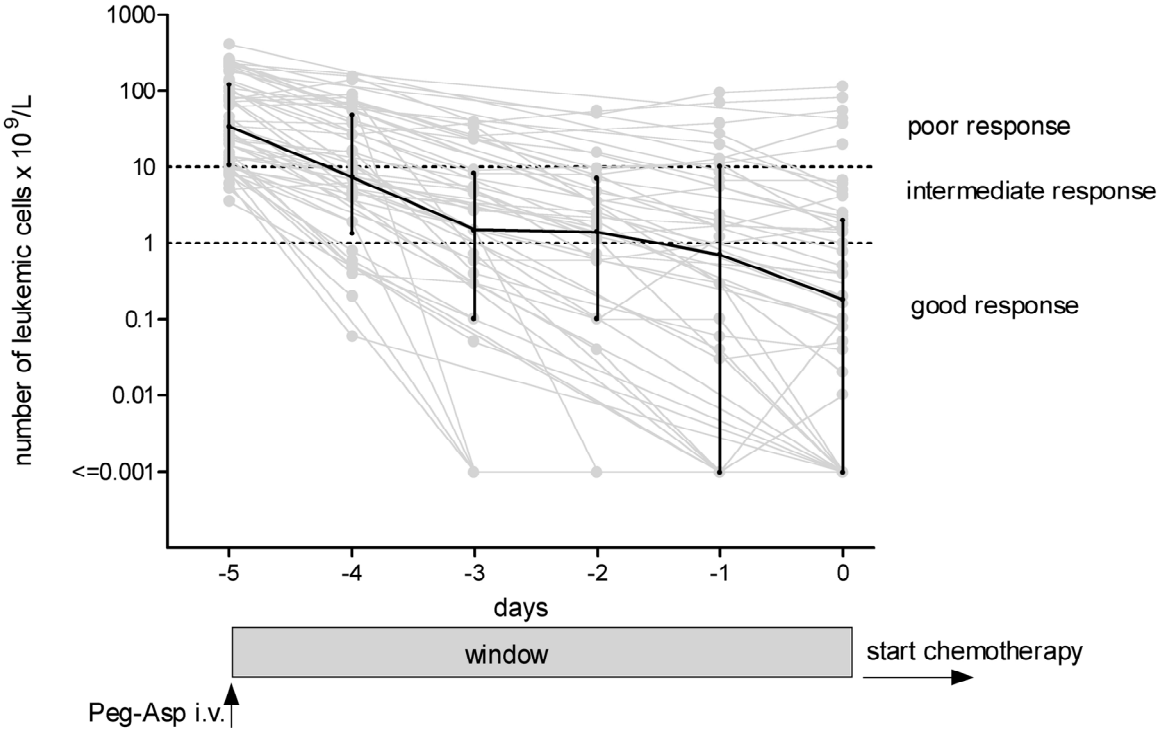


Figure 3.1 Clinical response to one dose of 1000 IU/m^2 PEG-Asp
PEG-Asp was administered intravenously at day -5, i.e. 5 days upfront regular chemotherapy. A good clinical response to PEG-Asparaginase was defined as < 1 x 10^9/L leukemic cells in the peripheral blood at day 0, an intermediate response was defined as 1 - 10 x 10^9/L, and a poor response as > 10 x 10^9/L leukemic cells in the peripheral blood at day 0. The data of individual patients are given in gray, medians with ranges (25th and 75th percentiles) are drawn in black.

In vitro cytotoxicity of L-Asparaginase was measured in 41 leukemic cell samples. Children with a poor or intermediate *in vivo* response to PEG-Asparaginase treatment had a median LC50 value of 1.0 IU/ml compared to 0.04 IU/ml for children with a good clinical response. So poor and intermediate responders were *in vitro* 25 fold more resistant to L-Asparaginase (P = 0.02) than children with a good *in vivo* response (Figure 3.2). In concordance with earlier studies,[33] T-ALL cells were significantly more *in vitro* resistant to L-Asparaginase than precursor B-ALL cells (LC50 median 1.22 IU/ml versus 0.10 IU/ml; P < 0.001). Only one 8-year old girl with T-ALL and an initial WBC of 132 x 10^9 /L had a poor clinical response (WBC 25 x 10^9 /L at day 0, absolute leukemic cell count 19.3 x 10^9 /L), whereas her blasts were *in vitro* sensitive to L-Asparaginase. She still is in continuous complete remission.

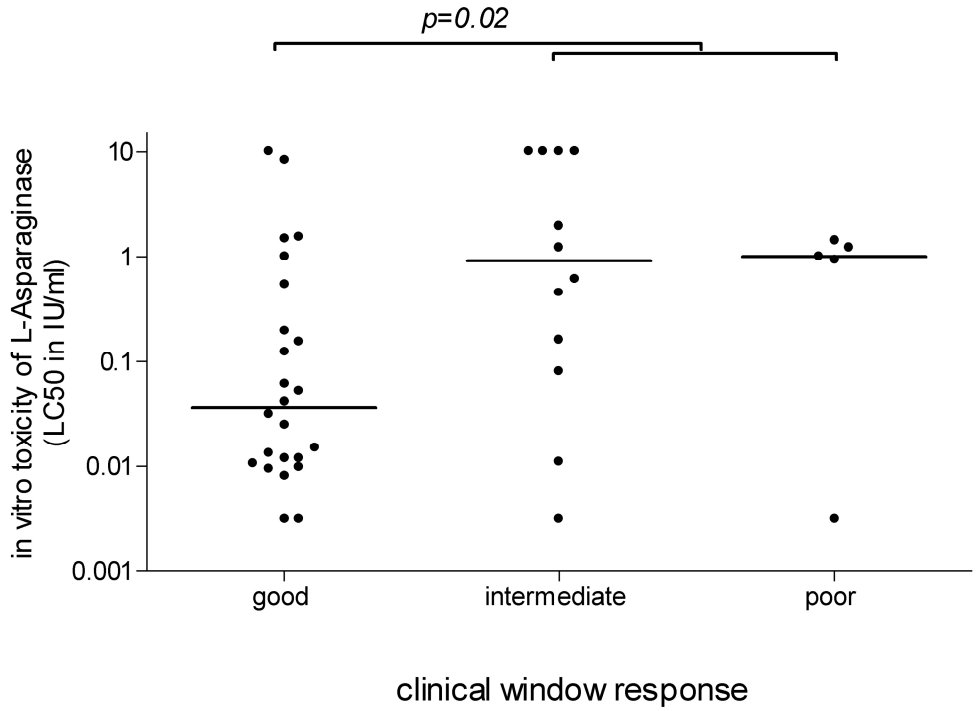


Figure 3.2 Comparison between in vivo and in vitro response to L-Asparaginase: *in 41 children in vitro cytotoxicity of L-Asparaginase (LC50 in IU/ml) was measured and related to the in vivo response to 1000 IU/m^2 PEG-Asparaginase given upfront regular chemotherapy. Good responders (leukemic cells at day 0 < 1 x 10^9/L) were significantly more sensitive to L-Asparaginase than intermediate responders (leukemic cells 1 - 10 x 10^9/L at day 0) (P = 0.03) and intermediate and poor responders together (leukemic cells > 1 x 10^9/L at day 0) (P = 0.02). The sensitivity to L-Asparaginase did not differ between the intermediate and poor responders.*

Apoptosis

Analysis of in vivo induced apoptosis by PEG-Asparaginase

We analyzed 25 patients for different apoptotic parameters in time (Figure 3.3). At diagnosis samples from 13 patients were available, immediately after the PEG-Asparaginase infusion samples from 21 patients, and on the consecutive days from 10 - 24 patients. The median percentage of ALL cells showing PS externalisation was 10.5 % before starting treatment (day -5). These values did not significantly differ in time (Figure 3.3A). The median percent of cells with changes in mitochondrial transmembrane potential was small at all time-points: before PEG-Asparaginase exposure 5.4% cells were DIOC6 positive, this did not change in time (*P* = NS) (Figure 3.3B). The median percentage of cells with cleaved (and hence activated) caspase 3 at diagnosis was 2.7% and remained low (*P* = NS) (Figure 3.3C). The median percent of cells with PARP inactivation at diagnosis was 2.9% and remained low (*P* = NS) (Figure 3.3D).

These data show that the drop in white blood cell count and number of leukemic cells (Figure 3.1) seen after PEG-Asparaginase administration was not simultaneously associated with an increase in *in vivo* apoptotic markers. None of the apoptotic markers was linked to immunophenotype or genotype.

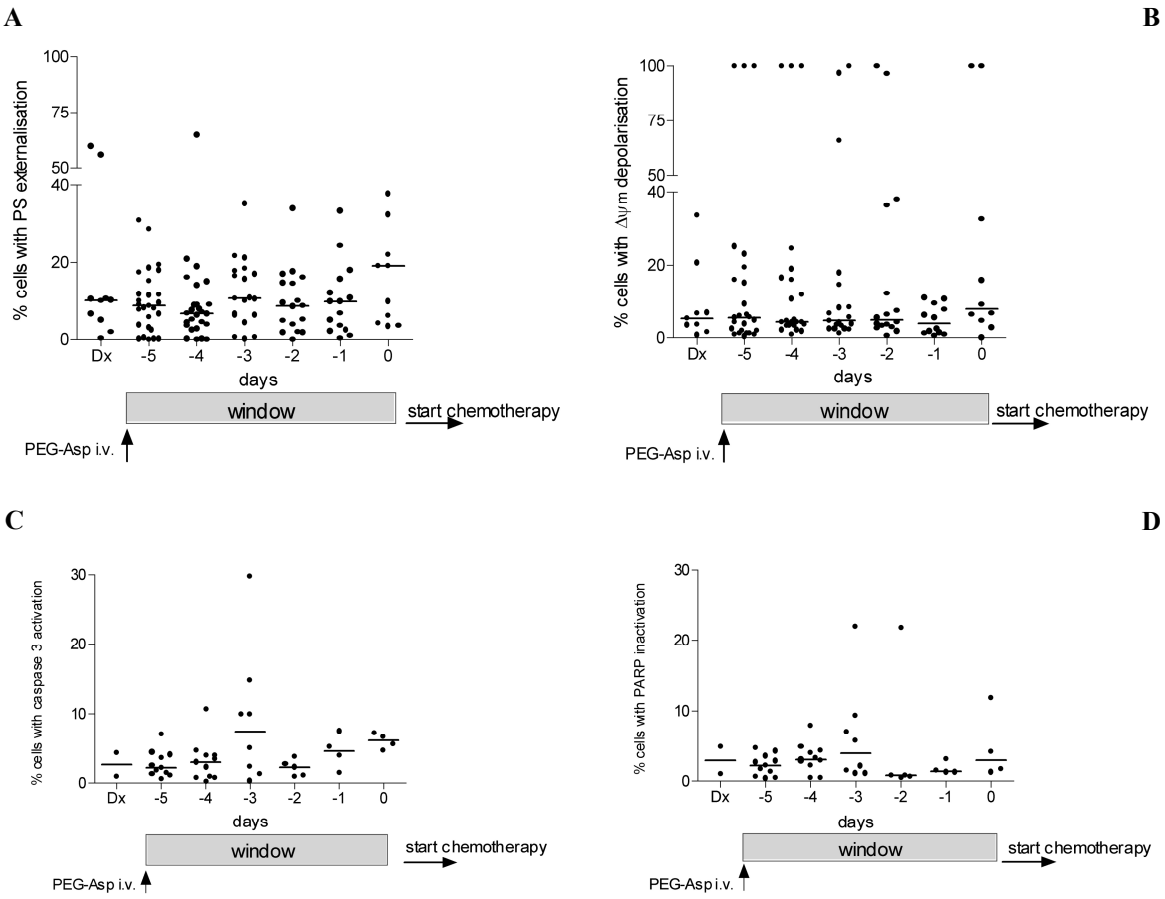


Figure 3.3A-D **In vivo induced apoptosis** *registered before and during a five day period after the administration of 1000 IU/m^2 PEG-Asparaginase upfront regular chemotherapy. Percent of cells with PS externalisation (Figure 3.3A), with $\Delta\psi$m depolarisation (Figure 3.3B), caspase 3 activation (Figure 3.3C) and PARP inactivation (Figure 3.3D) are shown*.

Analysis of in vitro induced apoptosis by L-Asparaginase

In contrast to data obtained after *in vivo* exposure, significant changes in apoptotic parameters were detected after *in vitro* exposure to L-Asparaginase (Figure 3.3E). This is in correspondance with earlier studies showing that activation of these apoptotic markers can be measured after *in vitro* exposure to L-Asparaginase.[19]

L-Asparaginase activity and levels of amino acids in serum

The median serum level of asparagine before treatment was 38.7 µM (p25th - 75th: 28.1 - 44.9 µM). In all patients serum asparagine levels decreased below the limit of detection of 0.2 µM after the administration of PEG-Asparaginase (P < 0.001) (Figure 3.4A and 3.4B). With the exception of two patients these asparagine levels remained below 0.2 µM until day 21, so for a total of at least 26 days. The L-Asparaginase activity in the serum was > 100 IU/L for a total of at least 15 days (Figure 3.4A). The level of serum aspartic acid (Figure 3.4B) increased after 1 hour of PEG-Asparaginase infusion from 7.1 µM (p25th - 75th: 4.1 -14.3 µM) to 22.1 µM (p25th - 75th: 15.5 - 47.3 µM) (P < 0.001), followed by a decrease to median 11.7 (p25th - 75th: 7.3 - 21.3 µM) and 15.5 µM (p25th - 75th: 9.6 - 23.5 µM) at day -3 and day 0. Compared to the level measured at 1 hour after the PEG-Asparaginase

infusion the serum aspartic acid levels were still increased at both time points (P = 0.02 at day -3 and P = 0.05 at day 0).

Most glutamine levels stayed beyond the upper limit of detection (> 250 µM) after PEG-Asparaginase treatment (Figure 3.4B). However, glutamic acid levels significantly increased from median 87.6 µM ($p25^{th}$ - 75^{th}: 57.8 - 124 µM) before treatment to 310 µM ($p25^{th}$ - 75^{th}: 178 - 396 µM) one hour after therapy (P < 0.001), and to 239 µM ($p25^{th}$ - 75^{th}: 125 - 306 µM) at day -3 (P < 0.001) and 159 µM ($p25^{th}$ - 75^{th}: 127 - 300 µM) at day 0 (P < 0.001) (Figure 3.4B).

The serum peak levels of PEG-Asparaginase did not differ between clinical response groups. No correlation with age was observed.

Intracellular amino acids levels

In vivo PEG-Asparaginase exposure

Intracellular levels of 20 different amino acids were measured in leukemic cells of 19 children with newly diagnosed ALL and in peripheral blood cells of 9 healthy control children without bone marrow disease. The protein concentration of ALL cells was median 176 µg/ml (25^{th} - 75^{th} percentile: 146 - 226 µg/ml) (n = 9), which significantly differed from the protein content of mononuclear peripheral blood cells of normal controls (447 µg/ml; 25^{th} - 75^{th} percentile: 347 - 568 µg/ml) (P < 0.0001). The amino acid levels were therefore expressed as µmol per mg of cellular protein.

The intracellular levels for 16 out of 20 amino acids did not differ between the ALL cells at diagnosis and normal controls (Table 3.3). Aspartic acid (P < 0.05), glutamic acid (P < 0.05) and cystathionine (P < 0.01) were 2-fold higher in ALL compared to normal controls, whereas taurine levels were 5.4-fold lower in leukemic cells (P < 0.001). PEG-Asparaginase did not affect the intracellular amino acid levels in time (Table 3.3), as is shown also in Figure 3.5A for asparagine, aspartic acid, glutamine and glutamic acid.

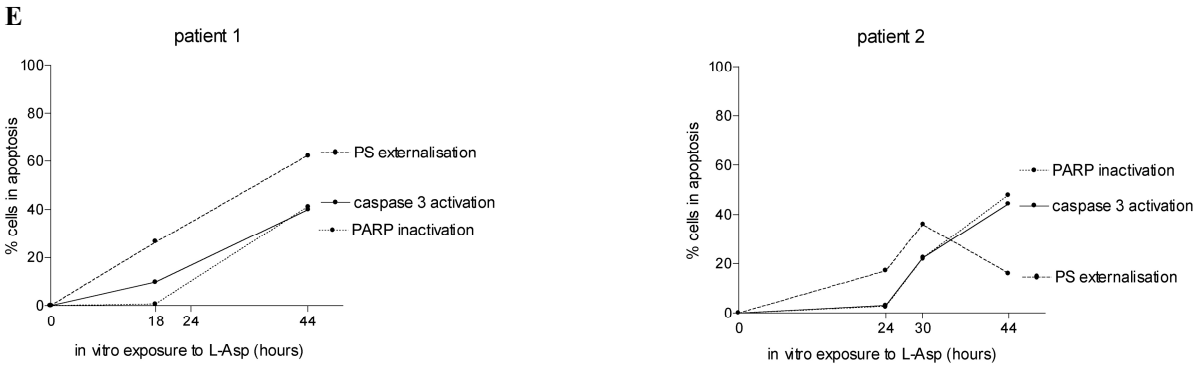


Figure 3.3E In vitro induced apoptosis *after exposure to 10 IU/ml L-Asparaginase over time for 2 patients.*

A

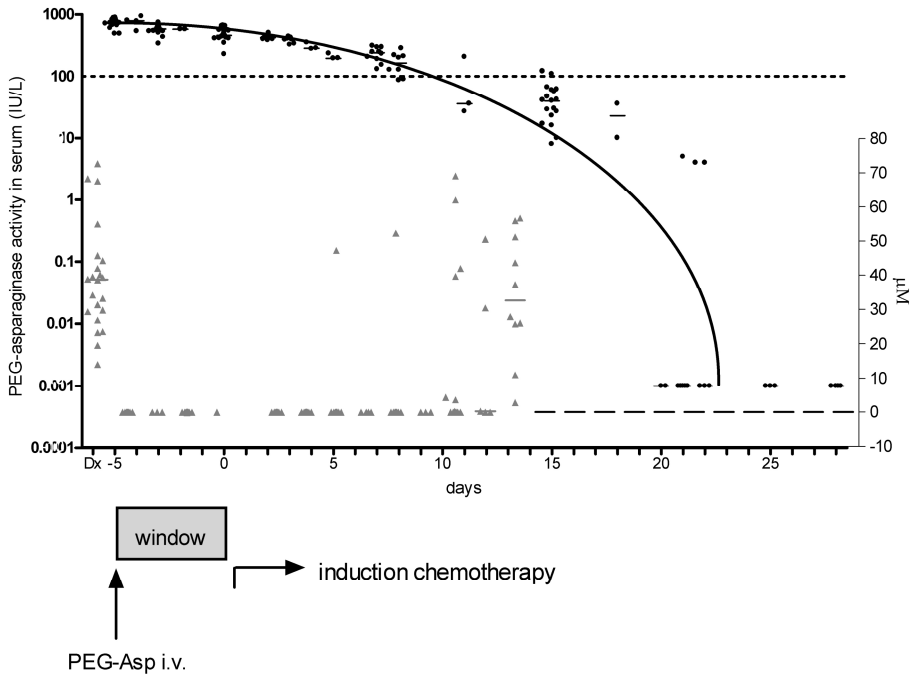


Figure 3.4A PEG-Asparaginase activity and asparagine levels
The effect of 1000 IU/m^2 PEG-Asparaginase administered i.v. 5 days upfront regular chemotherapy on serum asparagine levels in time. The levels of PEG-Asparaginase (left Y-axis in IU/L) are given in black, the levels of asparagine (right Y-axis in μM) in gray; the lower dotted line is the detection limit of asparagine, the upper dotted line is the reference L-Asparaginase activity level of 100 IU/L.

The intracellular amino acid levels at diagnosis and the *in vivo* response to PEG-Asparaginase did not correlate (Figure 3.6, Table 3.4). Results were borderline significant for leucine, but not significant if adjusted for multiple testing. One remarkable difference demonstrated valine, which was 2 times higher in non-responding patients.

Since amino acid levels did not change in time, the relation between clinical response and amino acid levels in time was not further evaluated.

In correspondence with the *in vivo* data, the levels of intracellular amino acids did not significantly differ between *in vitro* L-Asparaginase resistant and sensitive patients (Table 3.4).

In vitro L-Asparaginase exposure

In contrast to the lack of *in vivo* intracellular depletion of asparagine and glutamine (Figure 3.5A), significant intracellular depletion was detected after *in vitro* exposure to L-Asparaginase. Leukemic blasts of 6 patients were *in vitro* exposed to 0.1 IU/ml L-Asparaginase, 10 IU/ml L-Asparaginase and to culture medium only as control, and analyzed before exposure and during 72 hours after exposure. Significant intracellular asparagine depletion was observed with 0.1 IU/ml (data not shown) and 10 IU/ml of L-Asparaginase at all time points tested ($P < 0.05$) (Figure 3.5B). Only after exposure to 10 IU/ml of L-Asparaginase, intracellular glutamine levels declined significantly within 3 hours ($P < 0.001$), whereas no significant rise in glutamic acid could be observed (Figure 3.5B).

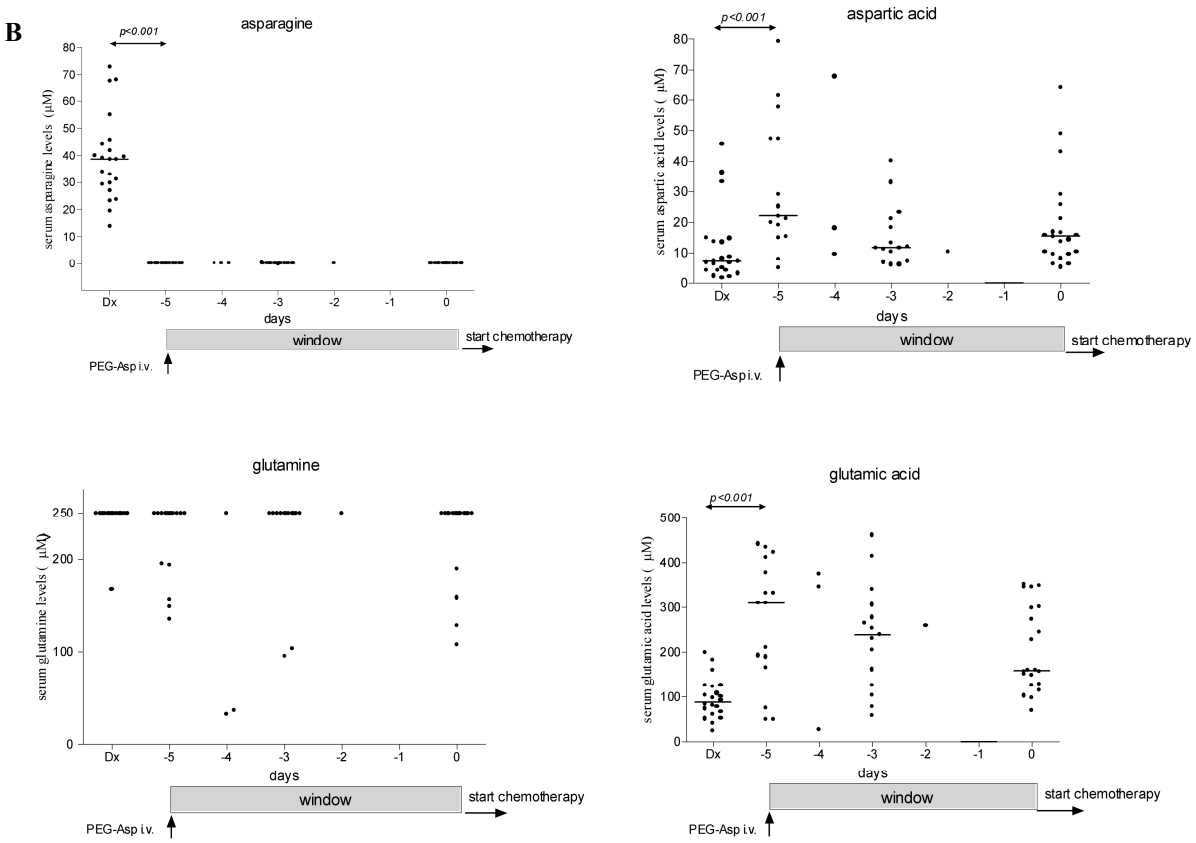


Figure 3.4B Effect of PEG-Asparaginase on serum amino acid levels
Asparagine, aspartic acid, glutamine and glutamic acid were measured in 24 children with newly diagnosed ALL before and after in vivo administration of one dose of 1000 IU/m^2 PEG-Asparaginase. Significant changes between the levels at diagnosis and 1 hour after the administration of PEG-Asparaginase are indicated by P < 0.001.

Clinical toxicity

No clinical toxicity related to PEG-Asparaginase was seen during the window phase. At day 29 of the induction phase in which patients received twice-weekly Paronal®, one patient out of 57 patients experienced a period of diabetes mellitus. Another child was diagnosed with transient hyperlipidemia. No clinical signs of pancreatitis, severe neurotoxicity or thrombotic events were observed.

Allergic reactions

Four infusions with Paronal® were planned during induction on day 29, 33, 36 and 40, and only the 30 high-risk patients were scheduled to receive 9 additional Paronal® infusions during intensification after 3 months.

No child demonstrated an allergic reaction to the first Paronal® infusion on day 28. One out of 57 children had a grade 3 allergic reaction on the second infusion. Treatment was switched to Oncaspar® and could not completely be finished. So 56/57 patients received the total of four Paronal® gifts during induction.

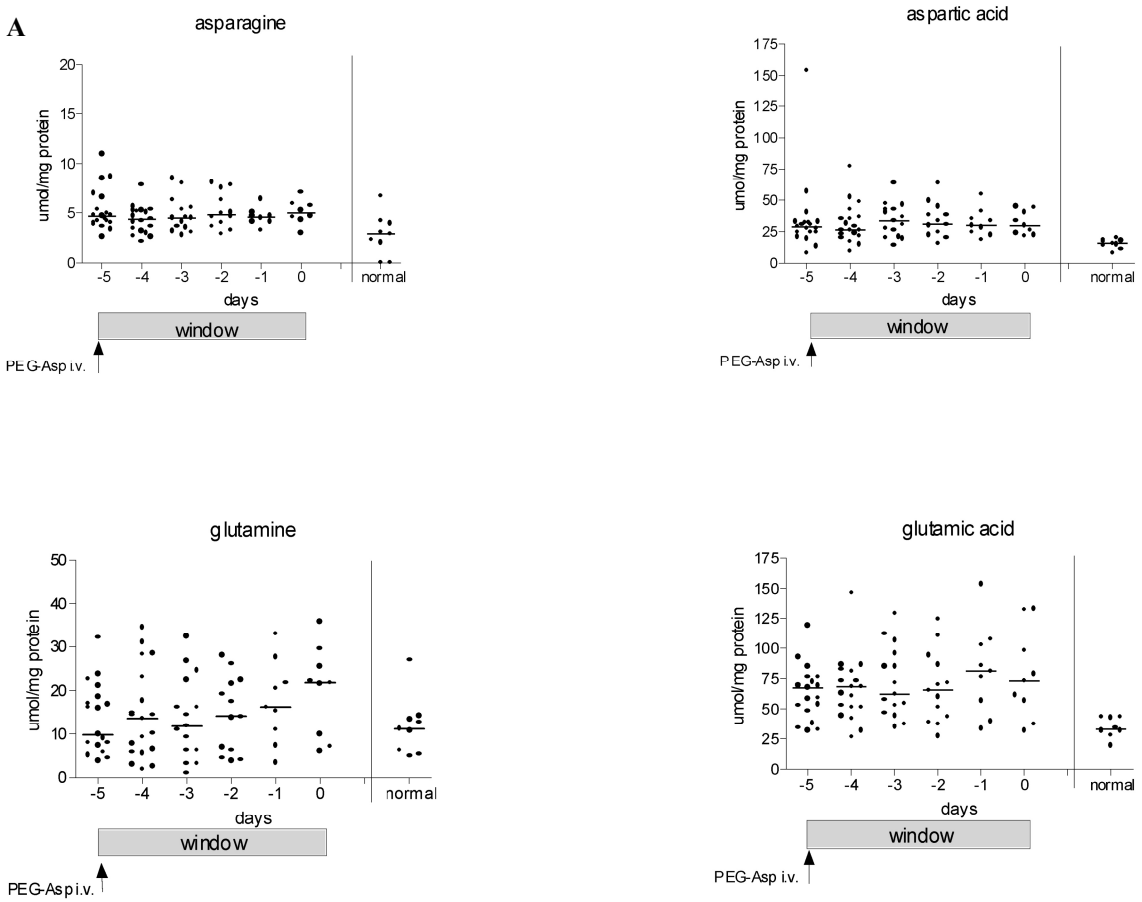


Figure 3.5A 4 intracellular amino acid levels in time in vivo
In vivo effect of one dose of 1000 IU/m^2 PEG-Asparaginase on serum amino acid levels of asparagine, aspartic acid, glutamine and glutamic acid measured in leukemic cells of 19 children with ALL compared to the levels found in peripheral blood cells of 9 healthy control cases. Data are given as μmol/mg of intracellular protein.

One of 30 high-risk patients relapsed within two months after diagnosis and changed protocol before the intensification phase. 15 of the remaining 29 (52%) children demonstrated an allergic reaction. 9 of these 15 could complete the intensification therapy by switching to Erwinase® or to Oncaspar®. The allergy rate of 52% was not higher than the 81% allergy rate in a group of 16 high-risk patients treated with the same ALL-9-HR protocol but without the upfront PEG-Asp window.

Changes in hemostasis (Table 3.5)
At diagnosis data point to enhanced thrombin generation. Consumption coagulopathy was monitored in 17 patients. On day 0, five days after the administration of 1000 IU/m^2 PEG-Asparaginase many coagulation proteins demonstrated a significant decrease. The decrease in coagulation factors on day 0 was not related to the clinical response to PEG- Asparaginase. The changes in coagulation parameters before Paronal® administration at day 29 and at the end of induction on day 43 (after 4 doses of Paronal®), demonstrated the same pattern as for patients who did not receive PEG-Asparaginase window treatment, as published recently.[34]

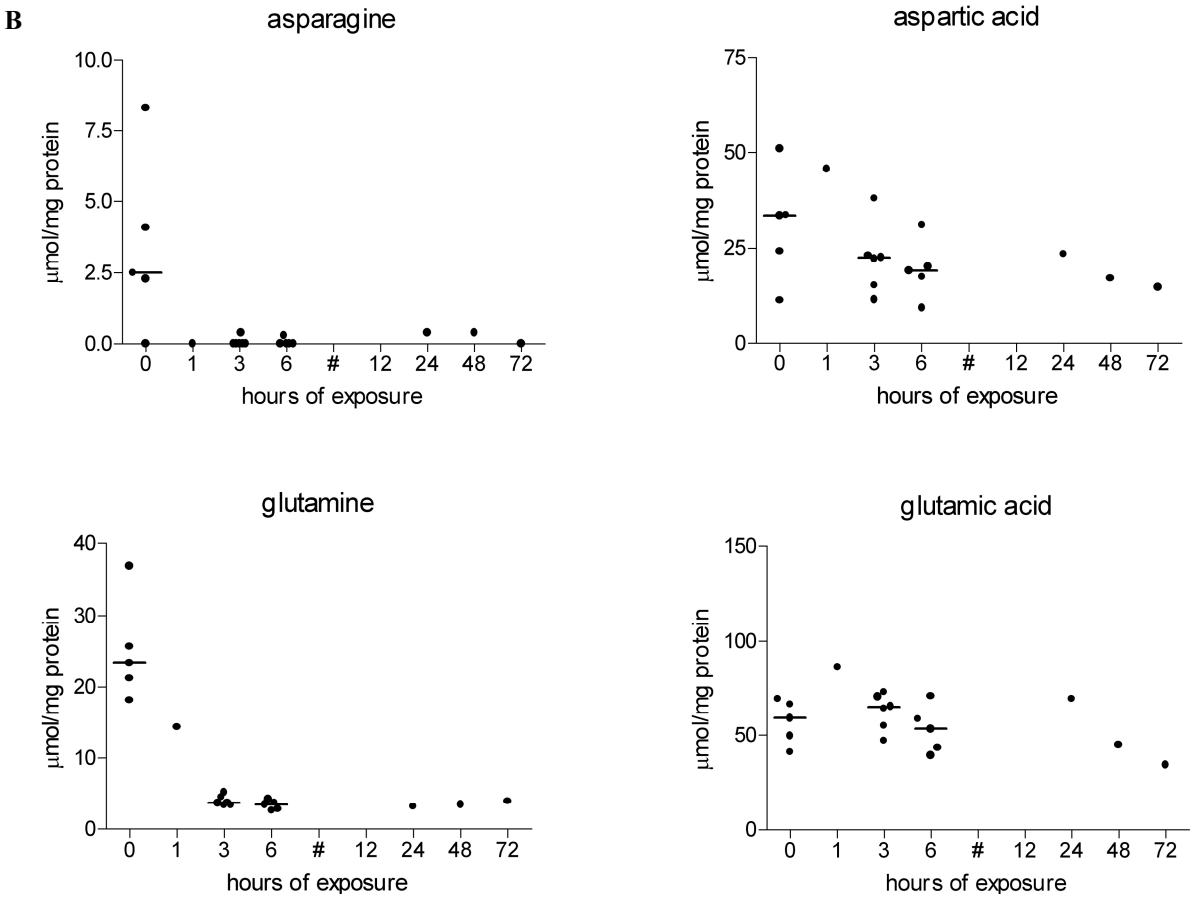


Figure 3.5B 4 intracellular amino acid levels in time in vitro
The effect of in vitro exposure to L-Asparaginase on intracellular amino acid levels of asparagine, aspartic acid, glutamine and glutamic acid of leukemic cells of 6 patients that were in vitro exposed to 10 IU/ml L-Asparaginase. Asparagine levels (P < 0.05) and glutamine levels (P < 0.001) decreased significantly before and after incubation with L-Asparaginase.

Outcome and prognostic factors

Survival analysis of *in vivo* response to PEG-Asparaginase showed that clinically good window responders had a more favourable outcome than clinically poor window responders (3-year EFS ± SE: 91 ± 6% for good, 81 ± 10% for intermediate and 33 ± 19% for poor responders (median follow-up 4.1 years, Figure 3.7A). The Hazard ratio for the good and intermediate responders compared to the poor responders was 6.4 (95% CI 1.81 - 22.86) (Table 3.6). The prognosis of good and intermediate responders was significantly higher than for the poor responder group (*P* = 0.004, univariate analysis). The *P*-value of the trend analysis for all three groups was 0.014.

Patients who were i*n vitro* sensitive or intermediate sensitive to L-Asparaginase had a 3-year EFS of 93 ± 7% and 100%, which is significantly different from the EFS of 53 ± 13% for the *in vitro* resistant patients (Figure 3.7B). The Hazard ratio for the good and intermediate sensitive patients compared to the resistant patients was 3.7 (95% CI 1.3 - 10.7); (*P* = 0.014) (Table 3.6). The *P*-value of the trend analysis for all three groups was 0.16.

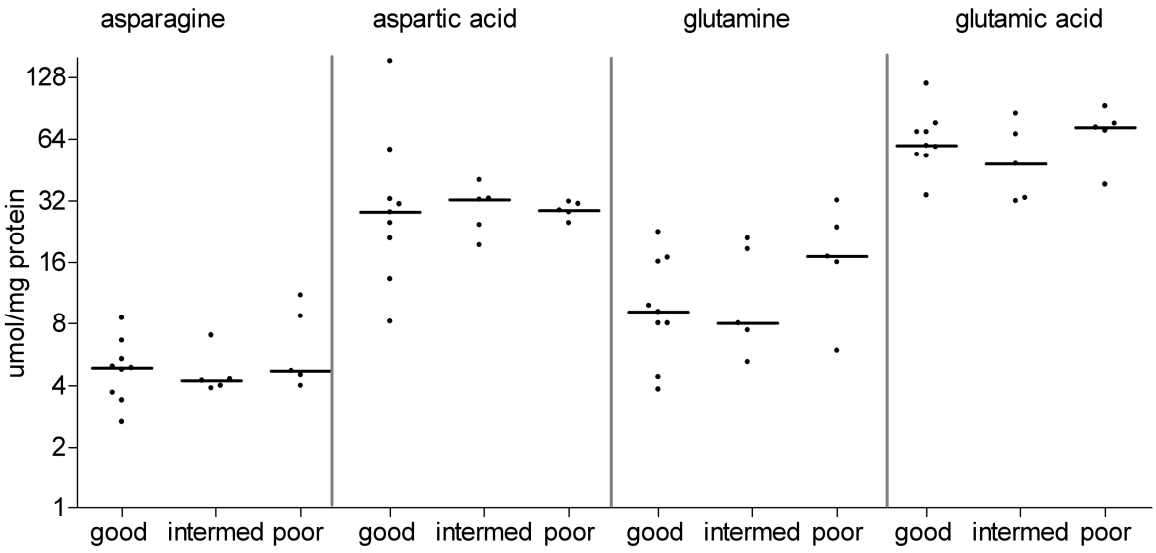


Figure 3.6 4 intracellular amino acids related to clinical response
Intracellular amino acid levels (in μmol/mg of intracellular protein) of asparagine, aspartic acid, glutamine and glutamic acid at diagnosis of 19 children with ALL, related to their clinical response to one dose of 1000 IU/m^2 PEG-Asparaginase 5 days later, as defined in Figure 3.1.

Of the other variables including age, sex, WBC, immunophenotype and genotype, only WBC (> or < 50 x 10^9 /L) at diagnosis was significantly related to outcome in the Cox univariate analysis (P = 0.02) (Table 3.6).

In a multivariate analysis, including clinical response, *in vitro* L-Asparaginase resistance, age, white blood cell count and immunophenotype, only the clinical response was an independent risk factor (P = 0.042) (χ2 trend 6.602, P = 0.0013).

DISCUSSION

In the present study we investigated the effect of a therapeutic window with one single dose of PEG-Asparaginase before start of combination chemotherapy on several pharmacodynamic and pharmacokinetic parameters, and on clinical response in pediatric ALL.

Clinical response

Monotherapy with 1000 IU/m^2 caused a gradual decrease of the leukemic cell burden in most patients within 5 days of treatment (Figure 3.1). Children with unfavorable characteristics like T-ALL or *BCR-ABL* / t(9;22)-positive ALL were *in vivo* more resistant to PEG-Asparaginase than children with a more favorable immunophenotype and genotype. This is in concordance with the fact that T-ALL cells were found to be *in vitro* more resistant to L-Asparaginase.[33] For *BCR-ABL* / t(9;22) the *in vivo* resistance to L-Asparaginase is a new finding. In the present study we also showed that *in vitro* resistance to L-Asparaginase is related to the clinical response to PEG-Asparaginase as single drug.

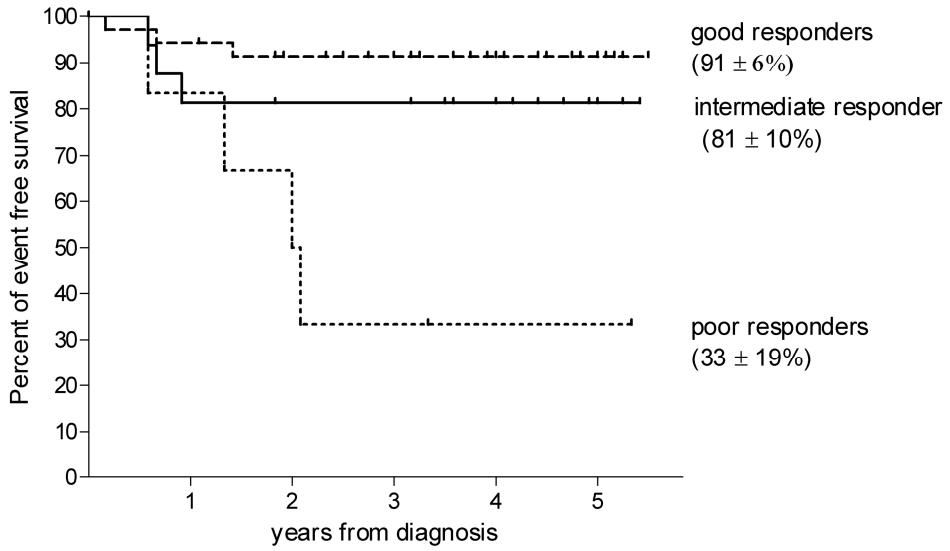


Figure 3.7A EFS and clinical response
Relationship between event free survival (EFS) and clinical response to the PEG-Asparaginase window. The prognosis differed between good and intermediate responders compared to poor responders (P < 0.01).

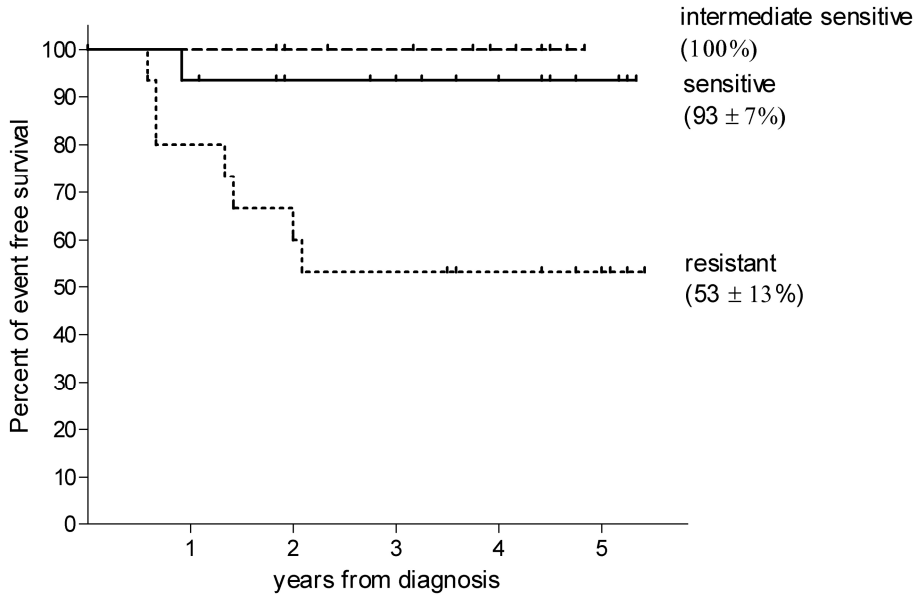


Figure 3.7B EFS and in vitro sensitivity
Relationship between event free survival (EFS) and in vitro sensitivity to L-Asparaginase. The prognosis differed between the sensitive and intermediate sensitive group compared to the group resistant to L-Asparaginase (P < 0.01).

Parameters of apoptosis

Apoptotic markers at diagnosis did not predict the clinical response. We were not able to demonstrate a change in the percentage of *in vivo* apoptotic cells after administration of PEG-Asparaginase. If however, ALL cells were *in vitro* exposed to L-Asparaginase, a significant increase in time of the apoptotic markers was found, confirming previous findings.[19] Together with the fact that there was a rapid decrease in leukemic cells upon *in vivo* PEG-Asparaginase exposure, these findings

indicate that apparently no apoptotic cells remain in the circulation *in vivo*. Most likely, these apoptotic cells are rapidly being removed by phagocytosis.[35]

L-Asparaginase activity in serum and intracellular amino acids

One dose of 1000 IU/m^2 PEG-Asparaginase immediately resulted in complete serum asparagine depletion with a concomitant rise in serum aspartic acid and glutamic acid. Our data are in correspondence with previous data observed in a larger group of patients treated with L-Asparaginase and confirm that PEG-Asparaginase will yield its pharmacodynamic effects for 2 - 4 weeks.[36] However, *in vivo* no changes were observed in the levels of intracellular amino acids (including asparagine, aspartic acid, glutamine and glutamic acid) directly after PEG-Asparaginase administration nor on successive days thereafter, except for valine. So far, no explanation can be given for the high level of valine in the non-responding group. In contrast, *in vitro* exposure to L-Asparaginase of ALL cells significantly changed the intracellular asparagine and glutamine levels that reflected the changes observed in serum suggesting that the surviving leukemic cells are capable of keeping the intracellular amino acid pools in balance, whereas those cells that are not capable of doing so die and are immediately removed from the blood circulation. Therefore the *in vivo* process of intracellular amino acid depletion in leukemic cells can not be measured which is in correspondence with the finding that no *in vivo* effect on apoptosis parameters could be measured. These data underline the fact that only *in vitro* studies have been published that show a relationship between amino acid depletion and apoptosis,[37,38] whereas literature on *in vivo* measurements is lacking.

Iwamoto *et al*.[39] recently described the protective effect of mesenchymal cells in L-Asparaginase cytotoxicity. They postulated that the interaction between ALL cells and the microenvironment in which these cells reside protected the leukemic cells from asparagine depletion by a high expression of asparagine synthetase in these mesenchymal cells. Surviving leukemic cells may therefore be rescued with asparagine produced by mesenchymal cells. There was no correlation between the upregulation of asparagine synthetase mRNA and the amount of asparagine in the leukemic cells (data not shown). This might support the hypothesis that asparagine and perhaps other amino acids are supplied to leukemic cells by the microenvironment.

Another possible explanation for the discrepancy of cellular asparagine levels *in vivo* and *in vitro* comes from the Italian group of Bussolati.[40,41] *In vitro* L-Asparaginase rapidly hydrolyzes asparagine and subsequently glutamine if asparagine is depleted.[42] Since the main amino group for *in vivo* asparagine synthesis is provided by glutamine, depletion of glutamine impacts endogenous asparagine synthesis. However, glutamine is abundantly present *in vivo,* and hence a donor for the amino group needed for asparagine synthesis is not lacking. This theory might also explain our finding that asparagine remains detectable in leukemic blasts after *in vivo* exposure to PEG-Asparaginase. Not only Boos *et al.*[42] but also Fine *et al.*[43] emphasized the fact that leukemic cell lines and primary

samples from leukemic patients are different from each other and cell line data cannot be extrapolated to primary patients' samples that easily.

At diagnosis the intracellular levels of glutamic acid and cystathionine were higher and taurine levels were lower in leukemic cells compared to normal peripheral blood mononuclear cells. Proliferating leukemic cells may produce more glutamic acid due to a fast turnover of glutamine to provide nitrogen and carbon needed for the synthesis of purines and pyrimidines energy metabolism.[44] It is known that malignant cells are more dependent on an exogenous source of asparagine and glutamine than normal cells.[45]

The high levels of cystathionine and low levels of taurine may be linked to each other. Leukemic cells were shown to have consistently lower taurine levels compared to normal lymphocytes and granulocytes.[46] A low activity of γ-cystathionase in leukemic cells might result in an abnormal methionine-cysteine pathway leading to higher levels of its starting product cystathionine and lower levels of the end product taurine.

Chakrabarti *et al*.[47] also tried to explain differences in L-Asparaginase resistance by differences in intracellular amino acid metabolism. They pointed to a hypothesis of Ryan and Keefer, that depletion of asparagine might result in decreased glycine and serine concentrations in L-Asparaginase sensitive but not in resistant tumor cells.[48,49] Asparagine is required for the transamination of glyoxylate resulting in glycine and serine depletion, which could have severe consequences for purine biosynthesis.[47] However, we did not find evidence to support these hypotheses since we found no difference in the serine or glycine content between *in vivo* good and poor responders to L-Asparaginase nor between *in vitro* L-Asparaginase sensitive and resistant leukemic cells.

It has also been suggested that L-Asparaginase treatment caused reduced incorporation of valine into proteins in L-Asparaginase sensitive cells but not in L-Asparaginase resistant tumor cells.[50] In our group valine levels before L-Asparaginase treatment were 2 times lower in *in vivo* good/intermediate responders compared to poor responders. However, no difference in the level of valine between *in vitro* L-Asparaginase resistant and sensitive ALL cells were observed in the present study.

Clinical toxicity, allergic reactions and changes in hemostasis

There was no acute toxicity of one dose of PEG-Asparaginase in this investigational window. One extra dose of PEG-Asparaginase did not lead to higher incidence of allergic reactions during induction treatment and also not during intensification therapy 3 months later. This is of importance since Silverman *et al*. proved that L-Asparaginase tolerance is an important prognostic factor in ALL.[2]

The single dose of PEG-Asparaginase in this study induced a fall in more than half of the coagulation proteins. We demonstrated that the serum asparagine depletion due to one PEG-Asparaginase infusion of 1000 IU/m^2 lasted about 4 weeks. This did not result in a persistent inhibition of coagulation protein synthesis. At day 29, so almost 5 weeks after PEG-Asparaginase administration, all coagulation parameters had recovered to normal or even increased levels. The

levels of all coagulation parameters were in the same range as investigated recently in children treated with the same protocol without the PEG-Asparaginase window.[34] Disturbances in hemostasis due to amino acid depletion are probably a local effect, i.e. in the liver only.[51,52]

Outcome and prognostic factors

Both the clinical response to PEG-Asparaginase window therapy and the *in vitro* sensitivity to L-Asparaginase are predictive for outcome. The prognostic relevance of the *in vitro* sensitivity has been shown previously.[5-8] In the present study we also show that the *in vivo* response to a window with L-Asparaginase predicts outcome, which is in concordance with the data of Asselin *et al*.[9] These results suggest that children with ALL with a poor clinical response to PEG-Asparaginase might benefit from a more intensive antileukemic therapy.

CONCLUSION

The clinical response to one dose of 1000 IU/m^2 PEG-Asparaginase intravenously as an investigational window is an independent prognostic marker related to outcome. Children with the favorable common/pre B ALL especially those with *TEL-AML1* positivity and hyperdiploidy show a good clinical response to PEG-Asparaginase and children with the prognostic unfavorable factors T-cell immunophenotype or *BCR-ABL* positivity have a relatively poor response to PEG-Asparaginase. The *in vivo* response to PEG-Asparaginase correlates well with *in vitro* sensitivity to L-Asparaginase. Intracellular changes in apoptotic features and amino acids in ALL cells can not be monitored *in vivo*; this may be explained by the fact that apoptotic cells are immediately removed from the circulation by phagocytosis. Otherwise it is possible that *in vivo* mesenchymal cells from the bone marrow supply leukemic blasts with asparagine in response to treatment with L-Asparaginase. Intracellular amino acids concentrations at diagnosis are not related to PEG-Asparaginase response. One additional dose of PEG-Asparaginase proved to be safe in children with ALL because it did not cause a higher incidence of allergic reactions to L-Asparaginase further on in treatment and it caused no severe toxicities.

Table 3.3 The effect of one single dose of PEG-Asp on intracellular amino acid levels in newly diagnosed ALL

	ALL patients						Normal controls
WINDOW days	-5 (before Asparaginase)	-4	-3	-2	-1	0	
Medians in µmol/mg (p25 - p75)							
Essential amino acids							
Isoleucine	5.6 (4.0 - 7.7)	6.0 (3.7 - 8.6)	6.1 (3.6 - 6.8)	6.3 (4.6 - 6.9)	6.2 (4.1 - 7.3)	7.0 (3.2 - 7.6)	5.0 (2.2 - 5.5)
Leucine	4.8 (3.0 - 7.9)	3.4 (2.7 - 6.5)	4.5 (3.1 - 5.7)	4.2 (3.5 - 7.3)	5.5 (4.0 - 6.2)	4.6 (2.7 - 7.0)	8.4 (4.4 - 8.8)
Lysine	4.9 (3.7 - 7.3)	4.6 (3.0 - 5.2)	5.0 (3.7 - 5.5)	3.6 (3.2 - 6.0)	5.3 (3.2 - 7.1)	4.5 (3.8 - 5.9)	7.7 (4.2 - 12.0)
Phenylalanine	3.3 (3.0 - 4.7)	3.6 (2.2 - 4.5)	3.8 (3.0 - 4.4)	4.7 (3.2 - 5.8)	3.9 (2.9 - 5.1)	4.6 (2.3 - 4.8)	3.1 (2.0 - 3.7)
Methionine	3.3 (2.9 - 4.5)	2.8 (2.4 - 3.1)	3.4 (3.1 - 4.9)	3.5 (2.9 - 4.2)	3.6 (2.7 - 4.6)	3.8 (2.3 - 4.0)	2.8 (2.3 - 3.1)
Threonine	5.5 (3.8 - 7.4)	5.5 (4.4 - 7.6)	5.6 (3.6 - 7.7)	6.3 (4.8 - 7.9)	6.9 (5.4 - 8.1)	6.1 (3.9 - 8.8)	7.1 (4.8 - 7.9)
Tyrosine	3.1 (2.6 - 4.7)	4.0 (3.2 - 5.9)	3.7 (3.1 - 4.1)	3.5 (2.8 - 4.1)	3.8 (3.2 - 4.6)	3.3 (2.5 - 4.0)	3.0 (2.1 - 3.6)
Valine	4.1 (3.2 - 5.9)	3.2 (2.9 - 4.3)	3.5 (3.0 - 4.1)	4.1 (3.0 - 5.5)	3.5 (3.0 - 4.4)	3.9 (2.7 - 4.4)	5.3 (2.8 - 6.7)
Histidine	3.0 (2.3 - 3.3)	2.7 (2.2 - 3.5)	3.1 (2.7 - 3.4)	3.2 (2.9 - 3.6)	2.9	3.1 (2.3 - 3.2)	2.4 (1.4 - 2.7)
Arginine	9.6 (5.9 - 12.0)	8.1 (5.6 - 9.9)	7.9 (5.7 - 9.9)	7.2 (5.2 - 11.0)	8.7 (7.3 - 9.6)	8.2 (5.8 - 11.0)	6.2 (4.1 - 8.1)
Non-essential amino acids							
Alanine	8.8 (5.8 - 11.7)	8.9 (5.9 - 24.3)	9.2 (5.8 - 12.5)	9.2 (7.4 - 13.4)	9.2 (5.9 - 12.2)	11.3 (6.3 - 12.7)	12.9 (8.1 - 18.0)
Asparagine	4.7 (4.0 - 6.6)	4.4 (3.1 - 5.3)	4.5 (3.5 - 5.6)	4.9 (3.8 - 6.9)	4.6 (4.1 - 4.9)	5.0 (4.5 - 5.8)	2.9 (1.0 - 4.1)
Aspartic acid	**28.8** (24.5 - 32.7)	**26.6** (22.0 - 36.5)	**33.4** (21.1 - 43.2)	**31.0** (23.5 - 41.5)	**29.9** (23.3 - 38.3)	**29.7** (23.1 - 42.4)	**15.7**[1] (12.6 - 18.2)
Glutamic acid	**67.7** (48.5 - 76.2)	**68.5** (51.2 - 81.2)	**62.0** (47.1 - 95.7)	**65.9** (41.2 - 90.6)	**81.1** (48.3 - 105)	**73.4** (47.2 - 116)	**33.1**[1] (29.8 - 43.4)
Glutamine	9.8 (47.4 - 18.6)	13.6 (5.7 - 23.1)	11.8 (6.3 - 22.5)	14.1 (5.5 - 22.1)	16.2 (9.2 - 24.7)	21.8 (8.5 - 27.7)	11.1 (5.8 - 13.8)
Cysthationine	**17.4** (13.8 - 22.6)	**21.0** (14.3 - 26.1)	**23.2** (17.7 - 30.7)	**24.2** (16.7 - 37.6)	**29.4** (19.7 - 42.6)	**28.2** (12.4 - 41.3)	**8.3**[2] (7.0 - 10.9)
Glycine	16.0 (11.4 - 20.7)	18.9 (14.1 - 24.4)	22.6 (16.0 - 29.6)	13.8 (12.5 - 21.1)	22.7 (17.0 - 35.7)	21.2 (16.2 - 31.9)	23.7 (16.8 - 25.4)
Proline	9.0 (6.8 - 16.2)	9.7 (6.8 - 14.1)	11.3 (6.9 - 14.0)	8.5 (6.1 - 14.1)	13.2 (9.0 - 15.6)	13.8 (9.2 - 15.6)	5.0 (1.7 - 7.1)
Serine	9.2 (6.0 - 11.0)	9.2 (4.9 - 11.6)	7.5 (4.3 - 11.6)	6.5 (5.8 - 9.9)	11.1 (9.7 - 13.2)	9.5 (9.1 - 13.2)	10.3 (6.9 - 12.5)
Taurine	**18.8** (10.2 - 47.3)	**22.2** (17.6 - 65.1)	**31.2** (16.0 - 71.8)	**32.9** (23.0 - 54.0)	**32.8** (18.7 - 49.1)	**27.1** (16.5 - 52.3)	**134**[3] (111 - 196)

Levels of 20 intracellular amino acids in µmol/mg cellular protein in leukemic blasts of 19 patients with ALL after one dose of PEG-Asparaginase, compared to the levels in peripheral blood cells of 9 normal controls. Data are given as medians with interquartile ranges. The amino acids that demonstrate significant different levels compared to normal are given in bold. Superscripts refer to P values: 1 = P ≤ 0.05, 2 = P ≤ 0.01 and 3 = P ≤ 0.001 for the comparison of patient data with normal controls.

Table 3.4 Intracellular amino acid levels at diagnosis related to in vitro and in vivo sensitivity

	In vitro **ASP resistant**	***In vitro*** **ASP sensitive**	***In vivo*** **poor responders**	***In vivo*** **intermediate/ good responders**
Medians in µmol/mg (p25 - p75)				
Essential amino acids				
Isoleucine	5.4 (3.6 - 7.3)	7.7 (6.3 - 8.6)	7.3 (5.0 - 11.5)	5.4 (4 - 7.1)
Leucine	4.9 (4.1 - 6.9)	4.5 (1.9 - 6.3)	8.7 (6.6 - 10.4)	4.1 (2.7 - 5.7)
Lysine	4.9 (3.4 - 7.3)	5.2 (3.8 - 6.1)	7.3 (5.8 - 8.9)	4.5 (3.2 - 6.1)
Phenylalanine	2.4 (1.7 - 4.7)	2.8 (2.0 - 3.4)	4.3 (3.9 - 4.8)	3.1 (2.7 - 4.2)
Methionine	1.5 (0.6 - 4.0)	1.8 (0.8 - 2.9)	4.1	3.0 (2.6 - 4.5)
Threonine	5.5 (4.3 - 7.4)	6.0 (4.8 - 8.3)	7.6 (2.3 - 9.4)	5.2 (3.3 - 6.4)
Tyrosine	2.6 (2.1 - 3.8)	0.9	4.9	3.0 (2.5 - 3.8)
Valine	3.3 (2.9 - 4.6)	4.4 (1.5 - 4.5)	6.45 (5.2 - 7.3)	3.5 (3.0 - 4.7)
Histidine	1.7 (1.0 - 3.0)	1.7 (1.2 - 3.1)	3.25	3.0 (2.3 - 3.3)
Arginine	10.9 (8.8 - 13.6)	10.4 (8.2 - 13.8)	13.1 (7.4 - 18.4)	9.3 (5.9 - 10.9)
Non-essential amino acids				
Alanine	9.6 (6.4 - 11.5	9.9 (7.0 - 14.5)	13.1 (7.8 - 15.6)	7.3 (5.8 - 11.3)
Asparagine	4.5 (4.1 - 7.5)	5.2 (4.1 - 8.7)	4.7 (4.3 - 9.9)	4.5 (3.8 - 6.0)
Aspartic acid	30.1 (28.4 - 33.0)	31.4 (23.4 - 43.9)	28.8 (26.6 - 31.4)	29.6 (20.2 - 36.7)
Glutamic acid	69.3 (53.2 - 81.1)	72.3 (59.3 - 95.1)	72.9 (54.5 - 84.4)	59.1 (41.3 - 73.0)
Glutamine	17.0 (7.5 - 25.0)	19.4 (8.6 - 28.0)	17.1 (11.0 - 28.0)	8.6 (6.3 - 17.8)
Cystathionine	25.0 (12.8 - 29.1)	25.0 (18.6 - 32.6)	24.3 (11.5 - 54.8)	16.3 (13.8 - 20.9)
Glycine	21.3 (12.3 - 27.8)	20.3 (16.1 - 26.1)	17.8 (7.5 - 21.4)	15.0 (10.3 - 22.1)
Proline	7.0 (5.3 - 14.0)	6.4 (5.3 - 10.8)	15.6 (7.5 - 22.5)	8.6 (6.6 - 11.8)
Serine	9.2 (6.0 - 12.6)	9.4 (6.8 - 14.9)	12.9 (9.5 - 16.1)	8.7 (5.5 - 10.7)
Taurine	25.0 (11.3 - 42.4)	17.5 (10.7 - 42.4)	20.5 (11.4 - 40.3)	18.8 (10.2 - 54.1)

Relationship between intracellular levels at diagnosis of 20 amino acids and in vitro sensitivity to L-Asparaginase or in vivo response to one dose of PEG-Asparaginase. Data are given as medians with interquartile ranges.

Table 3.5 Coagulation parameters of 57 patients before the window with PEG-Asparaginase at day -5, before starting ALL-9 induction therapy (day 0), and during 4 Paronal® infusions

	Day -5	Day 0	Day 29	Day 33	Day 36	Day 40	Day 43
Asparaginase	**PEG-ASP**		**Paronal®**	**Paronal®**	**Paronal®**	**Paronal®**	
Dose	1000 IU/m^2		6000 IU/m^2	6000 IU/m^2	6000 IU/m^2	6000 IU/m^2	
Screening							
APTT	32 (27 - 37)						
30 - 42 sec							
PT	13.9 (12.4 - 15.6)						
11.7 - 16 sec							
Thrombin time	27 (25.0 - 30.5)						
22 - 26 sec							
Procoagulants							
Fibrinogen	2.7 (2.1 - 3.9)	1.7^3 (1.3 - 1.9)	1.7 (1.4 - 2.2)	1.5 (1.1 - 1.7)	1.3 (1.1 - 1.6)	1.5 (1.2 - 1.8)	1.9 (1.3 - 2.9)
1.6 - 4.3 g/L							
F V	0.87 (0.64 - 1.19)	0.58 (0.46 - 0.76)	1.49^3 (1.20 - 1.84)	1.76 (1.23 - 1.94)	1.48 (1.26 - 1.76)	1.70 (1.07 - 2.06)	1.47 (1.04 - 1.93)
F II	0.69 (0.43 - 0.91)	0.62 (0.49 - 0.74)	1.19^3 (1.04 - 1.45)	1.07 (0.92 - 1.40)	1.10 (0.92 - 1.15)	1.03 (0.90 - 1.29)	1.06 (0.83 - 1.22)
F VII	0.66 (0.52 - 0.78)	0.79 (0.69 - 0.95)	1.09 (0.88 - 1.27)	1.42 (1.08 - 1.76)	1.40 (1.14 - 1.77)	1.39 (1.14 - 1.68)	1.29 (0.98 - 1.69)
F IX	1.17 (0.98 - 1.35)	0.42^3 (0.33 - 0.51)	1.86^3 (1.54 - 2.27)	0.96^3 (0.70 - 1.42)	1.02 (0.62 - 1.50)	0.76 (0.61 - 1.15)	0.99 (0.57 - 1.30)
F X	0.95 (0.70 - 1.26)	0.68^2 (0.53 - 0.75)	1.47^3 (1.16 - 1.79)	1.30 (0.98 - 1.57)	1.20 (0.95 - 1.33)	1.13 (0.84 - 1.35)	1.17 (0.74 - 1.30)
Anticoagulants							
AT	0.75 (0.66 - 0.86)	0.54^2 (0.48 - 0.65)	1.37^3 (1.30 - 1.55)	0.92^2 (0.82 - 1.02)	0.86 (0.69 - 1.08)	0.75 (0.64 - 1.00)	0.87 (0.65 - 1.03)
Prot C	0.71 (0.55 - 0.87)	0.53 (0.45 - 0.64)	2.09^3 (1.51 - 2.43)	1.25 (1.06 - 1.55)	1.38 (0.95 - 1.67	1.27 (1.00 - 1.41	1.15 (0.97 - 1.61
Prot S	0.72 (0.60 - 0.87)	0.37^3 (0.33 - 0.54)	1.03^3 (0.87 - 1.17)	0.63^3 (0.46 - 0.75)	0.57 (0.42 - 0.71)	0.59 (0.42 - 0.71)	0.60 (0.50 - 0.77)
Thrombin generation							
F1+2 pmol/L	576 (259 - 1067)	365 (211 - 588)	201^1 (130 - 290)	230 (167 - 293)	267 (178 - 344)	427 (189 - 623)	243 (161 - 362)
69 - 229 pmol/L							
TAT μg/L	13 (7.9 - 48.2)	5.2^3 (3.5 - 9.1)	5.0 (4.0 - 10.6)	4.9 (3.3 - 7.2)	4.6 (3.5 - 6.4)	5.1 (3.6 - 15.7)	5.5 (3.8 - 7.0)
1.5 - 4.1 μg/L							
Fibrinolysis							
Alpha-2-antipl	1.08 (1.02 - 1.20)	0.70^3 (0.62 - 0.86)	1.58^3 (1.42 - 1.64)	1.07^3 (0.94 - 1.13)	1.04 (0.83 - 1.19)	0.93 (0.73 - 1.19)	1.02 (0.81 - 1.47)
Plasminogen	1.01 (0.79 - 1.18)	0.56^3 (0.52 - 0.70)	1.22^3 (1.10 - 1.34)	0.76^3 (0.69 - 0.90)	0.74 (0.66 - 0.93)	0.75 (0.60 - 0.92)	0.79 (0.65 - 1.02)
PAP μg/L	641 (456 - 913)	255^3 (204 - 355)	292 (194 - 397)	139^3 (106 - 182)	157 (100 - 192)	157 (122 - 228)	181 (128 - 269)
80 - 450 μg/L							
D-dimer	0.42 (0.27 - 0.85)	0.35 (0.19 - 0.59)	0.11^3 (0.07 - 0.19)	0.09 (0.06 - 0.12)	0.11 (0.07 - 0.17)	0.10 (0.06 - 0.17)	0.12 (0.09 - 0.21)
0.1 - 0.55 mg/L							

Results of procoagulant and anticoagulant parameters, of parameters of thrombin generation and of fibrinolysis of 57 patients before the window with PEG-Asparaginase (day –5), before starting ALL-9 induction therapy (day 0), and related to 4 Paronal® infusions (days 29 - 43). Data represent percentages or as indicated otherwise. Normal reference ranges are 80 - 130%, or as indicated in italic. Data are given as medians with interquartile ranges. Superscripts refer to p values: 1 = P ≤ 0.05, 2 = P ≤ 0.01 and 3 = P ≤ 0.001 related to the previous measurement.

Table 3.6 Univariate analyses not stratified for treatment arm

	***P*-value**	**Hazard ratio**	**95% CI**	
Age[1]	0.918	0.9	0.2	4.3
Sex	0.214	0.4	0.1	1.8
WBC[2]	0.022	6.1	1.3	28.7
Immunophenotype[3]	0.344	1.9	0.5	7.0
Genotype[4]	0.115	5.3	0.7	41.7
Clinical response to PEG-Asparaginase[5]	0.004	6.4	1.8	22.9
L-Asparaginase sensitivity (in vitro)[6]	0.014	3.7	1.3	10.7

Results of univariate Cox proportional hazards regression analyses not stratified for ALL-9 treatment arm (standard risk / high risk). Only WBC, clinical response to the PEG-Asparaginase window therapy and in vitro sensitivity to L-Asparaginase are significantly related to outcome.
[1] < or > 10 years
[2] < or > 50 x 10^9/L
[3] T versus precursor B-ALL
[4] hyperdiploidy and TEL-AML1 compared to the others
[5] good and intermediate clinical responders compared to poor responders to PEG-Asp
[6] in vitro sensitive and intermediate sensitive compared to resistant to L-Asparaginase

REFERENCES

1. Capizzi RL, Bertino JR, Skeel RT, et al. L-asparaginase: clinical, biochemical, pharmacological, and immunological studies. Ann Intern Med. 1971;74:893-901.
2. Silverman LB, Gelber RD, Dalton VK, et al. Improved outcome for children with acute lymphoblastic leukemia: results of Dana-Farber Consortium Protocol 91-01. Blood. 2001;97:1211-1218.
3. Pieters R, Loonen AH, Huismans DR, et al. In vitro drug sensitivity of cells from children with leukemia using the MTT assay with improved culture conditions. Blood. 1990;76:2327-2336.
4. Yamada S, Hongo T, Okada S, Watanabe C, Fujii Y, Ohzeki T. Clinical relevance of in vitro chemoresistance in childhood acute myeloid leukemia. Leukemia. 2001;15:1892-1897.
5. Janka-Schaub GE, Harms DO, den Boer ML, Veerman AJ, Pieters R. [In vitro drug resistance as independent prognostic factor in the study COALL-O5-92 Treatment of childhood acute lymphoblastic leukemia; two-tiered classification of treatments based on accepted risk criteria and drug sensitivity profiles in study COALL-06-97]. Klin Padiatr. 1999;211:233-238.
6. Pieters R, Huismans DR, Loonen AH, et al. Relation of cellular drug resistance to long-term clinical outcome in childhood acute lymphoblastic leukaemia. Lancet. 1991;338:399-403.
7. Kaspers GJ, Veerman AJ, Pieters R, et al. In vitro cellular drug resistance and prognosis in newly diagnosed childhood acute lymphoblastic leukemia. Blood. 1997;90:2723-2729.
8. Den Boer ML, Harms DO, Pieters R, et al. Patient stratification based on prednisolone-vincristine-asparaginase resistance profiles in children with acute lymphoblastic leukemia. J Clin Oncol. 2003;21:3262-3268.
9. Asselin BL, Kreissman S, Coppola DJ, et al. Prognostic significance of early response to a single dose of asparaginase in childhood acute lymphoblastic leukemia. J Pediatr Hematol Oncol. 1999;21:6-12.
10. Ollenschlager G, Roth E, Linkesch W, Jansen S, Simmel A, Modder B. Asparaginase-induced derangements of glutamine metabolism: the pathogenetic basis for some drug-related side-effects. Eur J Clin Invest. 1988;18:512-516.
11. Broome JD. L-Asparaginase: discovery and development as a tumor-inhibitory agent. Cancer Treat Rep. 1981;65 Suppl 4:111-114.
12. Miller HK, Salser JS, Balis ME. Amino acid levels following L-asparagine amidohydrolase (EC.3.5.1.1) therapy. Cancer Res. 1969;29:183-187.
13. Muller HJ, Boos J. Use of L-asparaginase in childhood ALL. Crit Rev Oncol Hematol. 1998;28:97-113.
14. Bussolati O, Belletti S, Uggeri J, et al. Characterization of apoptotic phenomena induced by treatment with L-asparaginase in NIH3T3 cells. Exp Cell Res. 1995;220:283-291.
15. Andrulis IL, Argonza R, Cairney AE. Molecular and genetic characterization of human cell lines resistant to L-asparaginase and albizziin. Somat Cell Mol Genet. 1990;16:59-65.
16. Jousse C, Averous J, Bruhat A, Carraro V, Mordier S, Fafournoux P. Amino acids as regulators of gene expression: molecular mechanisms. Biochem Biophys Res Commun. 2004;313:447-452.
17. Appel IM, den Boer ML, Meijerink JP, Veerman AJ, Reniers NC, Pieters R. Up-regulation of asparagine synthetase expression is not linked to the clinical response L-asparaginase in pediatric acute lymphoblastic leukemia. Blood. 2006;107:4244-4249.
18. Holleman A, Cheok MH, den Boer ML, et al. Gene-expression patterns in drug-resistant acute lymphoblastic leukemia cells and response to treatment. N Engl J Med. 2004;351:533-542.
19. Holleman A, den Boer ML, Kazemier KM, Janka-Schaub GE, Pieters R. Resistance to different classes of drugs is associated with impaired apoptosis in childhood acute lymphoblastic leukemia. Blood. 2003;102:4541-4546.

20. Veerman AJ, Hahlen K, Kamps WA, et al. High cure rate with a moderately intensive treatment regimen in non-high-risk childhood acute lymphoblastic leukemia. Results of protocol ALL VI from the Dutch Childhood Leukemia Study Group. J Clin Oncol. 1996;14:911-918.

21. Yoshimoto T, Nishimura H, Saito Y, et al. Characterization of polyethylene glycol-modified L-asparaginase from Escherichia coli and its application to therapy of leukemia. Jpn J Cancer Res. 1986;77:1264-1270.

22. Slater RM, Smeets DF, Hagemeijer A, et al. Update of the cytogenetic study of childhood non-high-risk acute lymphocytic leukemia at diagnosis in protocol VI of the Dutch Childhood Leukemia Study Group. Haematol Blood Transfus. 1990;33:169-173.

23. Kaspers GJ, Veerman AJ, Pieters R, et al. Mononuclear cells contaminating acute lymphoblastic leukaemic samples tested for cellular drug resistance using the methyl-thiazol-tetrazolium assay. Br J Cancer. 1994;70:1047-1052.

24. Schrappe M, Reiter A, Zimmermann M, et al. Long-term results of four consecutive trials in childhood ALL performed by the ALL-BFM study group from 1981 to 1995. Berlin-Frankfurt-Munster. Leukemia. 2000;14:2205-2222.

25. Lanvers C, Vieira Pinheiro JP, Hempel G, Wuerthwein G, Boos J. Analytical validation of a microplate reader-based method for the therapeutic drug monitoring of L-asparaginase in human serum. Anal Biochem. 2002;309:117-126.

26. Lenda K, Svenneby G. Rapid high-performance liquid chromatographic determination of amino acids in synaptosomal extracts. J Chromatogr. 1980;198:516-519.

27. Appel IM, Pinheiro JP, den Boer ML, et al. Lack of asparagine depletion in the cerebrospinal fluid after one intravenous dose of PEG-asparaginase: a window study at initial diagnosis of childhood ALL. Leukemia. 2003;17:2254-2256.

28. Smith PK, Krohn RI, Hermanson GT, et al. Measurement of protein using bicinchoninic acid. Anal Biochem. 1985;150:76-85.

29. Appel IM, Hop WC, Pieters R. Changes in hypercoagulability by asparaginase: a randomized study between two asparaginases. Blood Coagul Fibrinolysis. 2006;17:139-146.

30. Monagle P, Barnes C, Ignjatovic V, et al. Developmental haemostasis. Impact for clinical haemostasis laboratories. Thromb Haemost. 2006;95:362-372.

31. Flanders MM, Phansalkar AR, Crist RA, Roberts WL, Rodgers GM. Pediatric reference intervals for uncommon bleeding and thrombotic disorders. J Pediatr. 2006;149:275-277.

32. Ries M, Klinge J, Rauch R. Age-related reference values for activation markers of the coagulation and fibrinolytic systems in children. Thromb Res. 1997;85:341-344.

33. Pieters R, den Boer ML, Durian M, et al. Relation between age, immunophenotype and in vitro drug resistance in 395 children with acute lymphoblastic leukemia--implications for treatment of infants. Leukemia. 1998;12:1344-1348.

34. Appel IM, Hop WCJ, van Kessel-Bakvis C, Stigter R, Pieters R. L-Asparaginase and the effect of age on coagulation and fibrinolysis in childhood acute lymphoblastic leukemia. submitted. 2007.

35. Durrieu F, Belloc F, Lacoste L, et al. Caspase activation is an early event in anthracycline-induced apoptosis and allows detection of apoptotic cells before they are ingested by phagocytes. Exp Cell Res. 1998;240:165-175.

36. Boos J, Werber G, Ahlke E, et al. Monitoring of asparaginase activity and asparagine levels in children on different asparaginase preparations. Eur J Cancer. 1996;32A:1544-1550.

37. Franek F, Fismolova I, Eckschlager T. Antiapoptotic and proapoptotic action of various amino acids and analogs in starving MOLT-4 cells. Arch Biochem Biophys. 2002;398:141-146.

38. Simpson NH, Singh RP, Perani A, Goldenzon C, Al-Rubeai M. In hybridoma cultures, deprivation of any single amino acid leads to apoptotic death, which is suppressed by the expression of the bcl-2 gene. Biotechnol Bioeng. 1998;59:90-98.

39. Iwamoto S, Mihara K, Downing JR, Pui CH, Campana D. Mesenchymal cells regulate the response of acute lymphoblastic leukemia cells to asparaginase. J Clin Invest. 2007;117:1049-1057.

40. Rotoli BM, Uggeri J, Dall'Asta V, et al. Inhibition of glutamine synthetase triggers apoptosis in asparaginase-resistant cells. Cell Physiol Biochem. 2005;15:281-292.

41. Tardito S, Uggeri J, Bozzetti C, et al. The inhibition of glutamine synthetase sensitizes human sarcoma cells to L: -asparaginase. Cancer Chemother Pharmacol. 2007.

42. Wagner A, Boos J. Unphysiological effects contributing to asparaginase toxicity in vitro. Am J Physiol. 1998;274:C1185-1186.

43. Fine BM, Kaspers GJ, Ho M, Loonen AH, Boxer LM. A genome-wide view of the in vitro response to l-asparaginase in acute lymphoblastic leukemia. Cancer Res. 2005;65:291-299.

44. Newsholme EA, Crabtree B, Ardawi MS. Glutamine metabolism in lymphocytes: its biochemical, physiological and clinical importance. Q J Exp Physiol. 1985;70:473-489.

45. Iiboshi Y, Papst PJ, Hunger SP, Terada N. L-Asparaginase inhibits the rapamycin-targeted signaling pathway. Biochem Biophys Res Commun. 1999;260:534-539.

46. Wakayama K, Besa EC, Baskin SI. Changes in intracellular taurine content of human leukemic cells. Nagoya J Med Sci. 1983;45:89-96.

47. Chakrabarti R, Schuster SM. L-asparaginase: perspectives on the mechanism of action and resistance. Int J Ped Hem/Oncol. 1997;4:597-611.

48. Ryan WL, Sornson HC. Glycine inhibition of asparaginase. Science. 1970;167:1512-1513.

49. Keefer JF, Moraga DA, Schuster SM. Comparison of glycine metabolism in mouse lymphoma cells either sensitive or resistant to L-asparaginase. Biochem Pharmacol. 1985;34:559-565.

50. Sobin LH, Kidd JG. A Metabolic Difference between Two Lines of Lymphoma 6c3hed Cells in Relation to Asparagine. Proc Soc Exp Biol Med. 1965;119:325-327.

51. Bushman JE, Palmieri D, Whinna HC, Church FC. Insight into the mechanism of asparaginase-induced depletion of antithrombin III in treatment of childhood acute lymphoblastic leukemia. Leuk Res. 2000;24:559-565.

52. Reinert RB, Oberle LM, Wek SA, et al. Role of glutamine depletion in directing tissue-specific nutrient stress responses to L-asparaginase. J Biol Chem. 2006;281:31222-31233.

4 chapter

Lack of asparagine depletion in the cerebrospinal fluid after one intravenous dose of PEG-Asparaginase: a window study at initial diagnosis of childhood ALL

Inge M. Appel
João Paulo V. Pinheiro
Monique L. den Boer
Claudia Lanvers
Nathalie C.M. Reniers
Joachim Boos
Rob Pieters

Leukemia 2003;17;2254-2256

INTRODUCTION

L-Asparaginase (L-Asp) plays a well-established role in the treatment of children with acute lymphoblastic leukemia (ALL). L-Asp is postulated to exert its antitumor activity by hydrolyzing asparagine (Asn) to aspartic acid and ammonia, thereby depleting the leukemic cells from Asn, leading to impaired protein synthesis and leukemic cell death. L-Asp may also act by depleting glutamine. Different preparations and ways of administering L-Asp result in different plasma activities. The half-life of *Erwinia*-L-Asp is shorter than that of *Escherichia coli* L-Asp, which is in turn shorter than that of the polyethylene glycol (PEG)-conjugated form of L-Asp.[1] PEG-Asp shows less immune response and a prolonged half-life of 5.7 days compared to 1.3 days for native L-Asp. These variations are related to differences in the extent of Asn depletion.[2]

Pharmacokinetics and pharmacodynamics of PEG-Asp are not well characterized in the cerebrospinal fluid (CSF). The L-Asp activity in the CSF is less than 1% of the corresponding plasma activity using native *E. coli*-L-Asp. Yet, L-Asp is believed to play a role in the prevention of meningeal leukemia probably by depleting the pool of Asn in the CSF. It is not known whether an incomplete depletion of Asn in the CSF results in a suboptimal antileukemic effect. In Rhesus monkeys and in a number of adult patients, Riccardi *et al*[3] demonstrated that, after native *E. coli* L-Asp, CSF-Asn levels were depleted to < 0.2 μM. Several groups showed that a plasma *E. coli*-L-Asp activity > 100 IU/L leads to an Asn depletion of < 0.2 μM in the plasma and CSF.[2,4] Müller *et al*[5] demonstrated that after one dose of 1000 IU/m^2 PEG-Asp intravenously (i.v.) as second-line treatment, the plasma L-Asp activity was still ≥ 100 IU/L after 14 days in 44/66 patients. Unfortunately, no CSF levels were measured in their study.

Recently, Avramis *et al*[6] showed that PEG-Asp (1 x 2500 IU/m^2) intramuscularly (i.m.) reached an L-Asp plasma activity > 100 IU/L accompanied by Asn levels < 3 μM during 3 - 14 days in 95% of the patients. CSF-Asn concentrations fell to 0.6 μM at day 28. In the present paper, we report on 24 newly diagnosed children with ALL treated in our center with a single dose of PEG-Asp (Oncaspar™) 1000 IU/m^2 i.v., 5 days before starting induction chemotherapy according to the ALL-9 study of the Dutch Childhood Leukemia Study Group (DCLSG).

MATERIAL AND METHODS

The bone marrow, blood and CSF were obtained at diagnosis. From day -5 till day 0, peripheral blood samples were collected daily, and later on twice a week. At day 0, a second lumbar puncture (LP) was performed at the start of combination induction therapy (dexamethasone, vincristine, and intrathecal triple therapy during the first 4 weeks, daunorubicine only in case of initial high-risk criteria). A third CSF sample was drawn at day 15.

The quantification of L-Asp activity was performed by incubating the samples with an excess amount of L-aspartic acid β-hydroxamate (AHA) at 37 °C. L-Asp

hydrolyzed AHA to L-aspartic acid and hydroxylamine, which was detected at 710 nm after condensation with 8-hydroxychinoline and oxidation to indooxine. This method allowed the quantification of 2.5 IU/L L-Asp in human serum with coefficients of variation for intra- and interday variability of 1.98 - 8.77% and 1.73 - 11.0%, and an overall recovery of 101 ± 9.92%.[7] Asn levels in the plasma and CSF were measured using the RP-HPLC technique following precolumn derivation with *o*-phthaldialdehyde and fluorescence detection according to Lenda and Svenneby.[8] The lower limit of detection (LOD) was 0.2 µM.

RESULTS AND DISCUSSION

All patients reached an L-Asp activity ≥ 100 IU/L for at least 10 days (Figure 4.1). A peak level of 744 ± 132 IU/L (mean ± SD) was reached 1 h after the PEG-Asp infusion, declining to 483 ± 101 IU/L (mean ± SD) after 5 days and to 212 ± 66 IU/L (mean ± SD) on day 7, 12 days after the PEG-Asp administration; on day 15 of the treatment schedule, 20 days after the PEG-Asp infusion, L-Asp activity had declined to 39 ± 28 IU/L (mean ± SD). Avramis showed that the mean peak of PEG-Asp activity was 1000 IU/L when measured 5 days after a dose of 2500 IU/m^2 i.m. was given to children with ALL, declining to about 100 IU/L on day 24.[6]

We also analyzed all CSF samples for L-Asp activity and detected no activity above the limit of quantification (2.5 IU/L).

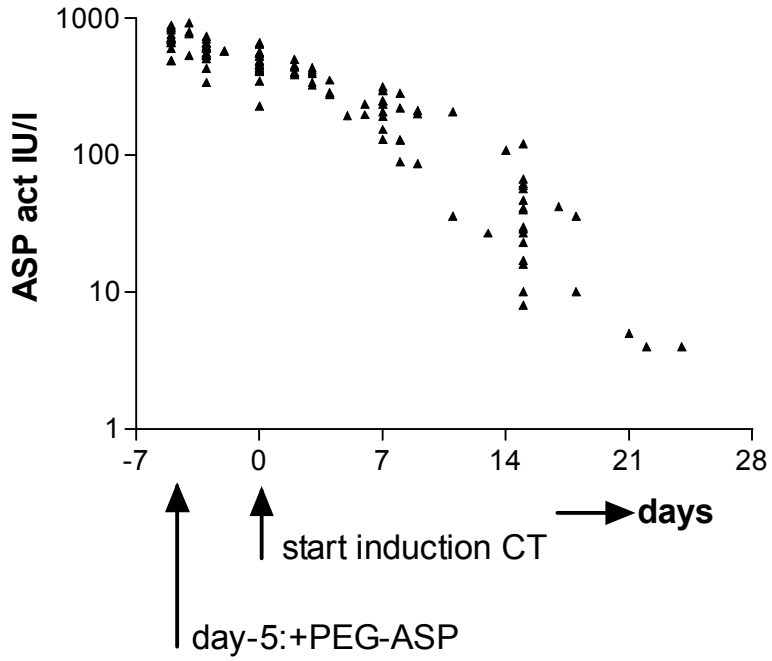


Figure 4.1 Plasma L-Asparaginase activity levels
Sequential analysis of plasma L-Asparaginase activity (IU/L) after 1000 IU/m^2 of PEG-Asp i.v. given 5 days before starting combined induction chemotherapy (CT).

In all patients, plasma Asn levels declined below the LOD of 0.2 μM (Figure 4.2A). From the point of view of peripheral treatment intensity, the results of Asn plasma concentrations after 1000 IU/m^2 PEG-Asp indicate a treatment intensity comparable to that observed with native unpegylated L-Asp (10,000 IU/m^2).[2]

Riccardi *et al*[3] showed that Asn depletion in CSF was only achieved after plasma Asn depletion. However, no complete Asn depletion in the CSF occurred in our patient group (Figure 4.2B): pretreatment starting levels at day -5 ranged from 3.5 to 7.2 μM (mean 5.1 ± 1.1 μM), decreasing to a mean concentration of 1.58 ± 0.66 μM at day 0 just before starting induction chemotherapy. At day 14 (19 days after the administration of PEG-Asp), the mean CSF Asn concentration was 2.2 ± 0.67 μM. The Asn concentration never dropped below the LOD (0.2 μM). We also spiked CSF samples with L-Asp, incubated the samples at 37 °C and analyzed them for Asn by HPLC. No Asn was detected in the spiked samples; thus, we are sure that we determined Asn in the CSF samples. CSF-Asn concentrations in the study of Avramis fell from a median pretreatment level of 2.3 to 1.1 μM on day 7 and 0.6 μM on day 28,[6] demonstrating that i.m. PEG-Asp also does not fully deplete CSF Asn. These Asn levels are still high above the detection limit of 0.01 μM in their study. The study of Avramis differs from our study in terms of the dose of PEG-Asp (2500 *vs* 1000 IU/m^2), the route of PEG-Asp (i.m. *vs* i.v.), and concomitant chemotherapy (prednisone p.o., vincristine i.v., and intrathecal cytarabine/methotrexate *vs* none in the first 5 days). The influence of concomitant antileukemic treatment on the pharmacokinetic and pharmacodynamic effects of L-Asp is not clear.

It has been suggested that the CSF-Asp activity never exceeds 0.2% of the L-Asp activity in plasma.[3] One explanation for the lack of Asn depletion in CSF may be that the pegylated form of L-Asp results in CSF-Asp levels that are even less than 0.2% of the plasma activity. We also analyzed all CSF samples for PEG-Asp activity and detected no activity above the limit of quantification (2.5 IU/L). Another explanation could be that the central nervous system is capable of synthesizing Asn locally despite the depletion of the systemic Asn pool. To maintain sufficient amino acids in the CSF, a net amino-acid entry from blood to CSF against a concentration gradient has been demonstrated in sheep.[9]

So, despite the fact that a dose of 1000 IU/m^2 PEG-Asp results in plasma Asn levels < 0.2 μM, the human body is still capable of maintaining the CSF Asn levels. Although PEG-Asp may have the advantage of fewer injections, this might be counterbalanced by less effective killing of blasts in the CSF. It is yet unknown whether an impaired depletion of Asn in CSF by PEG-Asp has an effect on the incidence of CNS relapse in this group of patients.

ACKNOWLEDGEMENTS

We thank Medac (GmbH, Germany) for the gift of PEG-Asp. We gratefully acknowledge the laboratory of the University Children's Hospital Muenster, Germany, for their technical support in this study.

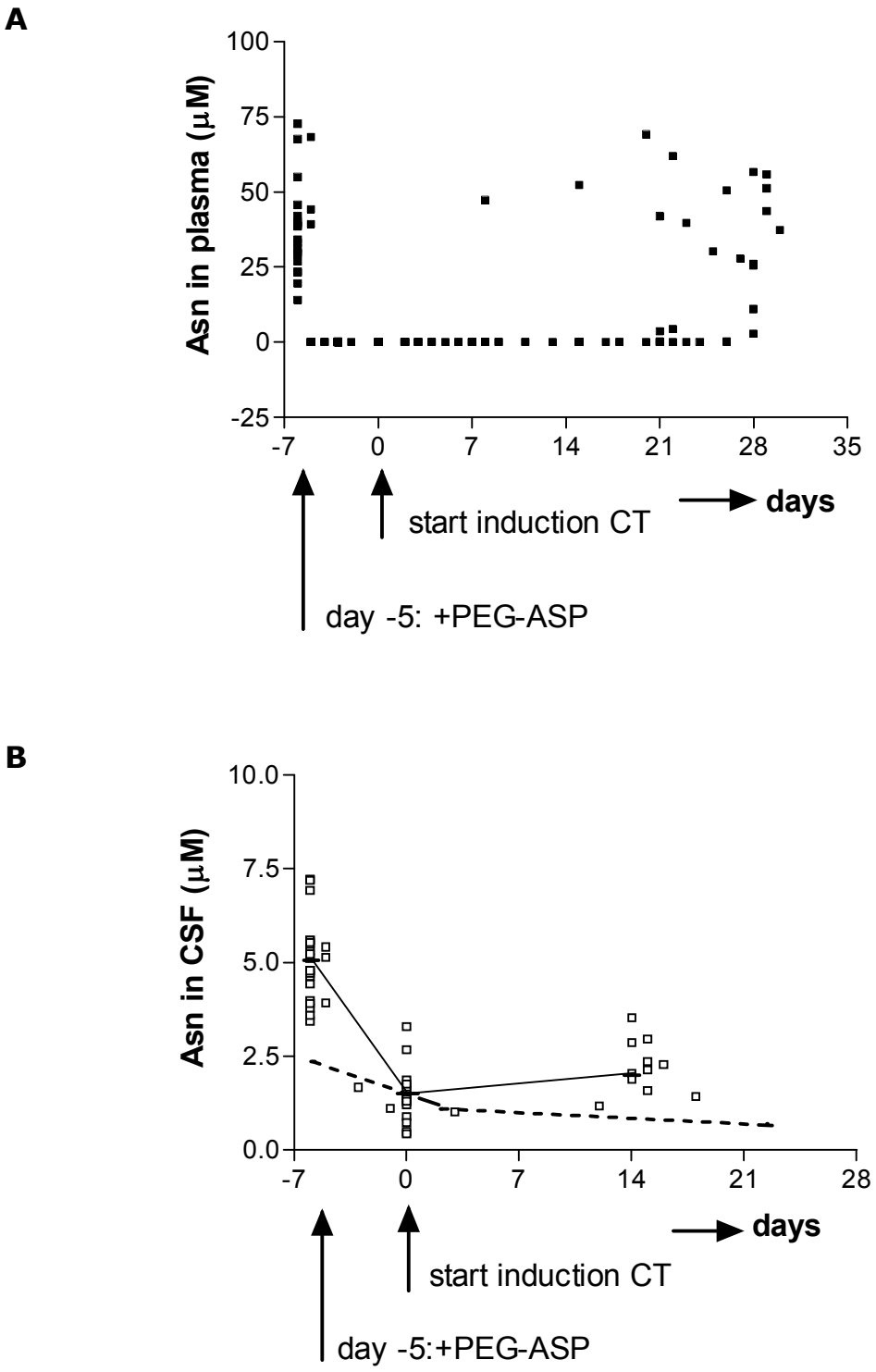


Figure 4.2 Asparagine levels in time

(A) Sequential analysis of plasma (■) asparagine levels (µM) after 1000 IU/m^2 of PEG-Asp i.v. given 5 days before starting combined induction chemotherapy (CT).
(B) Sequential analysis of CSF (□) asparagine levels (µM) after 1000 IU/m^2 of PEG-Asp i.v. given 5 days before starting combined induction chemotherapy (CT). The dotted line depicts the results of Avramis after 2.500 IU/m^2 PEG-Asp given i.m. and combined with induction chemotherapy during the first 5 days.

REFERENCES

1. Asselin BL, Whitin JC, Coppola DJ, Bernal SD, Leavitt PR, Gelber RD, et al. Comparative pharmacokinetic studies of three asparaginase preparations. J Clin Oncol. 1993;11:1780-1786.
2. Boos J, Werber G, Ahlke E, Schulze-Westhof P, Nowak-Gottl U, Wurthwein G, et al. Monitoring on asparaginase activity and asparaginase levels in children on different asparaginase preparations. Eur J Cancer. 1996;32A:1544-1550.
3. Riccardi R, HolcenbergJS, Glaubiger DL, WoodJH, Poplack DG. LAsparaginase pharmacokinetics and asparagine levels in cerebrospinal fluid of Rhesus monkeys and humans. Cancer Res. 1981;41:4554-4558.
4. Rizzari C, Zucchetti M, Conter V, Diomede L, Bruno A, Gavazzi L, et al. L-Asparagine depletion and L-asparaginase activity in children with acute lymphoblastic leukemia receiving i.m. or i.v. Erwinia C or E. Coli L-asparaginase as first exposure. Ann Oncol. 2000;11:189-193.
5. Müller HJ, Loning L, Horn A, Schwabe D, Gunkel M, Schrappe M, et al. Pegylated asparaginase (Oncaspar) in children with ALL: drug monitoring in reinduction according to the ALL/NHL-BFM 95 protocols. Br J Hematol. 2000;110:379-384.
6. Avramis VI, Sencer S, Periclou AP, Sather H, Bostrom BC, Cohen LC, et al. A randomized comparison of native Escherichia coli asparaginase and polyethylene glycol conjugated asparaginase for treatment of children with newly diagnosed standard risk acute lymphoblastic leukemia: a Children's Cancer Group study. Blood. 2002;99:1986-1994.
7. Lanvers C, Pinheiro JPV, Hempel G, Wuerthwein, Boos J. Analytical validation of a microplate reader based method for the therapeutic drug monitoring of L-asparaginase in human serum. Anal Biochem. 2002;309:117-126.
8. Lenda K, Svenneby G. Rapid high-performance liquid chromatographic determination of amino acids in synaptosomal extracts. J Chromatogr. 1980;198:516-519.
9. Segal M. Transport of nutrients across the choroid plexus. Microsc Res Technol. 2001;52:38-48.

II PART

L-ASPARAGINASE AND HEMOSTASIS

5 chapter

Changes in hypercoagulability by L-Asparaginase: a randomized study between two L-Asparaginases

Inge M. Appel
Wim C.J. Hop
Rob Pieters

Blood Coagulation and Fibrinolysis 2006;17:139-146

ABSTRACT

Alterations in hemostasis have frequently been observed in children with acute lymphoblastic leukemia. Thrombotic events are well documented in patients receiving L-Asparaginase as a single agent or in combination with other chemotherapeutic drugs. The present prospective, randomized study evaluated the effect of two different L-Asparaginase preparations, native *Escherichia coli* L-Asparaginase (Crasnitin; Bayer AG, Leverkusen, Germany; n = 10) and L-Asparaginase derived from *Erwinia chrysanthemi* (Erwinase®; Porton Pruducts, London, UK; n = 10) on the changes in parameters concerning hypercoagulability. Patients were randomized to receive a total of eight doses of 10 000 IU/m^2 L-Asparaginase intravenously with intervals of 3 days during induction therapy. Before starting L-Asparaginase treatment all patients had already demonstrated an increased thrombin generation shown by the elevated levels of prothrombin F1+2 and thrombin antithrombin complex (TAT), presumably due to therapy with prednisone, daunorubicin and vincristine. A significant decrease in alpha$_2$-antiplasmin and plasminogen levels was measured in the *E. coli* L-Asparaginase but not in Erwinase-treated patients. Increased thrombin generation combined with a decrease in α_2-antiplasmin and plasminogen levels may lead to a state of increased risk for thrombosis due to a delay in fibrin elimination in *E. coli* L-Asparaginase-treated patients only.

INTRODUCTION

L-Asparaginase has proven to be an important element in the treatment of childhood acute lymphoblastic leukemia (ALL). As a side effect, L-Asparaginase can diminish synthesis of several coagulation factors and inhibitors[1] that can lead to an increased risk for thrombosis.[2-4] This suggests that the balance between coagulation and fibrinolysis is shifted towards fibrin formation and deposition.

The changes observed in coagulation parameters in patients treated with L-Asparaginase have not, however, been consistent in different series of patients,[5-7] which can be related to differences in age, genetic predisposition for hypercoagulability, co-administration of other chemotherapy and the product of L-Asparaginase used. Due to a shorter half life the same dosage of Erwinase® (Porton Pruducts, London, UK) shows less antileukemic activity than various kinds of *Escherichia coli* L-Asparaginase.[8,9] It is suggested that different *E. coli* L-Asparaginase preparations influence fibrinolytic proteins in a different way.[10] In a previous study we saw that decreased coagulation factor synthesis in particular antithrombin was in part counterbalanced by the effect of prednisone.[11,12] The imbalance in coagulation factors was less pronounced in children treated with Erwinase® than in children treated with *E. coli* L-Asparaginase. The European Organization for Research and Treatment of Cancer Children's Leukemia Group (EORTC-CLG) randomized 700 children with ALL for *E. coli* L-Asparaginase or Erwinase®. Abnormalities in hemostasis, not further specified, were more frequent in the *E. coli* L-Asparaginase than in the *Erwinia* arm of the study (30.2% versus 11.9%, $P < 0.0001$).[7]

Several highly sensitive and specific tests for activation of the hemostatic system are available (Figure 5.1). Measurement of the activation peptide prothrombin fragment 1+2 (F1+2) provides information on the cleavage of the prothrombin molecule by activated factor X. The protease inhibitor complex thrombin antithrombin (TAT) reflects the *in vivo* thrombin generation process. Activation of the fibrinolytic system leads to plasmin formation, which lyses cross-linked fibrin to fibrin degradation products. This will generate increased levels of plasma D-dimer. The plasmin-α_2-antiplasmin (PAP) complex level will be raised in case of increased fibrinolytic activity due to binding of the active proteolytic enzyme plasmin by α_2-antiplasmin (α_2-AP).

In order to study the effects of different kinds of L-Asparaginases, we first analyzed the data of a randomized study comparing *E. coli* L-Asparaginase (Crasnitin®; Bayer, Leverkusen, Germany) and L-Asparaginase derived from *Erwinia chrysanthemi* (Erwinase®) in childhood ALL. We studied the effects of L-Asparaginase on thrombin generation measuring F1+2 and TAT and on fibrin degradation by measuring α_2-AP, plasminogen and PAP, D-dimers and fibrinogen.

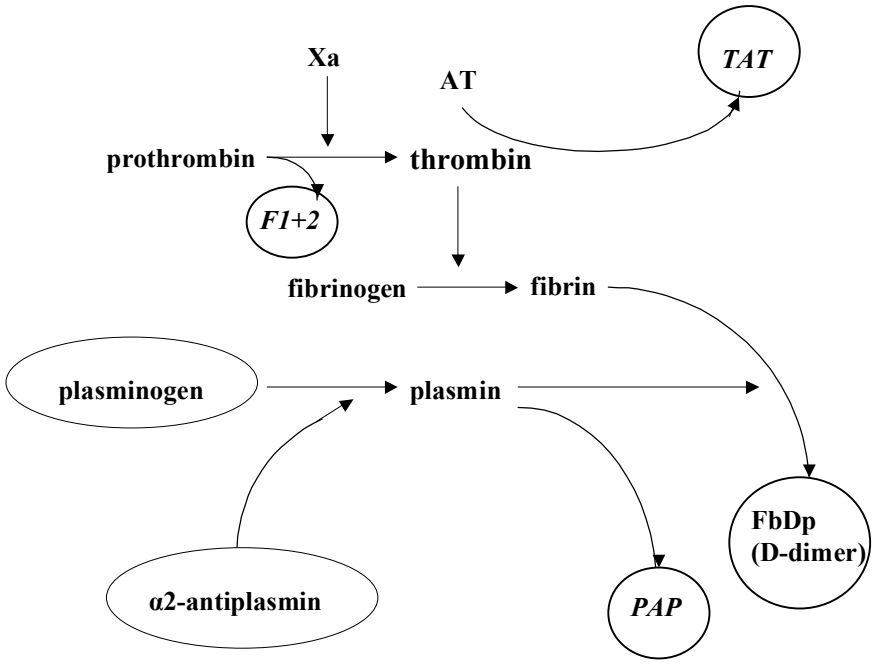


Figure 5.1 Diagram on thrombin generation and fibrinolysis
Diagram showing the biochemical steps in the generation of stable inactive enzyme-inhibitor complexes. Prothrombin fragment 1+2 (F1+2) is generated by the cleavage of prothrombin by activated FX. Thrombin antithrombin complexes (TAT) reflect the thrombin generation process. Lysis of fibrin will generate fibrin degradation products (FbDp) which in turn increase the levels of D-dimer. The binding of plasmin by alpha-2-antiplasmin leads to the formation of the plasmin-alpha-2-antiplasmin complex (PAP).

METHODS

Patients

Twenty children, consecutively admitted to our hospital with newly diagnosed ALL between June 1989 and December 1990, were entered in the study. There were 10 boys and 10 girls, varying in age from 10 months to 12.3 years (mean 6.1 years). The characteristics of the 20 patients are given in Table 5.1. The groups did not differ significantly with respect to the given characteristics. The induction treatment was according to the ALL-7 protocol of the Dutch Childhood Oncology Group, formerly the Dutch Childhood Leukemia Study Group,[13] which is based upon the Berlin-Frankfurt-Munster (BFM) 86 protocol[14] (Figure 5.2). After written informed consent the patients were randomized on day 18 for *E. coli* L-Asparaginase or Erwinase® administered every 3 days starting at day 19 as 10 000 IU/m^2 in a 1-h infusion given at 1400-h each time. A total of eight doses were given during induction. No transfusions with fresh frozen plasma or antithrombin concentrate were administered to the children. The local ethical committee approved the study.

Table 5.1 Characteristics of 20 patients at diagnosis

	Escherichia coli **L-Asparaginase-treated children n =10**	***Erwinia*** **L-Asparaginase-treated children n =10**
Male/female	5/5	5/5
age (years)	5.5 (2.5 - 9.4)	5.1 (0.8 - 12.6)
Hb (mmol/l)	5.4 (4.3 - 7.5)	4.3 (2.6 - 9.1)
WBC (10^9/l)	10.8 (4.3 - 38.7)	62.8 (4.6 - 585)
Thr (10^9/l)	81 (16 - 310)	37 (< 10 - 141)
Immunological phenotyping		
Null ALL	0	1
Common ALL	7	5
Pre B ALL	2	0
T ALL	1	4

Data given are number of patients or median (range). Ten patients treated with E. coli L-Asparaginase and 10 treated with Erwinia L-Asparaginase. The immunological phenotyping is included. Hb, hemoglobin; WBC, white blood count; Thr, trombocytes; ALL, acute lymphoblastic leukemia.

Samples

A circadian variation of the fibrinolytic activity in blood has only been demonstrated for tissue-type plasminogen activator (tPA) and plasminogen activator inhibitor (PAI).[15] Other coagulation parameters vary little or not at all during the day. In order to eliminate any influence of circadian variation, we planned all measurements at 1400-h. Blood samples were collected before each L-Asparaginase administration at 1400-h from the infusion line (Venisystems Butterfly 21 gauge; Abbott, Ireland). The venous blood samples were collected in cups containing 3.8% trisodium citrate. The samples were chilled immediately in an ice bath, centrifuged at 20.000 rpm for 30 min at + 4 °C. The supernatant was withdrawn and stored at -80 °C until the time of analysis.

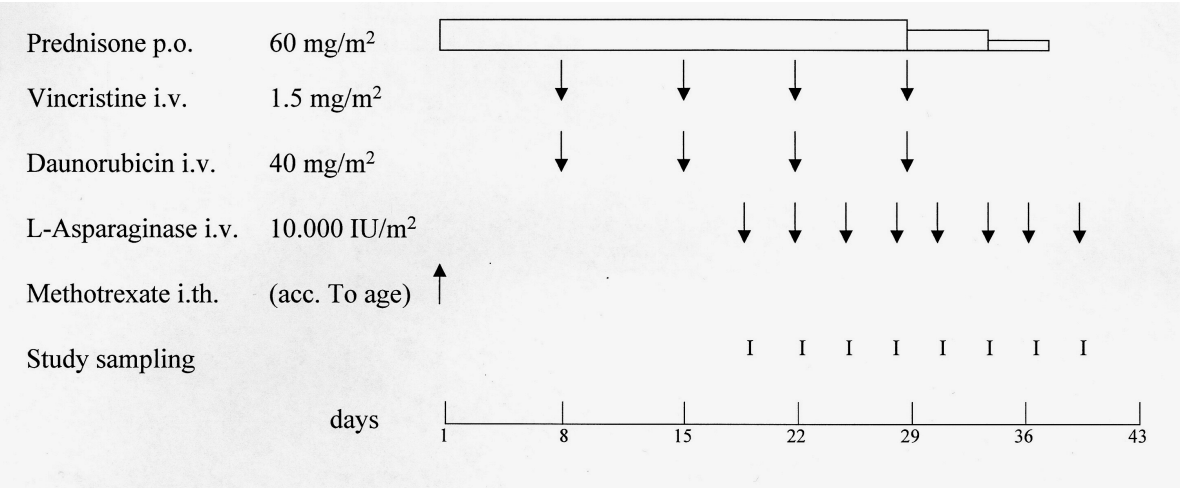


Figure 5.2 DCOG-ALL-7 induction therapy
First part of the induction therapy for acute lymphoblastic leukemia according to the DCOG-ALL-7 study. Methotrexate intrathecal was admitted according (acc.) to age.

Coagulation assays

The values of activation markers like F1+2, TAT, PAP and D-dimer are similar in children and adults, as was demonstrated by Ries *et al*.[16] So we were able to adapt the reference values for adults as provided by the manufacturers.

F1+2 was measured using a commercial solid phase enzyme-linked immunosorbent-assay (Enzygnost F1+2; Dade Behring GmbH, Marburg, Germany) following the manufacturer's instructions.[17] We adopted the reference values (5^{th} to 95^{th} percentile) provided by Dade Behring, 0.44 - 1.11 nmol/l.

TAT was determined with a commercial solid phase enzyme-linked immunosorbent assay (Enzygnost TAT, Behringwerke AG, Marburg, Germany) following the manufacturer's instructions.[18] We constructed our own pediatric reference values on 62 healthy children between 1 and 15 years old; reference values for TAT plasma levels were median 2.7 µg/l (5^{th} to 95^{th} percentile 1.1 - 4.3 µg/l). These reference values were in line with the values as determined by Ries *et al*.[16]

Alpha2-antiplasmin (α_2-AP) was determined in a kinetic test.[19] The normal reference interval lies between 80 and 120% (Dade Behring GmbH).

Plasminogen was also determined in a kinetic test.[20] The normal reference interval lies between 75 and 140% (Dade Behring GmbH).

PAP was measured by a sandwich enzyme immunoassay of Dade Behring GmbH following the manufacturer's instructions.[21] The normal reference range (percentile 2.5 - 97.5) of this enzyme immunoassay lies between 120 and 700 µg/l (Dade Behring GmbH) as determined in 466 healthy adults. There are no differences in normal values between children and adults.[16]

D-Dimer levels were measured using a commercial enzyme-linked immunoassay (Asserachrom D-Di; Diagnostica Stago, Asnières, France) following manufacturer's instructions.[22,23] The lower limit of sensitivity was 500 ng/ml, levels above the 500 ng/ml point to an increased breakdown of fibrin monomers.

Fibrinogen was examined according to the Clauss method.[24] Normal values range between 2 and 4 g/l.

Determinations of PAP and D-dimer were only done at day 19, before the first L-Asparaginase administration, at day 25 after two L-Asparaginase administrations and at day 34, after five infusions of L-Asparaginase.

Statistics

Repeated measurements analysis of variance (SAS PROC MIXED; SAS Institute, Cary, North Carolina, USA) was used to evaluate the differences between and within both experimental groups. This method was used after logarithmic transformation of TAT, F1+2 and D-dimer to obtain approximate normal distributions. $P = 0.05$ (two sided) was considered the limit of significance. The Mann Whitney *U* test was used to calculate differences in the various parameters at day 19 between the two groups of patients.

RESULTS

Groups were comparable regarding various baseline coagulation/fibrinolytic parameters on day 19 at the start of L-Asparaginase treatment.

Prothrombin fragment 1+2

To obtain approximate normal distributions these data were analyzed after logarithmic transformation. All patients demonstrated high levels of F1+2 before the start of L-Asparaginase therapy (Figure 5.3). During and after administration of L-Asparaginase, no significant differences of F1+2 in time evolved in comparison with the values before starting L-Asparaginase on day 19. Also within each of the two groups analyzed separately no differences at the various time points compared with day 19 were found ($P > 0.1$). Adjusted for baseline values at day 19, the mean value of F1+2 at the various time points was 0.08 nmol/l lower in the *E. coli* L-Asparaginase-treated patients than in the Erwinase®-treated children, but this did not reach statistical significance ($P = 0.1$). The mean values of F1+2 varied between 1.3 (SEM 0.1) and 2.3 nmol/l (SEM 0.8) in the *E. coli* L-Asparaginase-treated group and between 1.5 (SEM 0.1) and 2.9 nmol/l (SEM 1.1) in the Erwinase®-treated children at the different time points (Figure 5.3).

Thrombin antithrombin

To obtain approximate normal distributions these data were analyzed after logarithmic transformation. High levels of TAT were already found in all 20 patients before starting L-Asparaginase (Figure 5.3). Within each of the two groups no significant changes of TAT occurred during treatment ($P > 0.1$). The TAT levels at the various time points did not significantly differ between the *E. coli* L-Asparaginase-treated patients and the Erwinase®-treated children. The mean values of TAT ranged between 6.6 (SEM 1.5) and 27 (SEM 19.3) µg/l in the *E. coli* L-Asparaginase-treated children and between 7.3 (SEM 1.4) and 55.4 (SEM 36) µg/l in the Erwinase®-treated group (Figure 5.3).

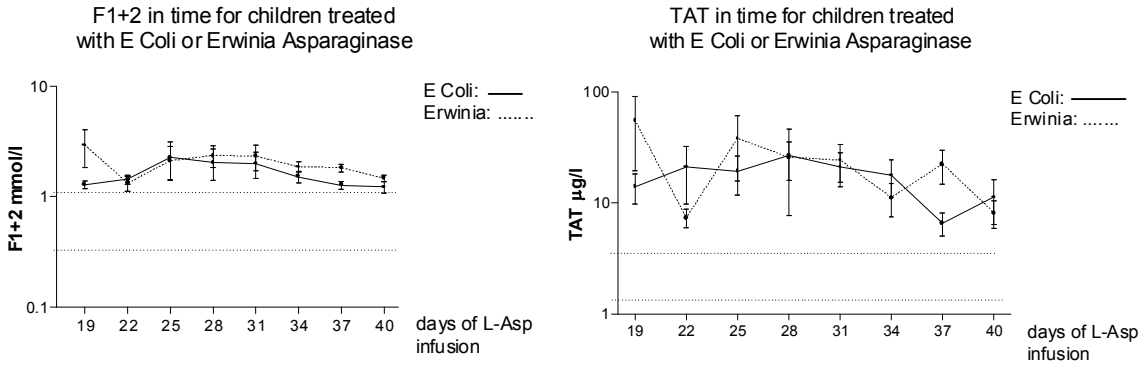


Figure 5.3 Parameters of trombin generation

Geometric means ± Standard error over time of prothrombin fragment 1+2 (F1+2) in mmol/L and thrombin-antithrombin complexes (TAT) in µg/L for 10 patients treated with Escherichia coli L-Asparaginase and 10 patients treated with Erwinase® monitored from the first infusion with L-Asparaginase 10.000 IU/m^2 intravenously until the last infusion. Range of normal values of F1+2: 0.44 - 1.11 mmol/L, range of normal values of TAT: 1.1 - 4.3 µg/L.

Alpha2-antiplasmin

The mean α_2-AP values in the *E. coli* L-Asparaginase-treated patients declined from 110% (SEM 3.6) before starting L-Asparaginase to 88% (SEM 4.6) at day 25, before starting the third L-Asparaginase infusion, and thereafter values stayed stable (Figure 5.4). Using RmANOVA, adjusted for baseline values at day 19, the mean values of α_2-AP at the various time points were 18.2 units lower in the children treated with *E. coli* L-Asparaginase ($P < 0.001$) than in the children treated with Erwinase®. In the Erwinase®-treated patients the mean α_2-AP values did not differ from baseline values at day 19 but stayed within the normal range with mean values ranging from 97% (SEM 3.4) to 106% (SEM 4.2) (Figure 5.4).

Plasminogen

In the *E. coli* L-Asparaginase group plasminogen levels started at a mean of 102% (SEM 6) and decreased to 72% (SEM 6) before the third L-Asparaginase administration and thereafter mean values remained stable between 74 and 79% (Figure 5.4). In the Erwinase®-treated patients the mean plasminogen value before starting L-Asparaginase therapy was 89% and mean values stayed between 84% (SEM 5.9) and 89% (SEM 5.5) (Figure 5.4). Adjusted for baseline values at day 19, the mean value of plasminogen at the various time points was 17.5 units lower in the *E. coli* L-Asparaginase-treated children ($P = 0.013$) than in the children treated with Erwinase®.

Plasmin-alpha2-antiplasmin

In the *E. coli* L-Asparaginase group the mean PAP values at day 19, 25 and 34 were respectively 233 (SEM 25.7), 145 (SEM 35) and 178 (SEM 33) µg/l. The corresponding values for the Erwinase®-treated children were, respectively, 298 (SEM 72), 289 (SEM 53) and 287 (SEM 46) µg/l as demonstrated in Figure 5.4. Using RmANOVA it was found that the mean value of PAP at the various time points adjusted for baseline values at day 19 was 120 µg/l lower in the *E. coli* L-Asparaginase group ($P = 0.04$) than in the Erwinase® group.

D-Dimer

To obtain approximate normal distributions these data were analyzed after logarithmic transformation. In the *E. coli* L-Asparaginase group the mean D-dimer values at day 19, 25 and 34 were, respectively, 162 (SEM 14.5), 152 (SEM 43) and 214 (SEM 100) ng/ml (Figure 5.4). The corresponding values for the Erwinase®-treated children were, respectively, 238 (SEM 58), 202 (SEM 41) and 264 (SEM 71) ng/ml. All individual values were within the normal range pointing to no degradation of fibrin to fibrin monomers. No active fibrinolysis was monitored. Adjusted for baseline values at day 19 the mean value of D-dimer at the various time points did not significantly differ between the *E. coli* L-Asparaginase-treated children and the Erwinase®-treated patients ($P = 0.4$).

Fibrinogen

Mean values at day 19 started in the *E. coli* L-Asparaginase-treated group at 1.4 g/l (SEM 0.2) with a nadir of 0.9 g/l (SEM 0.1) at day 25 to gradually increasing values of 1.5 g/l (SEM 0.16) at day 40. In the Erwinase®-treated children fibrinogen levels start at day 19 at 1.0 g/l (SEM 0.1), gradually increasing to 2.3 g/l (SEM 0.2) at day 40.

During the study in none of the 20 patients did any clinical signs of serious bleeding or thrombotic events occur.

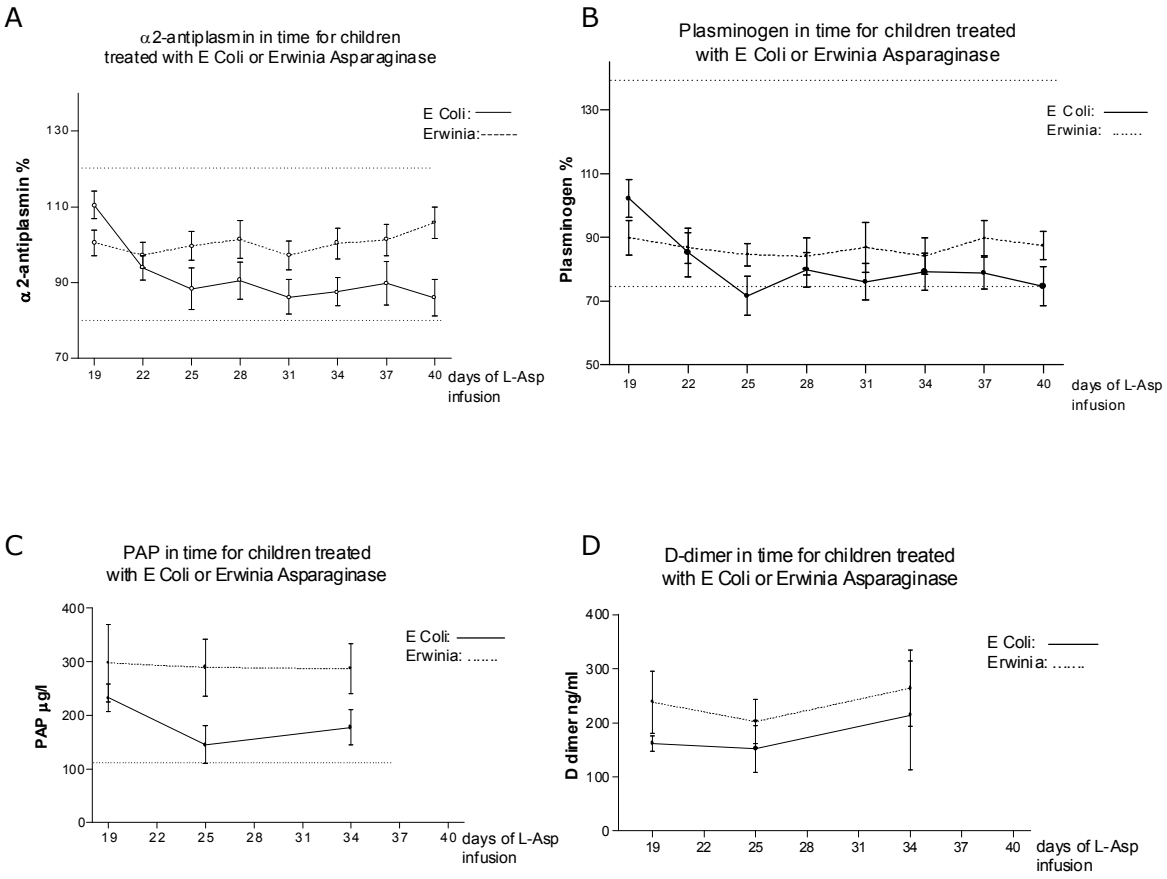


Figure 5.4 Parameters of fibrinolysis

Means ± SEM over time of (A) α2-antiplasmin (%) and (B) of plasminogen (%) for 10 patients treated with E. coli L-Asparaginase and 10 patients treated with Erwinase® monitored from the first infusion with L-Asparaginase 10.000 IU/m^2 intravenously until the last infusion. Adjusted for baseline values at day 19, the mean values of α2-antiplasmin are significantly lower in the E. coli L-Asparaginase-treated children (P = 0.001) compared with the Erwinia-treated children. The mean values of plasminogen are significantly lower in the E. coli L-Asparaginase- treated children (P = 0.013) than in the Erwinase®-treated children. Range of normal values of α2-antiplasmin: 80 - 120%; range of normal values of plasminogen: 75 - 140%.
(C) Means ± SEM in time of plasminogen-α2-antiplasmin (PAP) in µg/l monitored from the first infusion, the third and the last infusion with L-Asparaginase 10.000 IU/m^2 intravenously. A significant decrease in PAP is found in E. coli L-Asparaginase-treated children between day 19 and day 25 (P = 0.03). Range of normal values: 120 - 700 µg/L.
(D) Geometric means ± SEM in time of D-Dimer in ng/ml monitored from the first infusion, the third and the last infusion with L-Asparaginase 10.000 IU/m^2 intravenously. Normal values < 500 ng/ml.

DISCUSSION

Children suffering from ALL are at risk for increased bleeding tendencies as well as for thrombotic events. Activation of the coagulation system can be attributed to the malignant disease.[4] Additionally, chemotherapeutic regimens contain several antileukemic drugs that can influence the balance between bleeding and thrombosis. The most important drugs that influence the coagulation system are prednisone and L-Asparaginase.

In a former study we demonstrated that the largest changes in coagulation proteins were monitored in the first part of ALL induction treatment consisting of intravenous vincristine, prednisone, daunorubicin and intrathecal methotrexate. During the second phase of induction treatment when L-Asparaginase was added, only minor additional changes in plasma coagulation factors were found.[11,12] A remarkable shortening of the APTT most likely due to a rise in factor VIII and IX, together with a progressive hypofibrinogenemia and a significant rise in both the AT and protein C activities, were responsible for an unstable balance between coagulation and bleeding factors before L-Asparaginase was added to the treatment. All these changes could probably be attributed to the use of prednisone.[25,26] We did not find an additional effect of L-Asparaginase on the coagulation proteins that are involved in fibrin formation.[11]

The changes observed in coagulation proteins are related to the product and the dose of L-Asparaginase used.[5,6,27,28] We now know that different L-Asparaginase preparations manifest different pharmacokinetic and pharmacodynamic properties. The influence on fibrinolytic proteins will be different.[10] In the present randomized prospective study we analyze the process of thrombin generation and fibrinolysis by two different L-Asparaginase preparations. All patients demonstrate increased thrombin generation already before starting L-Asparaginase, pointing to a state of hypercoagulability demonstrated by elevated levels of F1+2 as well as TAT (Figure 5.3). The administration of L-Asparaginase, either generated from native *E. coli* or from *Erwinia chrysanthemi*, does not lead to a further increase or any decrease in the levels of TAT and F1+2. As prednisone therapy continues during the second part of ALL induction treatment the raised levels of F1+2 and TAT might be attributed to the concomitant use of corticosteroids.[25] Considering that the half-lives of F1+2 and TAT are respectively 90 and 3 minutes and that both parameters were taken at the same time for each investigation, we conclude that thrombin generation is not clearly influenced by the administration of L-Asparaginase in the way it was given in this study. The collection of blood samples from metal butterfly needles is comparable with a direct venipuncture and not to the situation with peripheral catheters *in situ*.[29] Therefore a reliable interpretation of F1+2 and TAT complexes is possible, pointing to a state of hypercoagulability in all patients, presumably due to the concomitant use of prednisone.

The statistically significant decline in both α_2-AP and plasminogen in the *E. coli* L-Asparaginase-treated children in relation to the parallel reduction in PAP levels points in this study to a decrease in synthesis of α_2-AP and plasminogen,

respectively. This decline does not occur in the Erwinase®-treated children (Figure 5.4). The presumed effect of prednisone on α_2-AP and plasminogen levels is negligible, as is demonstrated by the normal values of both proteins before starting L-Asparaginase. Patients on long-term steroid therapy demonstrate a hypercoagulable and hypofibrinolytic state due to elevations of several coagulation factors and due to elevations of PAI-1.[30] This hypofibrinolytic state will possibly be induced by prednisone in the same way in all our patients. So, from this randomized study, we can conclude that the decrease in PAP levels, due to a decrease in α_2-AP and plasminogen synthesis, will cause a delay in fibrin elimination and therefore might lead to a hypercoagulable state in the *E. coli* L-Asparaginase-treated patients only and not in the Erwinase®-treated children.

The D-dimer levels stayed within the normal range during L-Asparaginase treatment. Statistical analysis reveals no difference on the various time points between *E. coli* L-Asparaginase and Erwinase®-treated children (Figure 5.4). The normal values of D-dimer even at baseline can be explained by hypofibrinogenemia in all 20 patients induced by initial prednisone therapy. This implies that active fibrinolysis after the introduction of L-Asparaginase potentially leading to fibrin degradation is not observed irrespective of the differences in pharmacokinetics between these two L-Asparaginases.

The present study shows different effects of different kinds of L-Asparaginase on the coagulation system.[8] The two L-Asparaginase preparations differ in half-life with different influences on the amino acid metabolism. A dose-dependent influence of L-Asparaginase preparations on protein synthesis has also been found. The half life of native *E. coli* L-Asparaginase is 1.24 days whereas the half life of Erwinase® is 0.65 days.[8,31] The effects measured in our study are the result of 10,000 IU/m^2 of L-Asparaginase administered every 3 days. In the Gruppo Italiano Malattie Ematologiche dell'Adulto (GIMEMA) study[32] *E. coli* L-Asparaginase was given daily in a lower dose of 6000 IU/m^2 and also at this dose an increase in hypercoagulability parameters during treatment with *E. coli* L-Asparaginase was found. Nowak-Gottl[33] demonstrated less changes in hemostasis in children treated with 2500 IU/m^2 of the native *E. coli* L-Asparaginase preparation compared with 5000 IU/m^2 of the same L-Asparaginase. In another non-randomized study they showed that the downregulation of coagulation proteins like fibrinogen, plasminogen and α_2-AP was more pronounced in children treated with Medac L-Asparaginase (Medac Therapeutics, Wedel, Germany) than in children treated with the original native product of Bayer (Crasnitin®) or Erwinase®.[10] In their report a significant enhanced thrombin generation also with increased levels of D-dimers was found. So, different effects on the coagulation system are not only found between L-Asparaginase generated from *E. coli* or from *Erwinia chrysanthemi*, but also among different commercial *E. coli* preparations.

We conclude that the leukemia itself together with initial treatment with prednisone during ALL induction leads to a situation of increased thrombin generation as is demonstrated by increased F1+2 and TAT levels. Addition of L-Asparaginase to the induction therapy leads to a decreased fibrinolytic potential due to decreasing levels of α_2-AP and plasminogen in children treated with *E. coli*

L-Asparaginase but not in children treated with Erwinase®. Therefore, the use of prednisone in combination with *E. coli* L-Asparaginase (Crasnitin®, Bayer) leads to an increased risk for thrombosis in children with ALL. Ongoing studies have to clarify how new formulations like pegylated L-Asparaginase and recombinant L-Asparaginase influence the coagulation system, and such studies have to be combined with pharmacokinetic and pharmacodynamic data.

REFERENCES

1. Ramsay NK, Coccia PF, Krivit W, Nesbit ME, Edson JR. The effect of L-Asparaginase of plasma coagulation factors in acute lymphoblastic leukemia. Cancer. 1977;40:1398-1401.
2. Mitchell LG, Hoogendoorn H, Giles AR, Vegh P, Andrew M. Increased endogenous thrombin generation in children with acute lymphoblastic leukemia: risk of thrombotic complications in L'Asparaginase-induced antithrombin III deficiency. Blood. 1994;83:386-391.
3. Nowak-Gottl U, Wolff JEA, Kuhn N, Boos J, Lilieweiss V, Schwabe D, et al. Enhanced thrombingeneration, P-von Willebrand factor, P-fibrin, D-Dimer and P-plasminogen activator inhibitor 1: predictive for venous thrombosis in asparaginase treated children. Fibrinolysis. 1994;8:66-68.
4. Athale UH, Chan AKC. Thrombosis in children with acute lymphoblastic leukemia Part II. Pathogenesis of thrombosis in children with acute lymphoblastic leukemia: effect of the disease and therapy. Thromb Res. 2003;11:199-212.
5. Nowak-Gottl U, Wermes C, Junker R, Koch HG, Schobbes R, Fleischhack G, et al. Prospective evaluation of the thrombotic risk in children with acute lymphoblastic leukemia. Blood. 1999;93:1595-1599.
6. Kirschke R, Nurnberger W, Eckhof-Donovan S, Nurnberger I, Gobel U. Coagulation and fibrinolysis in children with acute lymphoblastic leukemia treated according to the COALL-05-92-protocol. Klin Padiatr. 1998;210:285-290.
7. Duval M, Suciu S, Ferster A, Rialland X, Nelken B, Lutz P, et al. Comparison of Escherichia coli-asparaginase with Erwinia-asparaginase in the treatment of childhood lymphoid malignancies: results of a randomized European Organisation for Research and Treatment of Cancer-Children's Leukemia Group phase 3 trial. Blood. 2002;99:2734-2739.
8. Asselin BL. The three aspraginases. Comparative pharmacology and optimal use in childhood leukemia. Adv Exp Med Biol. 1999;457:621-629.
9. Eden OB, Shaw MP, Lilleyman JS, Richards S. Non-randomised study comparing toxicity of Escherichia coli and Erwinia asparaginase in children with leukaemia. Med Pediatr Oncol. 1990;18:497-502.
10. Nowak-Gottl U, Kuhn N, Wolff JE, Boos J, Kehrel B, Rath B, et al. Inhibition of hypercoagulation by antithrombin substitution in E. coli L-Asparaginase-treated children. Eur J Haematol. 1996;56:35-38.
11. Risseeuw-Appel IM, Dekker I, Hop WC, Hahlen K. Minimal effects of E. coli and Erwinia asparaginase on the coagulation system in childhood acute lymphoblastic leukemia: a randomized study. Med Pediatr Oncol. 1994;23:335-343.
12. Risseeuw-Appel IM, Dekker I, Stigter RL, Hop WCJ, Hählen K. Progressive elevation of Antithrombin III levels during acute lymphoblastic leukemia induction treatment, not followed by a decrease in Antithrombin III after addition of Asparaginase: a randomized study between Escherichia Coli and Erwinia Asparaginase. Int J Ped Haematol/Oncol. 1996;3:66-74.
13. Kamps WA, Veerman AJP, van Wering ER, van Weerden JF, Slater R, van der Does-van den Berg A. Long term follow-up of Dutch Childhood Leukemia Study Group (DCLSG) protocols for children with acute lymphoblastic leukemia. Leukemia. 2000;14:2240-2246.
14. Reiter A, Schrappe M, Sauter S, Ludwig WD, Harbott J, Ritter J, et al. Treatment strategy and results of non-B acute lymphoblastic leukeima in the German-Austrian-multicenter trial ALL/NHL-BFM 86. Med Ped Oncol. 1992;20:390.
15. Adreotti F, Kluft C. Circadian variation of fibrinolytic activity in blood. Chronobiol-Int. 1991;8:336-351.
16. Ries M, Klinge J, Rauch R. Age-related reference values for activation markers of the coagulation and fibrinolytic system. Thromb Res. 1997;85:341-344.
17. Pelzer H, Schwartz A, Stuber W. Determination of human prothrombin activation fragment 1+2 in plasma with an antibody against a synthetic peptide. Thromb Haemost. 1991;65:153-159.

18. Pelzer H, Schwartz A, Heimburger N. Determination of human thrombin-antithrombin III complex in plasma with an enzyme-linked immunosorbent assay. Thromb Haemost. 1988;50:101-106.

19. Matsuda T, Ogawara M, Miura R, Seki T, Matsumoto T, Teramura Y, Nakamura K. Selective determination of alpha2-plasma inhibitor activity in plasma using chromogenic substrate. Thromb Res. 1984;33:379-388.

20. Knos M, Friberger P. Methods for plasminogen determination in human plasma and for streptokinase standardization. In: Davidson et al., editors. Progress in chemical fibrinolysis and thrombolysis. Edinburgh: Chuchill Livingstone; 1979. pp. 154-158.

21. Holvoet P, de Boer A, Verstreken M, Collen D. An enzyme limked immunosorbent assay (ELISA) for the measurement of plasmin-alpha 2-antiplasmin complex in human plasma: application to the detection of in vivo activation of the fibrinolytic system. Thromb Haemost. 1986;56:124-127.

22. Heaton DC, Billings JD, Hickton CM. Assessment of D-dimer assays for the diagosis of deep vein thrombosis. J Lab Clin Med. 1987;110:588-591.

23. Bick RL, Baker WF. Diagnostic efficacy of the D-dimer assay in disseminated intravascular coagulation. Thromb Res. 1992;65:785-790.

24. Clauss A. Determination of fibrinogen[in German]. Acta Haemat. 1957;17:237-246.

25. Mall V, Thomas KB, Sauter S, Niemeyer CM, Sutor AH. Effect of glucocorticosteroids, E. Coli- and Erwinia L-Asparaginase on hemostatic proteins in children with acute lymphoblastic leukemia. Klin Padiatr. 1999;211:205-210.

26. Dal Bo Zanon R, Fornasiero L, Boscaro M, Capellato G, Fabris F, Girolami A. Increased F VIII associated activities in Cushing's syndrome: a probable hypercoagulable state. Thromb Haemost. 1982;47:116-117.

27. Albertsen B, Schroder H, Ingerslev J, Jakobsen P, Avramis AI, Muller HJ, et al. Comparison of intramuscular therapy with Erwinia asparaginase and asparaginase Medac: pharmacokinetics, pharmacodynamics, formation of antibodies and influence on the coagulation system. Br J Haematol. 2001;115:983-990.

28. Nowak-Gottl U, Werber G, Ziemann D, Ahlke E, Boos J. Influence of two different Escherichia Coli Asparaginase preparations on fibrinolytic proteins in childhood ALL. Haematologica. 1996;81:127-131.

29. Hafner G, Schinzel H, Ehrental W, Wagner C, Konheiser U, Zotz R, et al. Influence of blood sampling from venipunctures and catheter systems on serial determinations of prothrombin activation fragment 1+2 and thrombin-antithrombin III complex. Ann Hematol. 1993;67:121-125.

30. Sartori TM, Maurizio PG, Sara P, Ugolino L, Annalisa A, Panagiotis T. Relation between long-term steroid treatment after heart transplantation, hypofibrinolysis and myocardial microthrombi. J Heart Lung Transplant. 1999;8:693-700.

31. Asselin BL, Whitin JC, Coppola DJ, Rupp IP, Sallan SE, Cohen HJ. Comparative pharmacokinetic studies of three asparaginase preparations. J Clin Oncol. 1993;11:1780-1786.

32. Leone G, Gugliotta L, Mazzucconi MG, De Stefano V, Belmonte MM, Dragoni F, et al. Evidence of a hypercoagulable state in patients with acute lymphoblastic leukemia treated with low dose of E. coli L-Asparaginase: a GIMEMA study. Thromb Haemost. 1993;69:12-15.

33. Nowak-Gottl U, Ahlke E, Schulze-Westhoff P, Boos J. Changes in coagulation and fibrinolysis in childhood ALL: a two-step dose reduction of one E. coli asparaginase preparation. Br J Haematol. 1996;95:123-126.

6 chapter

L-Asparaginase and the effect of age on coagulation and fibrinolysis in childhood acute lymphoblastic leukemia

Inge M. Appel
Wim C.J. Hop
Carla van Kessel-Bakvis
Rolinda Stigter
Rob Pieters

Submitted

ABSTRACT

Alterations in hemostasis are frequently observed in children with acute lymphoblastic leukemia (ALL).
In the present study we analyzed age-related disturbances in coagulation and fibrinolysis parameters during the induction phase of the DCOG-ALL-9 protocol. 64 children were classified by age into three groups (1 - 5, 6 - 10, 11 - 16 years), and studied during induction treatment of ALL including 4 weeks of dexamethasone, followed by 2 weeks tapering of dexamethasone during which 6,000 IU/m^2 Paronal® (total 4 doses) was administered intravenously twice weekly. Blood samples were collected immediately before each Paronal® infusion to analyze procoagulant (fibrinogen, F II, F V, F VII, F IX, F X) and anticoagulant factors (AT, protein C, protein S), parameters of thrombin generation (F1+2, TAT) and fibrinolysis (α_2-antiplasmin, plasminogen, PAP, D-dimer).
Children were in a hypercoagulable state after 4 weeks of dexamethasone due to upregulation of coagulation parameters. Upregulation was highest in the two youngest age groups. During Paronal® treatment the 11 - 16 year olds showed lower values in procoagulant and, even more, in anticoagulant factor levels compared to the younger children. Activation markers of thrombin generation and fibrinolysis did not change over time during the study period. The fibrinolytic potential was significantly more disturbed in children older than 11 years as a result of decreasing levels of α_2-antiplasmin and plasminogen during Paronal® treatment.
A more severe decline of anticoagulant and fibrinolytic parameters in children between 11 and 16 years of age underline that these children are at higher risk of thrombosis during ALL induction treatment.

INTRODUCTION

Alterations in hemostasis are frequently observed in children with acute lymphoblastic leukemia (ALL). Although the process of increased thrombin generation is already active at diagnosis,[1] thrombo-embolism, does not occur until anti-leukemic therapy is started, typically during the induction phase of therapy. The pathogenesis of this increased thrombotic risk is not fully understood. It includes a combination of variables related to the disease itself, its treatment and the host. As most drugs are given as combination chemotherapy, assessing how single agents affect the coagulation system is quite difficult.

L-Asparaginase and steroids have been studied most extensively in this respect. L-Asparaginase is an enzyme that converts the non-essential amino acid asparagine into aspartic acid and ammonia. The resulting inhibition in protein synthesis explains both for its antileukemic effect and its toxicity on the coagulation system. Different kinds of L-Asparaginase have different half-lives and different peak activities.[2,3] The extents of asparagine depletion, antileukemic effect and disturbance of the coagulation system are dependent on doses and type of L-Asparaginase preparation used.

Different investigators have shown that prednisone therapy is associated with higher F VIII, von Willebrand factor, F II and antithrombin (AT), and with lower fibrinogen levels.[1,4] Two trials in children with ALL[5,6] – randomizing dexamethasone versus prednisone – demonstrated no apparent differences in incidences of clinically overt thrombotic events.

In an earlier randomized study[7] we reported on the differences in hypercoagulability between two L-Asparaginase preparations: Crasnitin® (Bayer) and Erwinase® (Ipsen). Treatment with the *E. coli* L-Asparaginase Crasnitin® caused a delay in fibrin elimination and thus increased the risk of thrombosis, unlike Erwinase® treatment.

Andrew *et al.* as early as the late 1980's introduced the concept of developmental hemostasis,[8] which recently was confirmed by Monagle *et al.*[9] From age-related changes in the coagulation system this concept proposes that hemostasis in childhood is age-dependent, i.e. the youngest children are the best protected from thrombosis. In confirmation of this concept, Barry *et al*. and Moghrabi *et al.* pointed to an effect of age on the risk for symptomatic thrombo-embolic disease during Dana-Farber Cancer Institute ALL treatment protocols.[10,11] Patients aged 10 -18 years were more likely to experience L-Asparaginase related toxicity than were younger children (thrombotic events 11% versus 2%), even when stratified by risk group. Reports on changes in the coagulation system related to L-Asparaginase treatment in children of different age groups are lacking so far.

The current report analyses the effect of age on disturbances in coagulation and fibrinolysis in children with newly diagnosed ALL.

METHODS

Patients and therapy

Subjects eligible for this study were all children with newly diagnosed ALL consecutively admitted to our hospital between April 1997 and October 2000. The family history was checked for thrombophilia. Still, a possible familiar thrombophilic risk factor was not an exclusion criterion. Routine screening for thrombophilia was not done. None of the subjects had a history of bleeding or thrombotic disorders. Prior to instituting therapy informed consent was obtained from parents or guardians in accordance with the Declaration of Helsinki.

Therapy was according to the ALL-9 protocol of the Dutch Childhood Oncology Group based on the earlier ALL-6 treatment strategy.[12] Non-high risk ALL was defined as WBC < 50 x 10^9/L, no mediastinal mass, no t(9;22), no t(4;11) or MLL rearrangement, no T-cell phenotype and no central nervous system or testicular involvement. All other leukemias were defined as high risk. The induction phase for all patients is depicted in Figure 6.1. Four doses of Paronal® (6,000 IU/m^2, *E. coli* L-Asparaginase from Medac GmBH Germany) were administered intravenously in one-hour infusions twice a week.

All children had a central venous device. Minor allergic reactions (grade I or II) were counteracted by lowering the infusion rate. Consequently, before starting the next Paronal® infusion, these patients were given an antihistaminic drug, e.g. clemastine, and eventually steroids.

Sampling

During induction on days 29, 33, 36 and 40, blood samples were collected from the venous device immediately before each Paronal® infusion and on day 43. Blood samples were collected in tubes containing 3.8% trisodium citrate, chilled immediately in an ice bath and centrifuged at 20,000 rpm for 30 minutes at +4 ºC. The supernatant was withdrawn and stored at -80 ºC until the time of analysis.

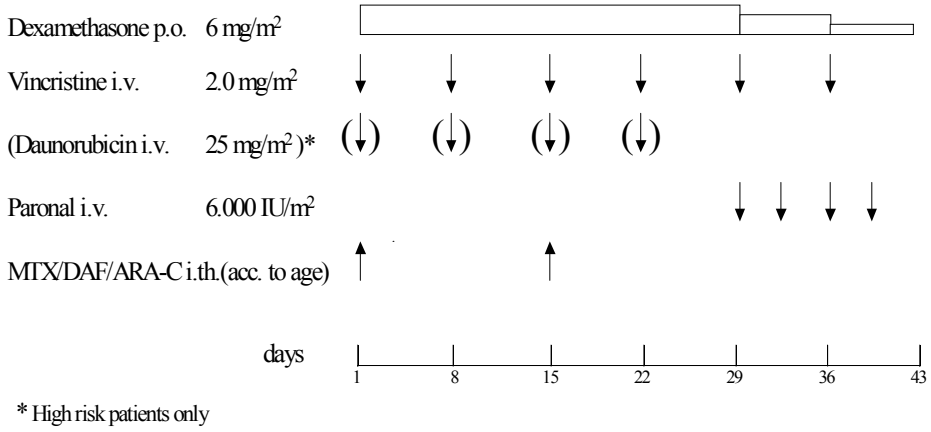


Figure 6.1 Induction therapy according to the DCOG-ALL-9 study

Coagulation assays
All coagulation assays were done with commercially available reagents and methods, as described previously.[7] All assays were performed on the Sysmex CA 1500 from Dade Behring (Germany). Reagents were supplied by Stago (France) or Dade Behring (Marburg, Germany).

Procoagulants
Factors II, V, VII, IX and X were measured in a one-stage assay using commercially available deficient plasma (Dade Behring, Marburg, Germany).

Anticoagulants
Antithrombin (AT) and protein C activities were measured functionally by a chromogenic assay (Dade Behring), protein C antigen by an enzyme-linked immunoadsorbent assay (Asserachrom, Stago, France). Total and free protein S levels were measured by quantitative ELISA (Asserachrom, Stago).

Thrombin generation
The fibrinogen concentration was measured according to the "Clauss" method;[13] normal reference ranges 2 - 4 g/L. The activation markers F1+2 and TAT were measured using commercially available ELISA techniques (Dade Behring). We adopted the reference values (5^{th} - 95^{th} percentile) for F1+2 provided by Dade Behring: 0.44 - 1.11 nmol/l. We constructed our own pediatric reference values for TAT on 62 healthy children between 1 and 15 years old: median 2.7 µg/l (5^{th} - 95^{th} percentile: 1.1 - 4.3 µg/l).

Fibrinolysis
Alpha-2-antiplasmin (α_2-AP) and plasminogen were determined in a kinetic test (Dade Behring). For the activation marker PAP, a commercially available ELISA technique was used (Dade Behring). The normal reference range (2.5^{th} - 97.5^{th} percentile) is 120 - 700 µg/l (Dade Behring) as determined in 466 healthy adults. There are no differences in normal values between children and adults.[14]

D-dimer levels were measured using an enzyme-linked immunoassay (Biopool, Ireland). The lower limit of sensitivity was 0.5 mg/ml, levels > 0.5 mg/ml point to an increased breakdown of fibrin monomers.

Normal values
In this manuscript we compared our data with reference values of Monagle *et al.*[9] except for protein S. We only measured antigen levels because of the interference of protein S activity levels with high factor VIII levels or hyperbilirubinemia. Stago data reported by Monagle and colleagues are compared with our Dade data.

Statistics
In concordance with previous studies, our results are reported for children aged 1 to 5 years, 6 to 10 years, and 11 to 16 years. Only for protein S values for boys and girls separately were calculated.[15,16]

The results are expressed as means and ranges (= minimum-maximum) so as to enable comparison with the data of Monagle *et al.* TAT, F1+2, PAP and D-dimer were transformed logarithmically in order to approximate normal distributions; for these variables data are given as medians (minimum-maximum). Repeated Measurements Analysis of Variance (SAS PROC MIXED) was used to evaluate differences between and within the different age groups. Interaction terms for age groups and time were used to evaluate whether the profiles of mean values over time (measurement days) of the 3 age groups deviated from parallelism. In case of significant time effects, comparisons were made with the first measurements on day 29. $P = 0.05$ (two-sided) was considered the limit of significance.

Data performed with Dade reagents are also evaluated with the outcomes of the various parameters expressed as the percentage of the predicted mean value, taking account of age and gender, if such reference values were available from Monagle *et al*.

RESULTS

Clinical data

After informed consent, 72 children consecutively admitted to our hospital with newly diagnosed ALL were enrolled in the study. Due to incomplete coagulation data, however, eight children were excluded from analysis. Characteristics of the remaining 64 children are shown in Table 6.1. All 64 completed the remission-induction phase. No septic periods occurred during the period analyzed. None of the children was treated with antithrombin concentrate or fresh frozen plasma.

One child, a nine-year-old boy, experienced a clinical thrombotic event. After a short period of severe headache he suddenly demonstrated a left sided hemiparesis one week after the last dose of Paronal®. An MR angiography showed a cerebral sinovenous thrombosis. At the time, AT was 65%, protein C activity 71%, protein C antigen 58%, protein S free 51% and total protein S 79%. He recovered completely.

Clinical complications such as pancreatitis, diabetes mellitus or hyperlipidemia were not observed. One child experienced an allergic reaction grade II to the fourth Paronal® infusion but after anti-allergic treatment the total dose could be administered.

Coagulation data

Tables 6.2 - 6.5 provide the results over time from before each Paronal® infusion on day 29, 33, 36, and 40 and from day 43 for the group as a whole and for the three different age groups. Except for 2 parameters, protein S total and log (TAT), the profiles over time did not significantly differ between the age groups. This points to a general parallel change of coagulation parameters over time for the separate age groups.

Table 6.1 Characteristics of 64 patients

Characteristics	n
Non high risk / high risk	48 / 16
Male / female	42 / 22
Age years	6.7 (1 - 16.7)*
WBC (x 10^9/L) at diagnosis	
< 50	50
50-100	9
>100	5
Age-group	
1-5 years (F / M)	35 (12 / 23)
6-10 years (F / M)	15 (5 / 10)
11-16 years (F / M)	14 (5 / 9)
Immunophenotyping	
c ALL	48
pre B ALL	12
T ALL	4

** mean (range)*

Procoagulants (Table 6.2)
Raw data of factor V demonstrated no change over time or between age group. All mean values except 2 were significantly higher as compared to mean reference values. Although there were significant differences between study days for the data expressed as % of mean (P = 0.02), none of the differences with day 29 was significant. Factors II, IX and X all declined significantly (P < 0.001) compared to day 29 values. In addition, there were significant differences between age groups. The same applies to the data expressed as percentages of the age specific normal reference values. Factor VII is the only protein whose level increased significantly over time for the group as a whole, and there were no significant differences between age groups.

Anticoagulants (Tables 6.3A and 6.3B)
All anticoagulants demonstrated a significant decline over time (P < 0.001). In addition, for all parameters there were significant effects of age group. The AT, protein C and S values generally were lower in the children aged 11 - 16 years as compared to others. AT is shown in Figures 6.2A and 6.2B: while mean levels were well above the mean reference value at day 29, all mean values were below at later days.

While free protein S levels demonstrated the same profile over time for the three age groups, this was not the case for total protein S (P = 0.005). However, within each age group all levels were significantly lower than those on day 29. No differences in free and total protein S between boys and girls were found (data not shown).

Table 6.2 Procoagulant factors

Paronal® 6000 IU/m²	Day 29 ↑	Day 33 ↑	Day 36 ↑	Day 40 ↑	Day 43	Raw data *P* days	*P age*	% of normal *P* days	*P age*
Procoagulants									
F V	**139**	**148**	**137**	**140**	**139**	**0.08**	**0.07**	**0.02**	**0.42**
1 - 5 years	140	157	146	142	150				
(97; 67 - 127%)	105 - 189	64 - 284	77 - 272	88 - 197	61 - 282				
6 - 10 years	141	146	136	153	142				
(99; 56 - 141%)	81 - 216	65 - 231	69 - 210	110 - 49	90 - 186				
11 - 16 years	136	120	113	121	117				
(89; 67 - 141%)	94 - 204	49 - 198	49 - 180	32 - 200	46 - 188				
F II	**134**	**120**	**113**	**116**	**112**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
1 - 5 years	139	126	120	122	120				
(89; 70 - 109%)	88 - 207	93 - 194	65 - 146	84 - 195	88 - 176				
6 - 10 years	132	114	114	123	120				
(89; 67 - 110%)	93 - 163	88 - 156	87 - 142	79 - 195	93 - 143				
11 - 16 years	128	108	91	89	88				
(90; 61 - 107%)	104 - 149	81 - 134	71 - 112	69 - 119	55 - 125				
F VII	**113**	**147**	**153**	**164**	**161**	**< 0.001**	**0.48**	**< 0.001**	**0.33**
1 - 5 years	112	142	150	157	153				
(111; 72 - 150%)	54 - 183	64 - 239	80 - 285	99 - 270	94 - 216				
6 - 10 years	119	159	157	182	188				
(113; 70 - 156%)	77 - 186	97 - 218	95 - 232	101 - 313	106 - 359				
11 - 16 years	113	141	156	161	157				
(118; 69 - 200%)	58 - 177	85 - 204	90 - 318	106 - 234	90 - 265				
F IX	**168**	**110**	**107**	**95**	**92**	**< 0.001**	**0.005**	**<0.001**	**< 0.001**
1 - 5 years	171	119	117	104	107				
(85; 44 - 127%)	82 - 240	46 - 200	26 - 217	43 - 186	49 - 216				
6 - 10 years	170	101	106	93	88				
(96; 48 - 145%)	87 - 222	49 - 183	40 - 153	42 - 159	15 - 136				
11 - 16 years	153	96	74	73	65				
(111; 64 - 216%)	41 - 212	50 - 153	40 - 112	38 - 131	30 - 100				
F X	**154**	**132**	**123**	**127**	**116**	**< 0.001**	**< 0.001**	**< 0.001**	**0.01**
1 - 5 years	163	145	136	139	126				
(98; 72 - 125%)	62 - 232	70 - 249	70 - 189	79 - 197	74 - 200				
6 - 10 years	145	119	117	124	124				
(97; 68 - 125%)	112 - 201	77 - 183	73 - 207	91 - 177	97 - 153				
11 - 16 years	137	110	90	98	87				
(91; 53 - 122%)	95 - 200	73 - 154	58 - 149	54 - 157	44 - 154				

Data (percentages) are given as means (minimum-maximum). Reference mean values with 95% reference ranges for the different age groups according to Monagle[9] are given in italics. P-values from ANOVA comparisons between study days of raw data and of data expressed as % of normal are denoted by p days, p age denotes ANOVA comparisons between the different age groups.

Table 6.3A Anticoagulant factors

Paronal® 6000 IU/m²	Day 29 ↑	Day 33 ↑	Day 36 ↑	Day 40 ↑	Day 43	Raw data *P* days	*P age*	% of normal *P* days	*P age*
Anticoagulants									
AT	**153**	**106**	**98**	**91**	**86**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
1 - 5 years	157	113	106	102	94				
(116; 101 - 131%)	103 - 202	78 - 142	72 - 144	71 - 147	62 - 138				
6 - 10 years	154	100	92	84	84				
(114; 95 - 134%)	130 - 183	83 - 117	71 - 116	59 - 109	56 - 112				
11 - 16 years	144	95	78	67	71				
(111; 96 - 126%)	118 - 168	71 - 120	88 - 95	42 - 87	42 - 114				
Prot C act	**172**	**123**	**114**	**110**	**100**	**< 0.001**	**0. 005**	**< 0.001**	**0. 07**
1 - 5 years	174	129	123	118	109				
(96; 65 - 127%)	100 - 329	45 - 216	55 - 171	78 - 188	53 - 186				
6 - 10 years	174	119	113	111	104				
(100; 71 - 129%)	132 - 258	82 - 186	68 - 177	71 - 162	69 - 147				
11 - 16 years	167	111	89	87	79				
(94; 66 - 118%)	84 - 228	42 - 142	39 - 165	34 - 138	37 - 144				

Data (percentages) are given as means (minimum-maximum).
A. Reference mean values with 95% reference ranges for the different age groups according to Monagle[9] are given in italics. P-values as in Table 6.2.

Table 6.3B Anticoagulant factors

Prot C ag	**150**	**107**	**101**	**99**	**92**	**< 0.001**	**0.008**
1 - 5 years	152	113	109	107	101		
	70 - 285	35 - 175	57 - 156	61 - 198	38 - 144		
6 - 10 years	152	102	97	99	94		
	108 - 194	57 - 158	63 - 144	58 - 137	76 - 120		
11 - 16 years	141	95	78	73	70		
	89 - 176	41 - 133	39 - 115	36 - 113	36 - 106		
Prot S free	**102**	**68**	**60**	**59**	**58**	**< 0.001**	**0.001**
1 - 5 years	104	73	65	65	64		
	11 - 154	37 - 124	28 - 94	40 - 101	24 - 114		
6 - 10 years	102	64	60	61	64		
	83 - 120	44 - 95	41 - 96	33 - 92	46 - 90		
11 - 16 years	98	59	46	42	41		
	74 - 142	42 - 81	35 - 59	28 - 53	22 - 61		
Prot S total	**106**	**83**	**79**	**79**	**81**	**< 0.001**	**< 0.001**
1 - 5 years	109	86	86	86	89		
	59 - 153	46 - 150	51 - 156	51 - 149	53 - 124		
6 - 10 years	107	78	76	80	88		
	86 - 145	58 - 96	54 - 93	50 - 100	77 - 98		
11 - 16 years	99	78	61	56	58		
	81 - 114	72 - 92	44 - 86	39 - 71	42 - 81		

B. Only raw data (percentages) are given. P-values from ANOVA comparisons between study days are denoted by p days, p age denotes ANOVA comparisons between the different age groups.

Table 6.4 Parameters of thrombin generation

Paronal® 6000 IU/m²	Day 29 ↑	Day 33 ↑	Day 36 ↑	Day 40 ↑	Day 43	Raw data *P* days	*P age*
Thrombin generation							
Fibrinogen g/L	**1.20**	**0.92**	**0.94**	**1.10**	**1.60**	**< 0.001**	**0.005**
1 - 5 years	1.34	1.08	1.15	1.29	1.64		
	0.1 - 2.6	0.1 - 2.0	0.1 - 2.5	0.1 - 2.4	0.5 - 2.7		
6 - 10 years	1.13	0.81	0.84	1.18	1.47		
	0.5 - 1.9	0.5 - 1.6	0.1 - 1.6	0.1 - 2.2	0.1 - 2.2		
11 - 16 years	0.93	0.51	0.45	0.60	1.57		
	0.6 - 1.6	0.1 - 1.0	0.1 - 0.9	0.1 - 0.7	0.1 - 5.9		
F1+2 nmol/L median	**1.10**	**1.08**	**1.19**	**1.09**	**1.15**	**0.14**	**0.33**
1 - 5 years	0.91	0.98	1.10	1.01	1.02		
	0.49 - 20.54	0.44 - 2.07	0.35 - 111.9	0.52 - 6.92	0.6 - 3.3		
6 - 10 years	1.32	1.16	1.47	1.25	1.47		
	0.6 - 3.99	0.55 - 2.36	0.45 - 2.78	0.56 - 127	0.58 - 3.13		
11 - 16 years	1.46	1.29	1.51	1.23	1.28		
	0.72 - 2.04	0.48 - 2.08	0.57 - 2.41	1.0 - 2.29	0.85 - 1.98		
TAT µg/L median	**6.5**	**4.3**	**4.0**	**3.2**	**4.8**	**< 0.001**	**0.31**
1 - 5 years	5.5	4.1	5.5	4.2	6.4		
	1.6 - 414	1.5 - 11.8	1.6 - 2157	1.9 - 148	2.0 - 46.3		
6 - 10 years	7.4	5.8	3.9	2.4	5.1		
	1.8 - 54.4	2.3 - 36	1.5 - 15.8	1.4 - 4.6	2.5 - 12.1		
11 - 16 years	6.5	3.5	3.0	3.2	3.3		
	2.6 - 27.7	2.1 - 8.6	1.7 - 5.2	2.0 - 7.3	2.2 - 9.0		

Data are given as means, or as medians when the distribution was skewed, and minimum-maximum. P-values from ANOVA comparisons between study days are denoted by p days, p age denotes ANOVA comparisons between the different age groups.

Thrombin generation (Table 6.4)

For fibrinogen differences were found between age groups (P = 0.005) as well as between study days (P < 0.001). Compared to general reference values, fibrinogen levels were decreased on day 29 in all age groups. They further declined significantly on day 33 - 36, and increased significantly (P < 0.001) to levels still below the normal reference levels on day 43.

Irrespective of age, F1+2 levels did not change over time during Paronal® treatment (P = 0.33). The profiles over time of mean logarithmically transformed TAT levels differed between age groups. Comparisons within each age group showed significant differences with day 29 for age group 6 - 10 years and 11 - 16 years. For both these groups values at day 36 and 43 were significantly lower.

Fibrinolysis (Table 6.5)

α_2-AP, plasminogen and PAP levels decreased significantly over time (P < 0.001). For α_2-AP and plasminogen also significant differences between age groups were found (p < 0.001), but not for PAP (P = 0.73). Irrespective of age, D-dimers demonstrated no changes over time.

Table 6.5 Parameters of fibrinolysis

Paronal® 6000 IU/m²	Day 29 ↑	Day 33 ↑	Day 36 ↑	Day 40 ↑	Day 43	Raw data *p* days	*p age*
Fibrinolysis:							
α_2-AP	**136**	**99**	**92**	**88**	**91**	**< 0.001**	**< 0.001**
1 - 5 years	142	108	1.03	98	104		
	101 - 282	68 - 198	54 - 135	50 - 141	65 - 174		
6 - 10 years	128	87	89	90	91		
	109 - 180	51 - 156	49 - 124	45 - 141	68 - 114		
11 - 16 years	127	85	64	58	66		
	95 - 198	40 - 129	36 - 99	37 - 82	40 - 115		
Plasminogen	**116**	**82**	**79**	**89**	**85**	**< 0.001**	**< 0.001**
1 - 5 years	123	91	87	94	95		
	108 - 234	57 - 153	48 - 145	50 - 142	59 - 144		
6 - 10 years	111	73	80	106	94		
	82 - 140	51 - 99	41 - 132	50 - 375	62 - 128		
11 - 16 years	105	67	55	57	60		
	81 - 131	48 - 97	41 - 75	33 - 77	43 - 84		
PAP µg/L median	**215**	**91**	**93**	**88**	**82**	**< 0.001**	**0.73**
1 - 5 years	199	97	128	101	83		
	51 - 945	17 - 553	28 - 272	4 - 330	37 - 306		
6 - 10 years	251	88	78	87	77		
	70 - 918	32 - 406	14 - 461	18 - 306	31 - 523		
11 - 16 years	252	73	95	58	85		
	3 - 1403	28 - 459	24 - 325	16 - 475	21 - 317		
D dimer mg/L median	**0.12**	**0.12**	**0.11**	**0.10**	**0.12**	**0.12**	**0.25**
1 - 5 years	0.10	0.12	0.09	0.09	0.11		
	0.03 - 0.47	0.05 - 0.23	0.05 - 0.23	0.03 - 0.33	0.07 - 0.52		
6 - 10 years	0.13	0.12	0.09	0.10	0.12		
	0.09 - 0.31	0.07 - 0.39	0.07 - 0.33	0.08 - 0.31	0.03 - 0.41		
11 - 16 years	0.18	0.12	0.16	0.13	0.17		
	0.05 - 0.47	0.03 - 0.24	0.05 - 0.24	0.06 - 0.28	0.06 - 0.22		

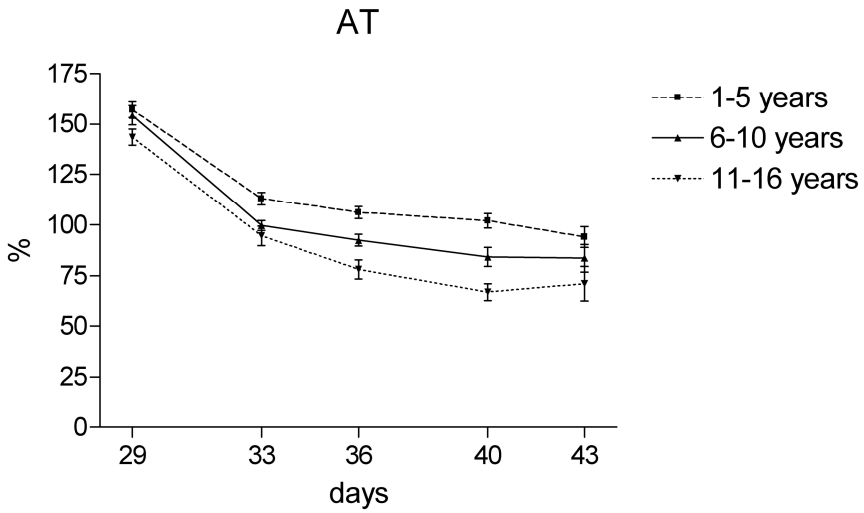
Data are given as means, or as medians when the distribution was skewed, and minimum-maximum. P-values as in Table 6.4.

DISCUSSION

More intensive thrombin generation and reduced antithrombotic potential are thought to be key factors for thrombo-embolic complications in children treated for ALL. Most of the thrombotic events occur during induction therapy.[17] In the present study we analyzed changes in coagulation parameters in children treated for ALL according to the DCOG-ALL-9 protocol. We report changes in the group as a whole and age dependent changes.

New data on developmental hemostasis have recently been published by Monagle *et al.*[9] We used these reference values, even though most of our coagulation parameters were determined with materials from a different manufacturer. Practically, results obtained with reagents from different manufacturers should give the same pattern over time, especially when calibrated against international standards. Therefore, we feel the use of analyzers/reagents from different companies is acceptable in pediatrics, provided good laboratory practice is adhered to.

A raw data



B % of normal reference values

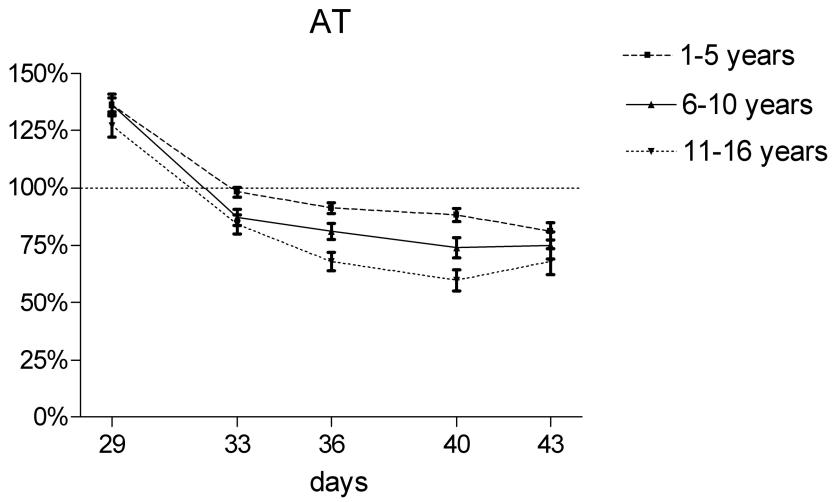


Figure 6.2 Plasma levels of AT in time
(A) Plasma levels in time of AT during the induction phase of the DCOG ALL-9 treatment schedule. Observed raw data are given as means ± SEM.
(B) Means ± SEM per age group with data expressed as percentage of the normal reference mean values per age group according to Monagle.[9]

The four week period of dexamethasone resulted in a hypercoagulable state on day 29 due to upregulation of all coagulation parameters except fibrinogen, as described earlier by Mall *et al.*[18] The underlying mechanism of rising levels of coagulation proteins with administration of corticosteroids remains to be unraveled. Some insight comes from an animal study performed by Sevaljevic *et al.*:[19] they demonstrated that corticosteroids downregulate fibrinogen on a transcriptional level.

In the present study, there were slight signs of thrombin generation but not of fibrinolysis before the first dose of Paronal®. When dexamethasone doses were tapered off from day 29 until day 43, treatment with four doses of 6,000 IU/m^2 of

Paronal® resulted in significant declining levels of nearly all coagulation proteins measured, most prominently in the children aged 11 - 16 years. A similar pattern was seen if data were expressed as percentages of age-related reference values. Patterns of decline and recovery of the coagulation protein levels were parallel for the three age groups. While pretreatment levels were reached by day 43 in the younger children, recovery took longer in the oldest group. This effect might in part be ascribed to different biological half-lives: F VII with a markedly short half-life hardly demonstrated changes in time, and AT and protein S with half-lives of about 60 hours tended to recover more slowly.

The activation markers F1+2 and D-dimer of thrombin generation and fibrinolysis showed no consistent change in any of the age groups, only slightly increased levels of TAT pointed to a persistent consumption of AT. Precursor B-ALL and T-ALL demonstrated the same TAT levels in the different age groups, in contrast to findings reported by Giordaneo *et al.*[20] PAP levels of the oldest group even declined to 58 μg/L. The decrease in α_2-AP and plasminogen in relation to the parallel reduction in PAP levels points to a decrease in synthesis of α_2-AP and plasminogen during L-Asparaginase treatment in induction.[7] A state of decreased fibrinolytic potential remained. The highest risk of thrombosis seemed to present itself at the end of Paronal® treatment for the children aged 11 - 16 years, seeing the more severe decline of anticoagulant and fibrinolytic parameters and the slower recovery to normal levels compared to the younger children.

Monagle and colleagues demonstrated a reduced risk of thrombosis without higher risk of bleeding in young children.[9] The fibrinolytic state found in younger children is comparable to that in adults.[14] Only adolescents have been found to have an impaired fibrinolytic response to venous occlusion when this was compared to the fibrinolytic response of adults.[21] Adolescents might therefore have a higher risk of thrombosis. Then, interference of a disease like ALL and treatment with steroids and L-Asparaginase might even increase the risk of thrombosis. As we demonstrated here, the younger the child with ALL, the better he/she will be protected from thrombo-embolic complications. Not only on account of physiological mechanisms, but also because steroids and L-Asparaginase have lesser impact on coagulation protein synthesis.

Extracellular amino acid depletion by L-Asparaginase influences the hepatic synthesis of proteins.[22] The influence of L-Asparaginase differed for the biosynthesis of various coagulation proteins in our study: most protein levels significantly decreased during L-Asparaginase treatment. Nevertheless, F V and F VII levels stayed in the same normal range or even increased during L-Asparaginase therapy. This might be due to increased production of F V by reticulo-endothelial cells when production by hepatocytes is impaired. F VII has the shortest half-life (4 - 7 hours) of the vitamin K dependent clotting factors. If enough vitamin K becomes available, the production of F VII would probably be adjusted immediately.

It is well known that different L-Asparaginase preparations have different pharmacokinetic and pharmacodynamic properties.[3,23,24] This is reflected by the observed variability in coagulation parameters following therapy with different

L-Asparaginase preparations, as we demonstrated in a randomized study.[7] Nowak-Gottl[25-28] reported that children treated with Paronal® show more changes than those treated with an L-Asparaginase product such as Crasnitin®. Regrettably, they did not measure activation markers like PAP.[29,30]

L-Asparaginase alone may not be a major determinant in thrombo-embolism, but other agents – especially steroids – were likely to modify the effects of L-Asparaginase on hemostasis. Higher doses of steroids, as dictated by the DFCI-protocols, lead to a much greater increase in levels of procoagulant factors and to suppression of the fibrinolytic potential.[31,32] These effects were thought to be responsible for the high incidences of thrombo-embolic events in the DFCI-studies. Not only higher doses of steroids but also the co-administration of L-Asparaginase might raise the risk of thromboembolic disease.[30] Monotherapy with L-Asparaginase even leads to a more severe fall in coagulation proteins compared to concomitant treatment with corticosteroids.[29,30]

There is discussion about the possible effects of hereditary prothrombotic risk factors.[33,34] Realizing that as such they have little importance in the development of thrombo-embolic events in childhood, we think that in a hypercoagulable situation due to antileukemic therapy with corticosteroids and L-Asparaginase they indeed may be of influence. This would as well explain the low rate of symptomatic thrombosis. We did not monitor these risk factors. Nevertheless, both the COALL study – even using high dose L-Asparaginase (45.000 IU/m^2) – and the PARKAA study (prophylactic antithrombin replacement in kids with ALL on L-Asparaginase) could not confirm a role of prothrombotic risk factors in the development of thromboses during their treatment regimens.[33,35]

Recently, Weyrich *et al.* identified that mammalian target of rapamycin (mTOR) inhibition induced platelet activation by thrombin and blocked clot retraction[36] The mTOR signaling pathway is a kinase, regulating cell growth. Possibly the inhibitory effect of L-Asparaginase on mTOR[37] leads as well to diminished clot retraction. This may explain that the risk for thrombosis by L-Asparaginase is in part counterbalanced by a diminished clot retraction.

CONCLUSION

We demonstrated that L-Asparaginase induced disturbances in coagulation and fibrinolysis in children with newly diagnosed ALL are age specific. Twice weekly doses of 6,000 IU/m^2 of Paronal® administered after four weeks of dexamethasone resulted in a higher risk of thrombosis for especially children aged 11 - 16 years.

REFERENCES

1. Athale UH, Chan AKC. Thrombosis in children with acute lymphoblastic leukemia Part II. Pathogenesis of thrombosis in children with acute lymphoblastic leukemia: effects of the disease and therapy. Thrombosis Research. 2003;111:199-212.
2. Avramis VI, Panosyan EH. Pharmacokinetic/Pharmacodynamic relationships of Asparaginase formulas. Clin Pharmacokinet 2005;44367-44393.
3. Asselin BL. The three aspraginases.Comparative pharmacology and optimal use in childhood leukemia. Adv Exp Med Biol. 1999;457:621-629.
4. Sartori TM, Maurizio PG, Sara P, Ugolino L, Annalisa A, Panagiotis T. Relation between long-term steroid treatment after heart transplantation, hypofibrinolysis and myocardial microthrombi. J Heart Lung Transplatation 1999;8:693-700.
5. Bostrom BC, Sensel MR, Sather HN, Gaynon PS, La MK, Johnston K, et al. Deaxamethasone versus prednisone and daily oral versus weekly intravenous mercaptopurine for patients with standard-risk acute lymphoblastic leukemia: a report from the Children's Cancer Group. Blood. 2003;101:3809-3817.
6. Mitchell CD, Richards SM, Kinsey SE, Lilleyman J, Vora A, Eden TOB. Benefit of dexamethasone compared to prednisolone for childhood acute lymphoblastic leukemia: results of the UK Medical Research Council ALL97 randomized trial. Br J Haematol. 2005;129:734-745.
7. Appel IM, Hop WC, Pieters R. Changes in hypercoagulability by asparaginase: a randomized study between two asparaginases. Blood Coagul Fibrinolysis. 2006;17:139-146.
8. Andrew M, Vegh P, Johnston M, Bowker J, Ofosu F, Mitchell L. Maturation of the hemostatic system during childhood. Blood. 1992;80:1998-2005.
9. Monagle P, Barnes C, Ignjatovic V, Furmedge J, Newall F, Chan A, et al. Developmental haemostasis. Impact for clinical haemostasis laboratories. Thromb Haemost. 2006;95:362-372.
10. Barry E, DeAngelo DJ, Neuberg D, Stevenson K, Loh ML, Asselin BL, et al. Favorable outcome for adolescents with acute lymphoblastic leukemia treated on Dana-Farber Cancer Institute Acute Lymphoblastic Leukemia Consortium Protocols. J Clin Oncol. 2007;25:813-819.
11. Moghrabi A, Levy DE, Asselin B, Barr R, Clavell L, Hurwitz C, et al. Results of the Dana-Farber Cancer Institute ALL Consortium Protocol 95-01 for children with acute lymphoblastic leukemia. Blood. 2007;109:896-904.
12. Veerman AJP, Hahlen K, Kamps WA, van Leeuwen EF, de Vaan GA, Solbu g, et al. High cure rate with moderate intensive treatment regimen in non-high risk childhood ALL. Results of protocol VI (1984-1988) from the Dutch Childhood Leukemia Study Group. J Clin Oncol. 1996;14:911-918.
13. Clauss A. Gerinnungsphysiol. Schnellmethode zur Bestimmung des Fibrinogens. Acta Haemat. 1957;17:237-246.
14. Ries M, Klinge J, Rauch R. Age-related reference values for activation markers of the coagulation and fibrinolytic system. Thrombosis Research. 1997;85:341-344.
15. Flanders MM, Phansalkar AR, Crist RA, Roberts WL, Rodgers GM. Pediatric reference intervals for uncommon bleeding and thrombotic disorders. J Pediatr. 2006;149:275-277.
16. Dykes AC, Walker ID, McMahon AD, Islam SI, Tait RC. A study of Protein S antigen levels in 3788 healthy volunteers: influence of age, sex and hormone use, and estimate for prevalence of deficiency state. Br J Haematol. 2001;113:636-641.
17. Caruso V, Iacoviello L, Di Castelnuovo A, Storti S, Mariani G, de Gaetano G, et al. Thrombotic complications in childhood acute lymphoblastic leukemia: a meta-analysis of 17 prospective studies comprising 1752 pediatric patients. Blood. 2006;108:2216-2222.

18. Mall V, Thomas KB, Sauter S, Niemeyer CM, AH S. Effect of glucocorticosteroids, E.Coli- and Erwinia L-Asparaginase on hemostatic proteins in children with acute lymphoblastic leukemia. Klin Padiatr. 1999;211:205-210.

19. Sevaljevic L, Macvanin M, Zakula Z, Kanazir DT, Ribarac-Septic N. Adrenalectomy and dexamethasone treatment alter the patterns of basal and acute phase response-induced expression of acute phase protein genes in rat liver. J Steroid Biochem Mol Biol. 1998;66:347-353.

20. Giordano P, Del Vecchio GC, Santoro N, Arcamone G, Coppola B, Altomare M, et al. Thrombin generation in children with acute lymphoblastic leukemia: effect of leukemia immunophenotypic subgroups. Pediatr Hematol Oncol. 2000;17:667-672.

21. Monagle P, Chan AK, Albisetti M, Vegh P, Andrew M, Mitchell L. Fibrinolytic system in adolescents: response to venous occlusion stress tests. Pediatr Res. 2003;53:333-337.

22. Bushman JE, Palmieri D, Whinna HC, Church FC. Insight into the mechanism of asparaginase-induced depletion of antithrombin III in treatment of childhood acute lymphoblastic leukemia. Leuk Res. 2000;24:559-565.

23. Müller HJ, Boos J. Use of L-Asparaginase in childhood ALL. Crit Rev in Oncol/Heamat. 1998;28:97-113.

24. Boos J, Werber G, Ahlke E, Schulze-Westhof P, Nowak-Gottl U, Wurthwein G, et al. Monitoring of Asparaginase Activity and Asparagine Levels in Children on Different Asparaginase Preparations. European Journal of Cancer 1996;32A:1544-1550.

25. Nowak-Gottl U, Werber G, Ziemann D, Ahlke E, Boos J. Influence of two different Escherichia coli asparaginase preparations on fibrinolytic proteins in childhood ALL. Haematologica. 1996;81:127-131.

26. Nowak-Gottl U, Ahlke E, Schulze-Westhoff P, Boos J. Changes in coagulation and fibrinolysis in childhood ALL: a two-step dose reduction of one E. coli asparaginase preparation. Br J Haematol. 1996;95:123-126.

27. Nowak-Gottl U, Kuhn N, Wolff JE, Boos J, Kehrel B, Rath B, et al. Inhibition of hypercoagulation by antithrombin substitution in E. coli L-asparaginase-treated children. Eur J Haematol. 1996;56:35-38.

28. Nowak-Gottl U, Ahlke E, Klosel K, Jurgens H, Boos J. Changes in coagulation and fibrinolysis in childhood acute lymphoblastic leukaemia re-induction therapy using three different asparaginase preparations. Eur J Pediatr. 1997;156:848-850.

29. Mitchell L, Hoogendoorn H, Giles AR, Vegh P, Andrew M. Increased endogenous thrombin generation in children with acute lymphoblastic leukemia: risk of thrombotic complications in L'Asparaginase-induced antithrombin III deficiency. Blood. 1994;83:386-391.

30. Appel IM, van Kessel-Bakvis C, Stigter R, Pieters R. Influence of two different regimens of concomittant treatment with asparaginase and dexamethasone on hemostasis in childhood acute lymphoblastic leukemia. Leukemia. 2007;21:2377-2380.

31. van Giezen JJ, Brakkee JG, Dreteler GH, Bouma BN, Jansen JW. Dexamethasone affects platelet aggregation and fibrinolytic activity in rats at different doses which is reflected by their effect on arterial thrombosis. Blood Coagulation and Fibrinolysis. 1994;5:249-255.

32. Patrassi GM, Sartori MT, Livi U, Casonato A, Danesin C, Vettore S, et al. Impairment of fibrinolytic potential in long-term steroid treatment after heart transplantation. Transplantation. 1997;64:1610-1604.

33. Mauz-Korholz C, Junker R, Gobel U, Nowak-Gottl U. Prothrombotic risk factors in children with acute lymphoblastic leukemia treated with delayed E. coli asparaginase (COALL-92 and 97 protocols). Thromb Haemost. 2000;83:840-843.

34. Nowak-Gottl U, Heinecke A, von Kries R, Nurnberger W, Munchow N, Junker R. Thrombotic events revisited in children with acute lymphoblastic leukemia: impact of concomitant Escherichia coli asparaginase/prednisone administration. Thromb Res. 2001;103:165-172.

35. Mitchell LG, Andrew M, Hanna K, Abshire T, Halton J, Anderson R, et al. A prospective cohort study determining the prevalence of thrombotic events in children with acute lymphoblastic leukemia and a central venous line who are treated with L-asparaginase: results of the Prophylactic Antithrombin Replacement in Kids with Acute Lymphoblastic Leukemia Treated with Asparaginase (PARKAA) Study. Cancer. 2003;97:508-516.

36. Weyrich AS, Denis MM, Schwertz H, Tolley ND, Foulks J, Spencer E, et al. mTOR-dependent synthesis of Bcl-3 controls the retraction of fibrin clots by activated human platelets. Blood. 2007;109:1975-1983.

37. Iiboshi Y, Papst PJ, Kawasome H, Hosoi H, Abraham RT, Houghton PJ, et al. Amino Acid-dependent Control of p70 s6k. J Biol Chem. 1999;274:1092-1099.

7 chapter

Influence of two different regimens of concomitant treatment with L-Asparaginase and dexamethasone on hemostasis in childhood acute lymphoblastic leukemia

Inge M. Appel
Carla van Kessel-Bakvis
Rolinda Stigter
Rob Pieters

Leukemia 2007;21;2377-2380

INTRODUCTION

L-Asparaginase is an important drug in the treatment of childhood acute lymphoblastic leukemia (ALL). Its influence on the coagulation system has been extensively studied.[1] L-Asparaginase is an enzyme leading to serum depletion of asparagine and glutamine. Consequently, protein synthesis is depressed leading to coagulation protein deficiencies as a side effect of antileukemic treatment. The effect on antithrombotic proteins might induce an increased risk for thrombosis, while the effects on procoagulant proteins might increase the bleeding risk. L-Asparaginase is usually given in combination with other chemotherapeutic drugs among which especially corticosteroids may also influence the coagulation system. Corticosteroids may induce hypofibrinogenemia potentially leading to bleeding, but also increase the risk for thrombotic events by the induction of a hypofibrinolytic state in combination with an increase in procoagulant factors.[2] So, in general, it is difficult to analyze which coagulation disorders in children with ALL are due to L-Asparaginase and which are due to concomitant use of other drugs such as corticosteroids. In this study, we analyzed the influence of both these drugs (Paronal® and dexamethasone) in one group of children during two different parts of ALL therapy.

METHODS

During induction, according to the DCOG (Dutch Childhood Oncology Group)-ALL-9 treatment schedule,[3] 24 children with high-risk ALL were treated with among others dexamethasone from day 1 to day 29 followed by 15 days of tapering to zero and four doses of Paronal® (6000 IU/m^2) on days 29, 33, 36 and 40. During the intensification phase of this protocol, the treatment included one weekly dose of Paronal® (10000 IU/m^2) during 9 weeks and 7 days of dexamethasone once every 3 weeks during the same 9 weeks (Figure 7.1).

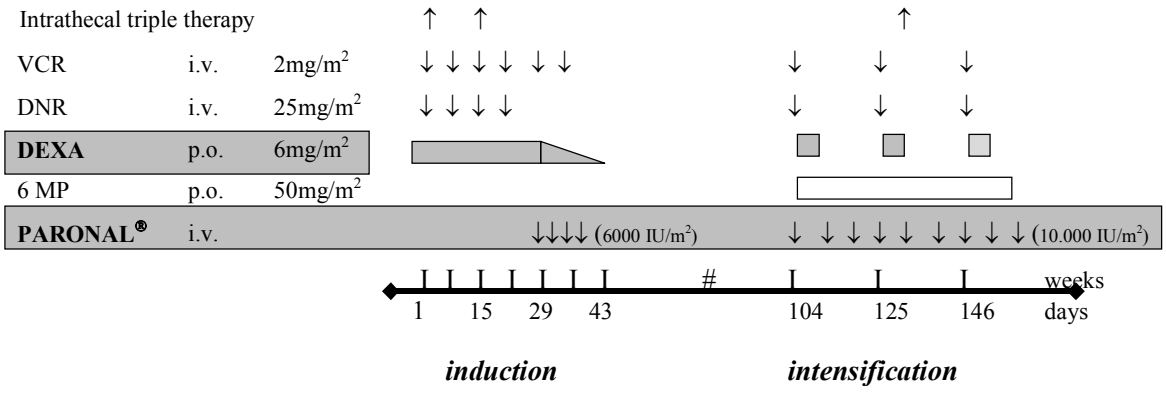


Figure 7.1 DCOG-ALL-9 treatment strategy
The induction and intensification part for only HR patients are given. Data are analyzed during induction treatment (days 29 - 43) and during intensification treatment (days 104 - 134).

Blood samples were collected immediately before each Paronal® infusion to analyze procoagulant (fibrinogen, F II, F V, F VII, F IX, F X) and anticoagulant factors (antithrombin, protein C, protein S) and parameters of thrombin generation (F1+2, thrombin-antithrombin (TAT) complex) and fibrinolysis (α2-antiplasmin, plasminogen, plasmin-α2 antiplasmin (PAP) complex, D-dimer).

RESULTS

After 4 weeks of dexamethasone on day 29, all procoagulant and anticoagulant factors demonstrated high levels, and a hypofibrinogenemia was observed (Figures 7.2 and 7.3). The four doses of Paronal® and tapering of the dose of dexamethasone (days 29 - 43) had the following effects on the procoagulant parameters:

- after an initial further decrease, fibrinogen levels stabilized at low levels or increased slightly (Figure 7.2A);
- F V levels stabilized at high levels (Figure 7.2B);
- F II, IX and X normalized (Figure 7.2C);
- only F VII increased to supranormal levels (Figure 7.2C);
- the anticoagulant parameters (AT, protein C and S) and the fibrinolytic potential (plasminogen and α2-antiplasmin) decreased significantly leading to a hypercoagulable state (Figures 7.3A and B);
- as a result of decreased protein synthesis of plasminogen and α2-antiplasmin, PAP too decreased significantly (Figure 7.3C);
- D-dimers demonstrated no changes, nor did TAT and F1+2, pointing to no signs of increased fibrinolysis or thrombin generation (Figure 7.3C).

In the intensification phase, only 11 children could be analyzed up to the sixth Paronal® infusion because at that point already 13 of 24 children had switched to another L-Asparaginase product (Erwinase or PEG-asparaginase) because of allergic reactions.
Interestingly, the effect of 1 weekly dose of L-Asparaginase with 1 week dexamethasone every 3 weeks during intensification was completely different compared to the effect during induction therapy, when dexamethasone was administered daily and L-Asparaginase twice a week:

- The levels of fibrinogen decreased significantly in relation to the 7-day administration of dexamethasone ($P < 0.0001$) (Figure 7.2A) and recovered during weekly L-Asparaginase administration in the absence of dexamethasone.
- F V did not demonstrate any durable changes (Figure 7.2B).
- F II, VII, IX and X increased during 1-week dexamethasone treatment and normalized afterwards during weekly administration of L-Asparaginase (Figure 7.2C).
- The levels of anticoagulant proteins and plasminogen and α2-antiplasmin hardly changed during this treatment phase (Figures 7.3A and B).
- No signs of increased thrombin generation and of fibrinolysis were monitored (Figure 7.3C).

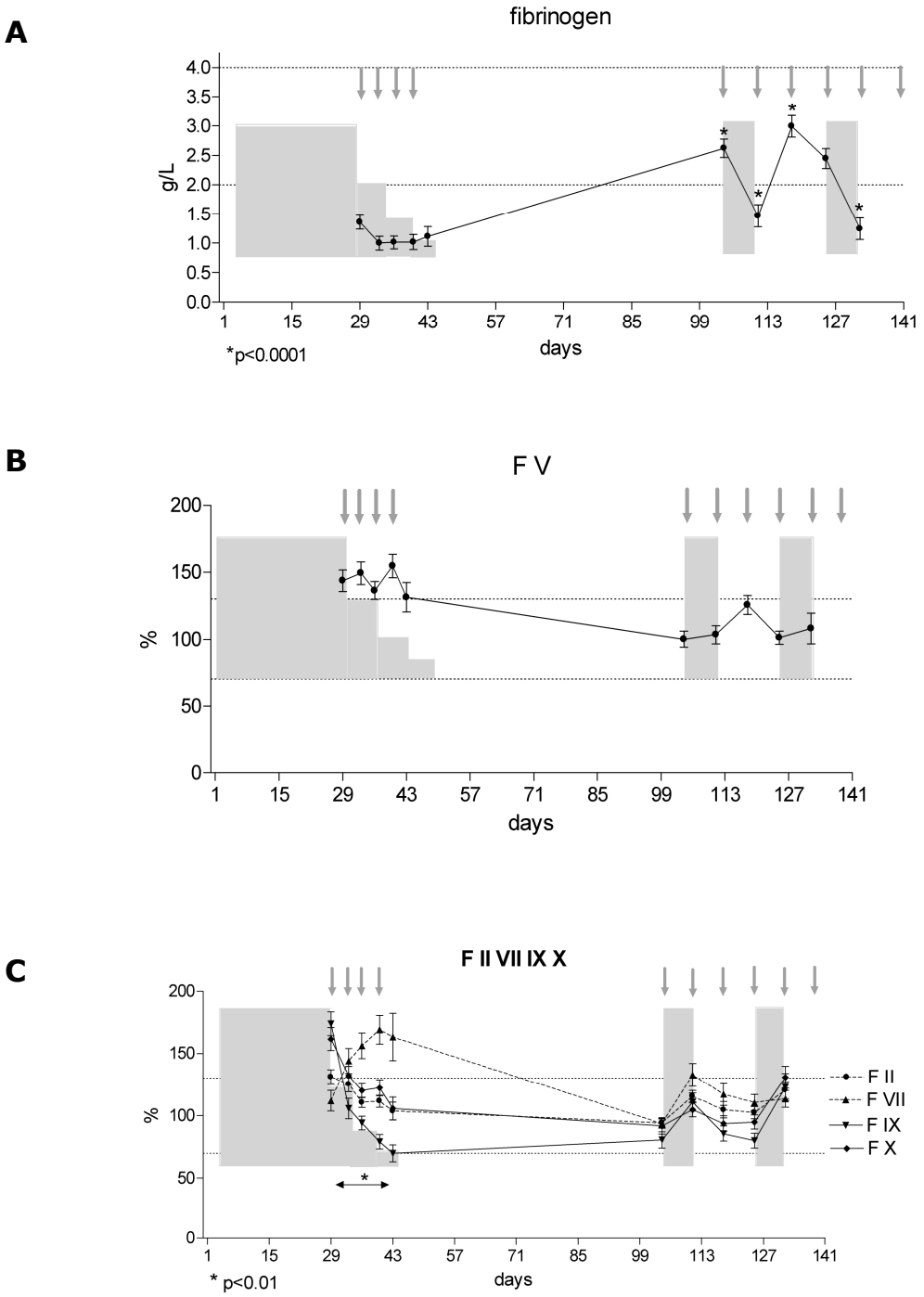


Figure 7.2 Plasma levels of procoagulant parameters during induction and intensification treatment

Plasma levels of fibrinogen (A), factor V (B) and vitamin K-dependent clotting factors (II, VII, IX and X) (C) during the induction and the intensification phase of the DCOG-ALL-9 treatment schedule.
During induction, dexamethasone 6 mg/m^2 was given for 4 weeks followed by tapering to zero in 15 days; Paronal® 6000 IU/m^2 was given twice a week on days 29, 33, 36 and 40. Intensification started at day 104 with Paronal® 10000 IU/m^{2}weekly and with 1 week dexamethasone 6 mg/m^2 every 3 weeks.
*The dexamethasone periods are drawn in gray, and arrows mark the Paronal® infusions. Data are given as means with standard error of the means. Normal reference ranges are marked with dotted lines. Differences between groups were calculated using one-way analysis of variance with Bonferroni's multiple comparison test to determine significance; P = 0.05 (two sided) was considered the limit of significance. The sign * refers to significant differences for comparison with previous measurements.*

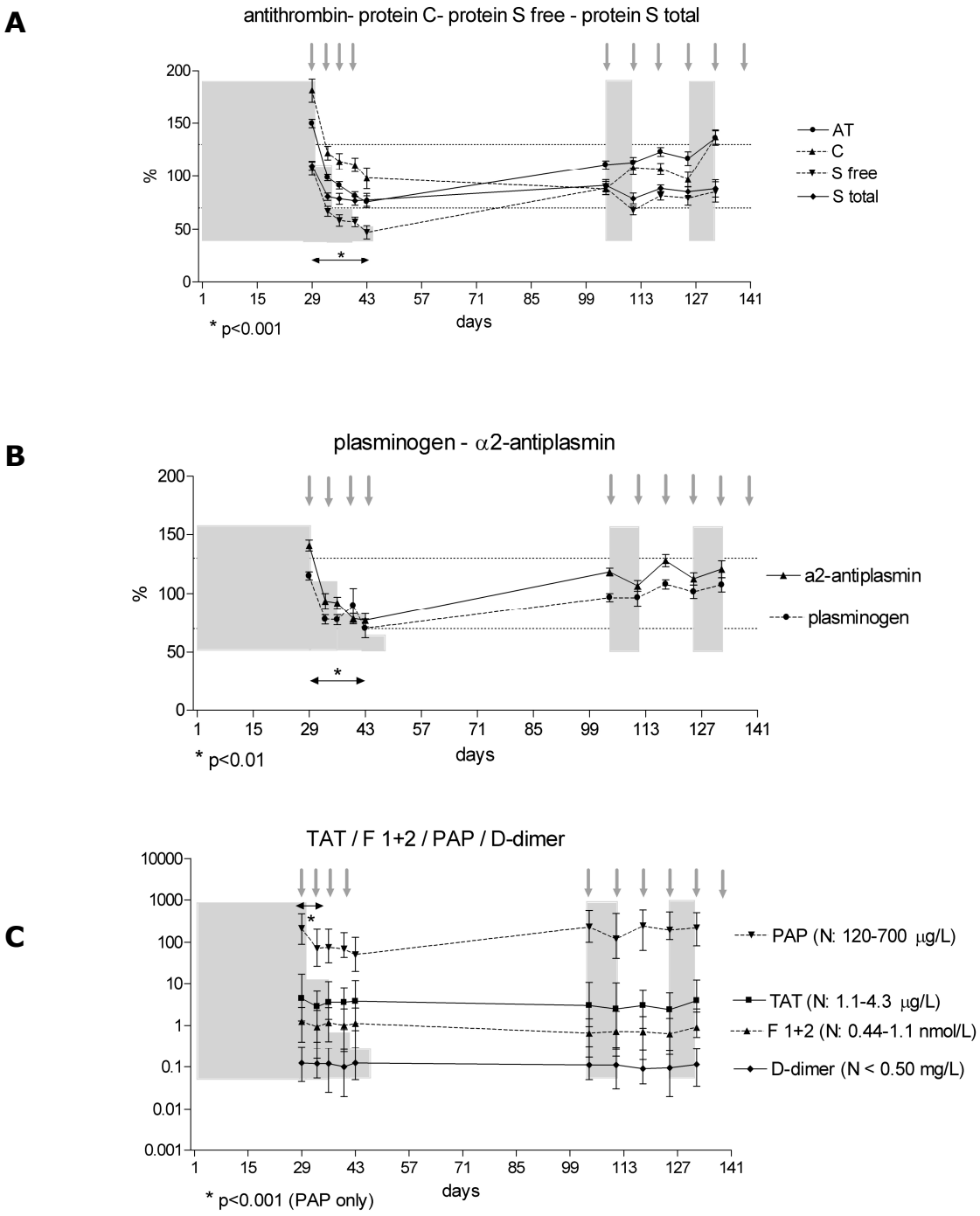


Figure 7.3 Plasma levels of anticoagulant parameters, parameters of fibrinolysis and of thrombin generation during induction and intensification

Plasma levels of anticoagulant proteins (antithrombin, protein C, free protein S and total protein S (A) and of plasminogen and α2-antiplasmin (B). Data are given as means with standard error of the means. Normal reference ranges are marked with dotted lines. For the activation markers thrombin–antithrombin complex, F1+2, plasmin–antiplasmin complex and D-dimers, data are given as medians with 25th and 75th percentiles (C). Normal reference ranges are shown between brackets.

*The dexamethasone periods are drawn in gray, and arrows mark the Paronal® infusions. Differences between groups were calculated using one-way analysis of variance, with Bonferroni's multiple comparison test to determine significance; P = 0.05 (two sided) was considered the limit of significance. The sign * refers to significant differences for comparison with previous measurements.*

DISCUSSION

The half-life of Paronal® has been calculated to be 23.0 ± 2.5 h.[4] It could be speculated that once a week 10,000 IU/m^2 of Paronal® would allow a recovery from diminished protein synthesis by L-Asparaginase, while twice weekly 6,000 IU/m^2 of Paronal® would lead to a cumulative effect with no time for recovery. But, it could also be hypothesized that the higher dose of Paronal® in the intensification phase would have led to prolonged inhibition of coagulation protein synthesis. Our data however, showed that the schedule of Paronal® once a week 10000 IU/m^2 resulted only in minor changes in coagulation proteins. One week of dexamethasone every 3 weeks in the intensification phase only, led to a significant decline in the level of fibrinogen (Figure 7.2A), which was followed a week later by a significant increase, whereas the combination of Paronal® and dexamethasone, as applied in induction, resulted in a severe decline in anticoagulant proteins and in a decreased fibrinolytic potential.

Based on these data, a hypercoagulable state was diagnosed during induction treatment only, whereas during intensification with a higher dose of Paronal® in a weekly schedule combined with only 1 week of dexamethasone every 3 weeks, the risk for thrombosis was minimal. We conclude that there is a crucial interaction between L-Asparaginase and dexamethasone in maintaining the balance between bleeding and thrombosis during therapy in childhood ALL and the effects on the coagulation system depend on the specific schedule of administration of these drugs.

REFERENCES

1. Athale UH, Chan AKC. Thrombosis in children with acute lymphoblastic leukemia. Part II. Pathogenesis of thrombosis in children with acute lymphoblastic leukemia: effects of the disease and therapy. Thromb Res. 2003;111:199-212.
2. van Giezen JJ, Brakkee JG, Dreteler GH, Bouma BN, Jansen JW. Dexamethasone affects platelet aggregation and fibrinolytic activity in rats at different doses which is reflected by their effect on arterial thrombosis. Blood Coagul Fibrinolysis. 1994;5:249-255.
3. Veerman AJ, Hahlen K, Kamps WA, Van Leeuwen EF, De Vaan GA, Solbu G, et al. High cure rate with a moderately intensive treatment regimen in non-high-risk childhood acute lymphoblastic leukemia. Results of protocol ALL VI from the Dutch Childhood Leukemia Study Group. I Clin Oncol. 1996;14:911-918.
4. Werber G. Pharmacokinetic/ pharmacodynamic monitoring of L-asparaginase in children. Hematology. 1995;70(Suppl II):130.

PART III

General Discussion and Summary

8 chapter

General discussion and perspectives

The main aim of this thesis was to study the pharmacokinetic and pharmacodynamic effects of one dose of PEG-Asparaginase in children with acute lymphoblastic leukemia (ALL) in order to get insight into the mechanisms of resistance to this drug. The second aim was to study which disturbances occur in coagulation parameters upon administration of L-Asparaginase.

PHARMACOKINETICS

Currently three preparations of L-Asparaginase are available. Each preparation, *E. coli* derived L-Asparaginase (Paronal®), its pegylated form (Oncaspar®) and the product from *Erwinia chrysanthemi* (Erwinase®), have different half-lives[1] and peak activities.[2] A L-Asparaginase activity of higher than 75 to 100 IU/L was shown to deplete asparagine from the serum of humans during several days.[2,3] Equivalent doses for optimal efficacy have been determined for different L-Asparaginase preparations, indicating that 1 dose of 1000-2500 IU/m^2 PEG-Asparaginase in 2 weeks is as effective as 5000 - 10.000 IU/m^2 *E. coli* L-Asparaginase every 3 days and 10.000 - 25.000 IU/m^2 *Erwinia* L-Asparaginase every 2 days in the same 2 weeks:[2,4-8]

1000 - 2500 IU/m^2 PEG-Asparaginase (Oncaspar®) once in 2 weeks
5000 - 10.000 IU/m^2 *E. coli* L-Asparaginase (Paronal®) once in 3 days for 2 weeks
10.000 - 25.000 IU/m^2 *Erwinia* L-Asparaginase (Erwinase®) once in 2 - 3 days for 2 weeks

The administration of 1000 IU/m^2 PEG-Asparaginase i.v. as monotherapy given upfront of regular chemotherapy in newly diagnosed children with ALL resulted into an enzyme activity of ≥ 100 IU/L for at least 10 days (this thesis). This resulted in a complete depletion of serum asparagine levels (≤ 0.2 μM) in all patients. However, no complete asparagine depletion in the cerebrospinal fluid (CSF) occurred. Similar data were recently also found by Rizzari *et al*.[9] who used the same dose of PEG-Asparaginase during ALL induction therapy. In a randomized study by Avramis *et al*. between native *E coli* L-Asparaginase (6000 IU/m^2 3 times per week) and PEG-Asparaginase (2500 IU/m^2 once) in children with standard risk ALL, CSF asparagine fell to median 0.6 μM on day 28 in the PEG-Asparaginase treated children and to 0.3 μM in the native *E. coli* L-Asparaginase treated group.[10] In relapsed ALL patients weekly 2500 IU/m^2 PEG-Asparaginase resulted in low but still detectable concentrations of CSF asparagine (0.2 - 3 μM).[11] Several groups have reported that sufficient CSF asparagine depletion (≤ 0.2 μM) was obtained when plasma L-Asparaginase activity was ≥ 100 IU/L.[6,12-14] Ahlke *et al.*[6] and Rizzari *et al.*[14] demonstrate complete CSF asparagine depletion (<0.2 μM) during 28 days using either 10.000, 5000 or 2500 IU/m^2 *E. coli* L-Asparaginase or 10.000 IU/m^2 *Erwinia* L-Asparaginase in comparable treatment schedules: 8 times L-Asparaginase at 3 day intervals. The activity of native L-Asparaginase in CSF is only about 0.2% of the L-Asparaginase activity in plasma.[15] Berg *et al.*[16] found

that 2500 IU/m^2 PEG-Asparaginase depleted asparagine in serum whereas variable amounts were still detected in the CSF of monkeys. The St Jude Children's Research Hospital Memphis analyzed the three different L-Asparaginase preparations at equivalent dosages in children with newly diagnosed ALL.[17] Median CSF asparagine concentrations were always higher in patients receiving PEG-Asparaginase compared to those receiving native *E. coli* L-Asparaginase or Erwinase®. It is important to notice that this was not a randomized study. Children received *E. coli* L-Asparaginase in induction, and PEG-Asparaginase in reinduction. Patients who developed clinical allergy to L-Asparaginase were switched to Erwinase®.

Combining our and previous results implies that L-Asparaginase and PEG-Asparaginase may not cross the blood-brain barrier. We assume that (PEG)-Asparaginase depletes the plasma asparagine pool and that CSF asparagine is primarily derived from the systemic asparagine pool.[15] It has been suggested that asparagine enters from the blood to the CSF against a concentration gradient[18] or that the central nervous system (CNS) may be capable of synthesizing asparagine locally despite depletion of the plasma asparagine pool.[19] It is not known whether an incomplete depletion of asparagine in the CSF results in a suboptimal antileukemic effect. However, depletion of asparagine in the CSF may be advocated to prevent meningeal leukemia. Treatment schedules including (PEG)-Asparaginase should therefore include additional CNS prophylactic therapy.

The importance of intensive L-Asparaginase therapy in pediatric ALL has been underlined by randomized studies of the Dana Farber Cancer Institute (DFCI)[20] and the European Organization for Research and Treatment of Cancer (EORTC).[21] Both studies randomized between native *E. coli* L-Asparaginase and *Erwinia* L-Asparaginase at identical dose schedules. The DFCI prescribed 25.000 IU/m^2 once weekly and the EORTC 10.000 IU/m^2 every 3 days of both drugs respectively. *Erwinia* L-Asparaginase was associated with less toxicity but a higher number of induction failures and relapses including those occurring in the CNS. Patients receiving *Erwinia* L-Asparaginase did not experience continuous asparagine depletion in contrast to children treated with *E. coli* L-Asparaginase. This may be explained by the fact that *Erwinia* L-Asparaginase has a shorter half-life than *E. coli* L-Asparaginase.[1] The data showed that a continuous depletion of asparagine is associated with a better clinical outcome than intermittent depletion.

The duration of L-Asparaginase treatment is also of prognostic importance.[20] Treatment with L-Asparaginase for more than 25 weeks during the reinduction phase of therapy resulted in a lower relapse rate than when children tolerated L-Asparaginase for a shorter period.[22-24]

Intensified extended use of L-Asparaginase therapy proved to compensate for treatment reduction in a study published by Pession *et al.*: standard risk patients treated according the BFM like treatment protocol received a treatment reduction in the second part of reinduction and were randomized for inclusion of 10.000 IU/m^2 native L-Asparaginase for 20 weeks.[23] The group with intensified L-Asparaginase treatment had a 10% rise in the 10-year disease free survival compared to the group without L-Asparaginase intensification.

Therefore, current and future treatment protocols of different study groups worldwide include intensified L-Asparaginase therapy. Also, plasma L-Asparaginase activity levels should be preferably monitored to adapt dosage in order to get continuous and complete asparagine depletion.

PHARMACODYNAMICS AND MECHANISM OF L-ASPARAGINASE RESISTANCE

Cell line studies showed that *in vitro* resistance to L-Asparaginase was mediated by upregulation of asparagine synthetase (AS) mRNA.[25,26] In this thesis we found that depletion of asparagine in plasma upon *in vivo* exposure to PEG-Asparaginase induced the level of AS mRNA in leukemic cells of children with ALL. However, both baseline and *in vivo* L-Asparaginase induced levels of AS mRNA did not differ between clinically good, intermediate and poor responders to this drug. Previous studies showed that *TEL-AML1* positive ALL patients are *in vitro* more sensitive to L-Asparaginase than *TEL-AML1* negative patients.[27,28] However, *TEL-AML1* positive cells expressed higher baseline levels of AS than their *TEL-AML1* negative counterparts and both subtypes of ALL did not differ in their capacity to upregulate AS upon exposure to L-Asparaginase.[29] In addition, the baseline AS mRNA level and *in vitro* L-Asparaginase resistance did correlate in *TEL-AML1* negative precursor B-ALL but not in *TEL-AML1* positive ALL.[29] Taken together these studies imply that AS levels or upregulation of these levels is not a major cause of L-Asparaginase resistance in pediatric ALL. However, recently Su *et al.* demonstrated in ALL cell lines that measurement of asparagine synthetase protein, rather than mRNA, might serve as an indicator of L-Asparaginase sensitivity.[30] Ongoing studies, using novel antibodies directed against asparagine synthetase, are addressing this issue in pediatric ALL.

Since AS is only one enzyme out of a complex network of enzymes involved in the metabolism of amino acids, we also studied whether changes occurred in the intracellular amount of individual amino acids that may point to causes of resistance to L-Asparaginase. We found that the intracellular amount of 20 amino acids did not change upon *in vivo* L-Asparaginase treatment of children with ALL. Remarkably, L-Asparaginase treatment did not affect the percent of apoptotic cells in the peripheral blood, despite the fact that the leukemic cell count decreased upon treatment. In contrast, *in vitro* exposure to L-Asparaginase resulted in an intracellular depletion of asparagine and induced apoptosis as was detected by phosphatidyl serine externalization (Annexin V-positivity), caspase 3 activation and PARP inactivation (Ref 31 and this thesis). Therefore, the fact that no increase in the percent of apoptotic cells was detected *in vivo* suggests that the apoptotic leukemic cells are immediately removed from the blood circulation by phagocytosis. The exposure of phosphatidyl serine at the outer cell membrane of apoptotic cells is known to attract macrophages for that purpose.[32]

The fact that no changes in the intracellular amino acid levels were detected in the remaining viable leukemic cells is intriguing. This suggests that the surviving and hence resistant leukemic cells have used a rescue mechanism since

L-Asparaginase clearly reduces intracellular asparagine levels when tested *in vitro*. It has been shown that the microenvironment may supply asparagine and other amino acids such as found by *in vitro* experiments using mesenchymal cells.[33] It is yet unknown whether this rescue mechanism also occurs *in vivo*. However, one can envision that targeting ALL cells by reducing the expression of asparagine synthetase of the bone marrow mesenchymal cells may improve the effectiveness of L-Asparaginase therapy. Another explanation for the unchanged intracellular amino acid levels is that leukemic cells may use glutamine, present in excess in the plasma, as source for the amino moiety needed for *de novo* asparagine synthesis. The observed upregulation of AS expression upon L-Asparaginase exposure may then be used to re-synthesize sufficient asparagine using plasma glutamine. In addition, other tissues (e.g. liver) may re-synthesize asparagine although this will be immediately depleted again when L-Asparaginase plasma levels are still high enough. *In vitro*, this rescue mechanism may not occur since glutamine is being depleted from the culture medium due to an excess of L-Asparaginase that is known to have some glutaminase activity too.[34]

Recent studies have shown that inhibition of glutamine synthetase potentiates the toxicity of L-Asparaginase in cell lines derived from rhabdomyosarcoma and osteosarcoma.[35] In principle, inhibition of glutamine synthetase may also trigger apoptosis of leukemic cells and may overcome L-Asparaginase resistance in childhood ALL. However, glutamine starvation induces severe toxic effects on other organ systems. Glutamine plays a crucial role as source of nitrogen and carbon for DNA synthesis, as source of energy between organs, and is used for urea synthesis in the liver, renal ammonia genesis and gluconeogenesis in both liver and kidney. Therefore, it is unlikely that inhibition of glutamine synthesis will be effective without side effects in a clinical setting.[36]

Of interest is that the baseline amino acid profile in ALL cells differed from normal cells. Aspartic acid, glutamic acid and cystathionine levels were higher, and taurine levels lower compared to normal peripheral blood mononuclear cells. In addition, the total protein content of leukemic cells was ~2-fold lower than normal lymphocytes. Proliferating leukemic cells may produce more aspartic acid and glutamic acid due to a fast turnover of asparagine and glutamine.[37] The high levels of cystathionine and low levels of taurine might be related to a low activity of γ-cystathionase in leukemic cells.[38] This enzyme converts cystathionine into cysteine (needed for taurine and glutathione production) and a lower cystathionase activity interferes with the methionine-cysteine metabolism pathway by accumulation of cystathionine. Glutathione plays an important role in the detoxification of antioxidants and drugs in cells. Previous studies showed, however, that the intracellular glutathione content is higher in leukemic cells than in controls[39] and that the glutathione level is not correlated with cellular resistance to L-Asparaginase or other drugs in pediatric ALL.[40,41] In this thesis we found that the baseline levels of amino acids in leukemic cells do not differ between *in vitro* L-Asparaginase sensitive and resistant cases nor between clinically good and poor responders to L-Asparaginase. Therefore, the observed difference in amino acid

levels between leukemic cells and normal lymphocytes presumably reflects a difference in metabolic activity and/or cell type.

Genome wide approaches have been used to identify genes and gene expression profiles associated with drug resistance of leukemic cells.[42,43] Gene expression patterns were identified that discriminated ALL cells resistant to single antileukemic drugs like L-Asparaginase. Over-expression of genes encoding ribosomal proteins was associated with L-Asparaginase resistance[42] and discordant sensitivity to vincristine.[43] L-Asparaginase has been shown to inhibit the mammalian target of rapamycin (mTOR) signaling pathway that is involved in ribosomal protein synthesis in leukemic cells.[44] Although ribosomal protein inhibitors might theoretically sensitize leukemic cells to L-Asparaginase they might on the other hand increase vincristine resistance due to the observed discordant response to these two drugs.[43] Ongoing clinical trials indicate that mTOR inhibitors may have clinical potential as anticancer agents.[45] However, the interference between mTOR pathway members is highly complex and therefore also the specificity of mTOR inhibitors is low.[46] Interestingly, not only asparagine but also glutamine starvation targets the mTOR signaling pathway suggesting that L-Asparaginase may inhibit this mTOR pathway also through its glutaminase activity.[44]

Response to L-Asparaginase in childhood ALL

In this thesis we showed that the *in vivo* response to a single dose of 1000 IU/m^2 PEG-Asparaginase in children with newly diagnosed ALL is an independent prognostic marker for long-term clinical outcome.[47] Previous studies showed that *in vitro* resistance to L-Asparaginase is linked to an unfavorable prognosis in pediatric ALL.[48-50] Not surprisingly, *in vivo* response to PEG-Asparaginase correlated well with the *in vitro* sensitivity to L-Asparaginase (this thesis). Favorable genotypes, e.g. *TEL-AML1* positive ALL and hyperdiploid ALL, are characterized by a high *in vitro* sensitivity to L-Asparaginase and by a good *in vivo* response to PEG-Asparaginase. Unfavorable subtypes like T-ALL and *BCR-ABL*/t(9;22) precursor B-ALL demonstrated a relative *in vivo* resistance to PEG-Asparaginase (this thesis). T-ALL cells were also shown to be relatively resistant to L-Asparaginase compared to B-lineage ALL.[51] The *in vivo* response or the *in vitro* resistance to PEG-Asparaginase might be used to stratify patients for risk-adapted L-Asparaginase dosage and administration schedules.

HEMOSTASIS

One of the main side effects of L-Asparaginase therapy are thrombo-embolic complications. These complications seem to be caused by a relationship between asparagine depletion and reduced protein synthesis in the liver. Consumption coagulopathy or a direct enzymatic breakdown of coagulation proteins[52] do not explain these complications.

At the time of diagnosis of ALL we monitored an increased thrombin generation and disseminated intravascular coagulation. This is in concordance with the findings of Mitchell *et al.*[53] and Uszinsky *et al.*[54] Cancer cells may directly synthesize procoagulant molecules and inflammatory cytokines. Malignant cells can interact with vascular endothelial cells to produce a prothrombotic state by comprising their anticoagulant properties and increasing the release of procoagulant proteins like F VIII from endothelial stores.[55] The window therapy with 1000 IU/m^2 PEG-Asparaginase resulted in a sharp decrease of all coagulation parameters due to protein synthesis inhibition by L-Asparaginase (this thesis). Native *E coli* L-Asparaginase[53] compared to the pegylated L-Asparaginase used in our studies yielded comparable coagulation disturbances as a risk factor for thrombosis.

Most of the thrombotic events occur during induction therapy.[56] The rate of thrombosis was 5.2% in a meta-analysis of 1752 children with ALL.[57] We analyzed two induction schedules: in the DCOG-ALL-7 study 10.000 IU/m^2 native *E. coli* L-Asparaginase (Crasnitin®) or *Erwinia* L-Asparaginase (Erwinase®) was given twice a week after 18 days of prednisone whereas in the DCOG-ALL-9 study 6000 IU/m^2 Paronal® was administered twice a week after 28 days of dexamethasone treatment. The different half-lives of these three L-Asparaginase products resulted in different effects on hemostasis: Erwinase® with a half-life of only 7 hours demonstrated the least effects on coagulation protein synthesis, whereas Crasnitin® administered in the same dose with a half-life of 18 hours and Paronal® with a half-life of 23 hours showed a significant decline in coagulation protein synthesis. Of all coagulation proteins measured only factor V demonstrated no severe impaired production. The fact that factor V is also produced by reticuloendothelial cells may counteract the effect of impaired production in the liver by hepatocytes due to L-Asparaginase therapy.[58] Randomized studies of the DFCI and the EORTC confirmed that native L-Asparaginase led to an increased risk for thrombo-embolic events compared to Erwinase® (but a decreased risk of leukemic relapse) if administered in the same dose schedule.[20,21]

The period of prednisone or dexamethasone treatment before the first infusion with L-Asparaginase differed between the two induction schedules we studied. More upregulation of coagulation proteins was noticed in the ALL-9-treatment schedule after 4 weeks of dexamethasone, compared to the ALL-7 schedule after 18 days of treatment with prednisone. Nevertheless, the tapering of corticosteroid treatment together with the twice-weekly administration of L-Asparaginase resulted in a hypercoagulable state in both schedules mainly due to a decrease in antithrombotic proteins and proteins involved in fibrinolysis. The interaction between L-Asparaginase and corticosteroids is crucial in disturbing the balance between the risk on bleeding and thrombosis. A multi-center prospective study of 300 patients of the German BFM and 120 patients of the German COALL study group confirmed these data: venous thromboembolism occurred in 10% of patients treated according to the BFM schedule during induction and in 1.7% during re-induction (total 11.7%), but thrombotic events occurred in only 0.8% during induction and in 1.7% during re-induction in the children treated according

to the COALL protocol (total 2.5%).[59] The difference in thrombotic incidence was due to differences in dose-schedule of steroids and L-Asparaginase. In BFM protocols L-Asparaginase is administered from early induction concurrent with steroids, like in the Dutch protocols. In the COALL regime L-Asparaginase is administered during consolidation without corticosteroids. One week only of dexamethasone every 3 weeks in combination with weekly L-Asparaginase during consolidation also caused much less hypercoagulability than the combination of bi-weekly L-Asparaginase with continuous dexamethasone treatment (this thesis).

It is known that older children are at higher risk for thrombo-embolic events.[20,22,60] We provided evidence that older children demonstrate more inhibition in coagulation protein synthesis compared to younger children after L-Asparaginase therapy (this thesis). These findings can explain why the risk of thrombosis or bleeding after L-Asparaginase therapy is age-dependent.[61-63]

The question remains why a relatively low rate of symptomatic thrombosis of 5% during ALL induction treatment is found[57] if we take notice of all coagulation disturbances found. Children with hereditary prothrombotic risk factors, i.e. factor V Leiden, the prothrombin variant, the MTHFR mutation, deficiencies of protein C, S or antithrombin are not at a high risk to develop thrombosis.[64] However, the combination of one of these risk factors together with a hypercoagulable situation due to antileukemic therapy with corticosteroids and L-Asparaginase may induce thrombotic events.[59,65] However, the COALL study, using high dose L-Asparaginase (45.000 IU/m^2) as well as the PARKAA study (prophylactic antithrombin replacement in kids with ALL on L-Asparaginase) could not confirm a role of prothrombotic risk factors in the development of thromboses during their treatment regimens.[65,66] Notably, the main goal of the PARKAA study was to determine if prophylactic treatment with antithrombin would reduce the incidence in thrombotic events. However, this appeared not to be the case.

The effect of L-Asparaginase on hemostatic protein synthesis was linked to depletion of free asparagine and glutamine in the liver.[58,67] Bushman proved that L-Asparaginase affected plasma levels of antithrombin by interfering with translation and/or secretion of the antithrombin protein in liver cells.[67] As coagulation proteins are mainly produced by the liver, disturbances in the synthesis of these proteins due to amino acid starvation are thus believed to be due to an effect of L-Asparaginase on protein synthesis in the liver.

The mTOR signaling pathway regulates cell growth by controling translation.[46] Rapamycin and L-Asparaginase inhibit the mTOR pathway, thereby interfering with the synthesis of ribosomal proteins.[68] Weyrich *et al.* recently identified that rapamycin inhibits platelet activation induced by thrombin and blocks clot retraction.[69] Possibly the inhibitory effect of L-Asparaginase on mTOR also diminishes the clot retraction. This may explain that the risk for thrombosis is in part counterbalanced by a diminished clot retraction, and thus accounts for the relatively low rate of symptomatic thrombosis during ALL induction treatment. To better understand the process of thrombin generation and fibrinolysis during L-Asparaginase therapy, further evaluation of these processes with newly developed techniques like modern thromboelastography might be considered.

In conclusion, the type and the dose of L-Asparaginase, the timing of concomitant corticosteroid therapy and the age of the patient, determine the effect of L-Asparaginase on the coagulation system. These factors should be taken into account when new treatment regimens are developed for children with ALL. Older children with one or more inherited prothrombotic defects receiving a combination of L-Asparaginase with concomitant corticosteroid treatment in induction therapy appear to be at highest risk of thrombosis. In our opinion, only for these children the use of low molecular weight heparins[70,71] should be investigated in order to decrease the risk of thrombotic complications.

REFERENCES

1. Asselin BL. The three asparaginases. Comparative pharmacology and optimal use in childhood leukemia. Adv Exp Med Biol. 1999;457:621-629.
2. Boos J, Werber G, Ahlke E, Schulze-Westhoff P, Nowak-Gottl U, Wurthwein G, et al. Monitoring of asparaginase activity and asparagine levels in children on different asparaginase preparations. Eur J Cancer. 1996;32A:1544-1550.
3. Avramis VI, Spence SA. Clinical pharmacology of asparaginases in the United States: asparaginase population pharmacokinetic and pharmacodynamic (PK-PD) models (NONMEM) in adult and pediatric ALL patients. J Pediatr Hematol Oncol. 2007;29:239-247.
4. Muller HJ, Beier R, Loning L, Blutters-Sawatzki R, Dorffel W, Maass E, et al. Pharmacokinetics of native Escherichia coli asparaginase (Asparaginase medac) and hypersensitivity reactions in ALL-BFM 95 reinduction treatment. Br J Haematol. 2001;114:794-799.
5. Muller HJ, Loning L, Horn A, Schwabe D, Gunkel M, Schrappe M, et al. Pegylated asparaginase (Oncaspar) in children with ALL: drug monitoring in reinduction according to the ALL/NHL-BFM 95 protocols. Br J Haematol. 2000;110:379-384.
6. Ahlke E, Nowak-Gottl U, Schulze-Westhoff P, Werber G, Borste H, Wurthwein G, et al. Dose reduction of asparaginase under pharmacokinetic and pharmacodynamic control during induction therapy in children with acute lymphoblastic leukaemia. Br J Haematol. 1997;96:675-681.
7. Abshire TC, Pollock BH, Billett AL, Bradley P, Buchanan GR. Weekly polyethylene glycol conjugated L-asparaginase compared with biweekly dosing produces superior induction remission rates in childhood relapsed acute lymphoblastic leukemia: a Pediatric Oncology Group Study. Blood. 2000;96:1709-1715.
8. Muller HJ, Beier R, da Palma JC, Lanvers C, Ahlke E, von Schutz V, et al. PEG-asparaginase (Oncaspar) 2500 U/m(2) BSA in reinduction and relapse treatment in the ALL/NHL-BFM protocols. Cancer Chemother Pharmacol. 2002;49:149-154.
9. Rizzari C, Citterio M, Zucchetti M, Conter V, Chiesa R, Colombini A, et al. A pharmacological study on pegylated asparaginase used in front-line treatment of children with acute lymphoblastic leukemia. Haematologica. 2006;91:24-31.
10. Avramis VI, Sencer S, Periclou AP, Sather H, Bostrom BC, Cohen LJ, et al. A randomized comparison of native Escherichia coli asparaginase and polyethylene glycol conjugated asparaginase for treatment of children with newly diagnosed standard-risk acute lymphoblastic leukemia: a Children's Cancer Group study. Blood. 2002;99:1986-1994.
11. Hawkins DS, Park JR, Thomson BG, Felgenhauer JL, Holcenberg JS, Panosyan EH, et al. Asparaginase pharmacokinetics after intensive polyethylene glycol-conjugated L-asparaginase therapy for children with relapsed acute lymphoblastic leukemia. Clin Cancer Res. 2004;10:5335-5341.
12. Gentili D, Conter V, Rizzari C, Tschuemperlin B, Zucchetti M, Orlandoni D, et al. L-Asparagine depletion in plasma and cerebro-spinal fluid of children with acute lymphoblastic leukemia during subsequent exposures to Erwinia L-asparaginase. Ann Oncol. 1996;7:725-730.
13. Dibenedetto SP, Di Cataldo A, Ragusa R, Meli C, Lo Nigro L. Levels of L-asparagine in CSF after intramuscular administration of asparaginase from Erwinia in children with acute lymphoblastic leukemia. J Clin Oncol. 1995;13:339-344.
14. Rizzari C, Zucchetti M, Conter V, Diomede L, Bruno A, Gavazzi L, et al. L-asparagine depletion and L-asparaginase activity in children with acute lymphoblastic leukemia receiving i.m. or i.v. Erwinia C. or E. coli L-asparaginase as first exposure. Ann Oncol. 2000;11:189-193.
15. Riccardi R, Holcenberg JS, Glaubiger DL, Wood JH, Poplack DG. L-asparaginase pharmacokinetics and asparagine levels in cerebrospinal fluid of rhesus monkeys and humans. Cancer Res. 1981;41:4554-4558.

16. Berg SL, Balis FM, McCully CL, Godwin KS, Poplack DG. Pharmacokinetics of PEG-L-asparaginase and plasma and cerebrospinal fluid L-asparagine concentrations in the rhesus monkey. Cancer Chemother Pharmacol. 1993;32:310-314.

17. Hak LJ, Relling MV, Cheng C, Pei D, Wang B, Sandlund JT, et al. Asparaginase pharmacodynamics differ by formulation among children with newly diagnosed acute lymphoblastic leukemia. Leukemia. 2004;18:1072-1077.

18. Segal MB. Transport of nutrients across the choroid plexus. Microsc Res Tech. 2001;52:38-48.

19. Milman HA, Cooney DA. The distribution of L-asparagine synthetase in the principal organs of several mammalian and avian species. Biochem J. 1974;142:27-35.

20. Moghrabi A, Levy DE, Asselin B, Barr R, Clavell L, Hurwitz C, et al. Results of the Dana-Farber Cancer Institute ALL Consortium Protocol 95-01 for children with acute lymphoblastic leukemia. Blood. 2007;109:896-904.

21. Duval M, Suciu S, Ferster A, Rialland X, Nelken B, Lutz P, et al. Comparison of Escherichia coli-asparaginase with Erwinia-asparaginase in the treatment of childhood lymphoid malignancies: results of a randomized European Organisation for Research and Treatment of Cancer-Children's Leukemia Group phase 3 trial. Blood. 2002;99:2734-2739.

22. Silverman LB, Gelber RD, Dalton VK, Asselin BL, Barr RD, Clavell LA, et al. Improved outcome for children with acute lymphoblastic leukemia: results of Dana-Farber Consortium Protocol 91-01. Blood. 2001;97:1211-1218.

23. Pession A, Valsecchi MG, Masera G, Kamps WA, Magyarosy E, Rizzari C, et al. Long-term results of a randomized trial on extended use of high dose L-asparaginase for standard risk childhood acute lymphoblastic leukemia. J Clin Oncol. 2005;23:7161-7167.

24. Amylon MD, Shuster J, Pullen J, Berard C, Link MP, Wharam M, et al. Intensive high-dose asparaginase consolidation improves survival for pediatric patients with T cell acute lymphoblastic leukemia and advanced stage lymphoblastic lymphoma: a Pediatric Oncology Group study. Leukemia. 1999;13:335-342.

25. Andrulis IL, Argonza R, Cairney AE. Molecular and genetic characterization of human cell lines resistant to L-asparaginase and albizziin. Somat Cell Mol Genet. 1990;16:59-65.

26. Aslanian AM, Fletcher BS, Kilberg MS. Asparagine synthetase expression alone is sufficient to induce l-asparaginase resistance in MOLT-4 human leukaemia cells. Biochem J. 2001;357:321-328.

27. Stams WA, den Boer ML, Beverloo HB, Meijerink JP, Stigter RL, van Wering ER, et al. Sensitivity to L-asparaginase is not associated with expression levels of asparagine synthetase in t(12;21)+ pediatric ALL. Blood. 2003;101:2743-2747.

28. Ramakers-van Woerden NL, Pieters R, Loonen AH, Hubeek I, Drunen van E, Beverloo HB, et al. TEL-AML1 gene fusion is related to in vitro drug sensitivity for L-Asparaginase in childhood acute lymphoblastic leukemia. Blood. 2000;96:1094-1099.

29. Stams WAG, den Boer ML, Holleman A, Appel IM, Beverloo HB, van Wering ER, et al. Asparagine synthetase expression is linked with L-Asparaginase resistance in TEL-AML1 negative, but not in TEL-AML1 positive pediatric acute lymphoblastic leukemia. Blood. 2005;105:4223-4225.

30. Su N, Pan YX, Zhou M, Harvey RC, Hunger SP, Kilberg MS. Correlation between asparaginase sensitivity and asparagine synthetase protein content, but not mRNA, in acute lymphoblastic leukemia cell lines. Pediatr Blood Cancer. 2007[Epub ahead of print].

31. Holleman A, den Boer ML, Kazemier KM, Janka-Schaub GE, Pieters R. Resistance to different classes of drugs is associated with impaired apoptosis in childhood acute lymphoblastic leukemia. Blood. 2003;102:4541-4546.

32. Shiratsuchi A, Nakanishi Y. Phosphatidylserine-mediated phagocytosis of anticancer drug-treated cells by macrophages. J Biochem (Tokyo). 1999;126:1101-1106.

33. Iwamoto S, Mihara K, Downing JR, Pui CH, Campana D. Mesenchymal cells regulate the response of acute lymphoblastic leukemia cells to asparaginase. J Clin Invest. 2007;117:1049-1057.

34. Bussolati O, Belletti S, Uggeri J, Gatti R, Orlandini G, Dall'Asta V, et al. Characterization of apoptotic phenomena induced by treatment with L-asparaginase in NIH3T3 cells. Exp Cell Res. 1995;220:283-291.

35. Tardito S, Uggeri J, Bozzetti C, Bianchi MG, Rotoli BM, Franchi-Gazzola R, et al. The inhibition of glutamine synthetase sensitizes human sarcoma cells to L: -asparaginase. Cancer Chemother Pharmacol. 2007.

36. Oehler R, Roth E. Regulative capacity of glutamine. Curr Opin Clin Nutr Metab Care. 2003;6:277-282.

37. Newsholme EA, Crabtree B, Ardawi MS. Glutamine metabolism in lymphocytes: its biochemical, physiological and clinical importance. Q J Exp Physiol. 1985;70:473-489.

38. Wakayama K, Besa EC, Baskin SI. Changes in intracellular taurine content of human leukemic cells. Nagoya J Med Sci. 1983;45:89-96.

39. Paydas S, Yuregir GT, Sahin B, Seyrek E, Burgut R. Intracellular glutathione content in leukemias. Oncology. 1995;52:112-115.

40. Maung ZT, Hogarth L, Reid MM, Proctor SJ, Hamilton PJ, Hall AG. Raised intracellular glutathione levels correlate with in vitro resistance to cytotoxic drugs in leukaemic cells from patients with acute lymphoblastic leukemia. Leukemia. 1994;8:1487-1491.

41. Kearns PR, Pieters R, Rottier MM, Pearson AD, Hall AG. Raised blast glutathione levels are associated with an increased risk of relapse in childhood acute lymphocytic leukemia. Blood. 2001;97:393-398.

42. Holleman A, Cheok MH, den Boer ML, Yang W, Veerman AJ, Kazemier KM, et al. Gene-expression patterns in drug-resistant acute lymphoblastic leukemia cells and response to treatment. N Engl J Med. 2004;351:533-542.

43. Lugthart S, Cheok MH, den Boer ML, Yang W, Holleman A, Cheng C, et al. Identification of genes associated with chemotherapy crossresistance and treatment response in childhood acute lymphoblastic leukemia. Cancer Cell. 2005;7:375-386.

44. Iiboshi Y, Papst PJ, Hunger SP, Terada N. L-Asparaginase inhibits the rapamycin-targeted signaling pathway. Biochem Biophys Res Commun. 1999;260:534-539.

45. Davies SP, Reddy H, Caivano M, Cohen P. Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem J. 2000;351(Pt 1):95-105.

46. Wullschleger S, Loewith R, Hall MN. TOR signaling in growth and metabolism. Cell 2006;124:471-484.

47. Appel IM, al e. Pharmacokinetic, pharmacodynamic and intracellular effects of PEG-Asparaginase in newly diagnosed childhood acute lymphoblastic leukemia: results from a single agent window study. 2007; submitted.

48. Pieters R, Huismans DR, Loonen AH, Hahlen K, van der Does-van den Berg A, van Wering ER, et al. Relation of cellular drug resistance to long-term clinical outcome in childhood acute lymphoblastic leukaemia. Lancet. 1991;338:399-403.

49. Den Boer ML, Harms DO, Pieters R, Kazemier KM, Gobel U, Korholz D, et al. Patient stratification based on prednisolone-vincristine-asparaginase resistance profiles in children with acute lymphoblastic leukemia. J Clin Oncol. 2003;21:3262-3268.

50. Hongo T, Yajima S, Sakurai M, Horikoshi Y, Hanada R. In vitro drug sensitivity testing can predict induction failure and early relapse of childhood acute lymphoblastic leukemia. Blood. 1997;89:2959-2965.

51. Pieters R, den Boer ML, Durian M, Janka G, Schmiegelow K, Kaspers GJ, et al. Relation between age, immunophenotype and in vitro drug resistance in 395 children with acute lymphoblastic leukemia--implications for treatment of infants. Leukemia 1998;12:1344-1348.

52. Nowak-Gottl U, Boos J, Wolff JE, Lill H, Veltmann H, Werber G, et al. Asparaginase decreases clotting factors in vitro: a possible pitfall? Int J Clin Lab Res. 1995;25:146-148.

53. Mitchell L, Hoogendoorn H, Giles AR, Vegh P, Andrew M. Increased endogenous thrombin generation in children with acute lymphoblastic leukemia: risk of thrombotic complications in L'Asparaginase-induced antithrombin III deficiency. Blood. 1994;83:386-391.

54. Uszynski M, Osinska M, Zekanowska E, Ziolkowska E. Children with acute lymphoblastic leukemia: is there any subgroup of children without elevated thrombin generation? A preliminary study utilizing measurements of thrombin-antithrombin III complexes. Med Sci Monit. 2000;6:108-111.

55. Sutherland DE, Weitz IC, Liebman HA. Thromboembolic complications of cancer: epidemiology, pathogenesis, diagnosis, and treatment. Am J Hematol. 2003;72:43-52.

56. Athale UH, Chan AKC. Thrombosis in children with acute lymphoblastic leukemia Part II. Pathogenesis of thrombosis in children with acute lymphoblastic leukemia: effects of the disease and therapy. Thrombosis Research. 2003;111:199-212.

57. Caruso V, Iacoviello L, Di Castelnuovo A, Storti S, Mariani G, de Gaetano G, et al. Thrombotic complications in childhood acute lymphoblastic leukemia: a meta-analysis of 17 prospective studies comprising 1752 pediatric patients. Blood. 2006;108:2216-2222.

58. Reinert RB, Oberle LM, Wek SA, Bunpo P, Wang XP, Mileva I, et al. Role of glutamine depletion in directing tissue-specific nutrient stress responses to L-asparaginase. J Biol Chem. 2006;281:31222-31233.

59. Nowak-Gottl U, Heinecke A, von Kries R, Nurnberger W, Munchow N, Junker R. Thrombotic events revisited in children with acute lymphoblastic leukemia: impact of concomitant Escherichia coli asparaginase/prednisone administration. Thromb Res. 2001;103:165-172.

60. Barry E, DeAngelo DJ, Neuberg D, Stevenson K, Loh ML, Asselin BL, et al. Favorable outcome for adolescents with acute lymphoblastic leukemia treated on Dana-Farber Cancer Institute Acute Lymphoblastic Leukemia Consortium Protocols. J Clin Oncol. 2007;25:813-819.

61. Andrew M, Vegh P, Johnston M, Bowker J, Ofosu F, Mitchell L. Maturation of the hemostatic system during childhood. Blood. 1992;80:1998-2005.

62. Monagle P, Barnes C, Ignjatovic V, Furmedge J, Newall F, Chan A, et al. Developmental haemostasis. Impact for clinical haemostasis laboratories. Thromb Haemost. 2006;95:362-372.

63. Ries M, Klinge J, Rauch R. Age-related reference values for activation markers of the coagulation and fibrinolytic system. Thrombosis Research. 1997;85:341-344.

64. Tormene D, Simioni P, Prandoni P, Franz F, Zerbinati P, Tognin G, et al. The incidence of venous thromboembolism in thrombophilic children: a prospective cohort study. Blood. 2002;100:2403-2405.

65. Mauz-Korholz C, Junker R, Gobel U, Nowak-Gottl U. Prothrombotic risk factors in children with acute lymphoblastic leukemia treated with delayed E. coli asparaginase (COALL-92 and 97 protocols). Thromb Haemost. 2000;83:840-843.

66. Mitchell LG, Andrew M, Hanna K, Abshire T, Halton J, Anderson R, et al. A prospective cohort study determining the prevalence of thrombotic events in children with acute lymphoblastic leukemia and a central venous line who are treated with L-asparaginase: results of the Prophylactic Antithrombin Replacement in Kids with Acute Lymphoblastic Leukemia Treated with Asparaginase (PARKAA) Study. Cancer. 2003;97:508-516.

67. Bushman JE, Palmieri D, Whinna HC, Church FC. Insight into the mechanism of asparaginase-induced depletion of antithrombin III in treatment of childhood acute lymphoblastic leukemia. Leuk Res. 2000;24:559-565.

68. Iiboshi Y, Papst PJ, Kawasome H, Hosoi H, Abraham RT, Houghton PJ, et al. Amino Acid-dependent Control of p70 s6k. J Biol Chem. 1999;274:1092-1099.

69. Weyrich AS, Denis MM, Schwertz H, Tolley ND, Foulks J, Spencer E, et al. mTOR-dependent synthesis of Bcl-3 controls the retraction of fibrin clots by activated human platelets. Blood. 2007;109:1975-1983.

70. Athale UH, Siciliano SA, Crowther M, Barr RD, Chan AKC. Thromboembolism in children with acute lymphoblastic leukemia treated on Dna-Farber Cancer Institute protocols: effect of age and risk stratification of disease. BJH. 2005;129:803-810.

71. Elhasid R, Lanir N, Sharon R, Weyl Ben Arush M, Levin C, Postovsky S, et al. Prophylactic therapy with enoxaparin during L-asparaginase treatment in children with acute lymphoblastic leukemia. Blood Coagul Fibrinolysis. 2001;12:367-370.

9 chapter

Summary
Samenvatting

SUMMARY

Acute lymphoblastic leukemia (ALL) is the most common cancer in children and adolescents. Treatment outcome has improved impressively through the years. In the 1990's the five-year event free survival rates generally ranged from 70% to 85%. Risk factors for treatment failure are age and leukocyte count at diagnosis, immunophenotype, genotype and early response to chemotherapy. Different clinical outcomes associated with various subtypes of ALL are partly attributed to drug sensitivity or resistance of leukemic cells due to differences in molecular pharmacodynamics. Drug resistance is an important cause of treatment failure. Therefore, a better understanding of the responsible mechanisms of resistance to chemotherapy in ALL is needed.

Together with vincristine, a corticosteroid and sometimes an anthracycline, L-Asparaginase forms the backbone of induction treatment for childhood ALL. L-Asparaginase is also an important component of reinduction and intensification phases of combination chemotherapy. Intensified L-Asparaginase therapy improved treatment outcome compared to that achieved with less intensive L-Asparaginase therapy.

PART I

Part I of this thesis focuses on the pharmacokinetics and pharmacodynamics of L-Asparaginase. L-Asparaginase hydrolyzes asparagine into aspartic acid and ammonia, which reduces the availability of asparagine for protein synthesis. Resistance to L-Asparaginase has been associated with upregulation of asparagine synthetase (AS) mRNA expression in cell line studies, but inconsistent data were found in primary patient samples. In **chapter two** we investigated whether baseline levels of AS mRNA before start of treatment and/or PEG-Asparaginase-induced changes in AS mRNA expression in leukemic cells were associated with the clinical response to PEG-Asparaginase given as monotherapy upfront combination chemotherapy in children with ALL. The baseline AS expression levels were in the same range as in lymphocytes of healthy controls. In all patients upregulation of AS mRNA in the leukemic cells occurred within 24 hours after *in vivo* PEG-Asparaginase exposure and thereafter the expression levels did not further change. However, the level of upregulation of AS was not associated with a poor clinical response to PEG-Asparaginase. In addition, baseline and L-Asparaginase induced changes in AS mRNA expression did not differ between *in vitro* L-Asparaginase resistant T and L-Asparaginase sensitive c/preB ALL cases. Moreover, *TEL-AML1* rearranged and hyperdiploid cases did not show an impaired *in vivo* upregulation of AS that might have explained their high *in vitro* sensitivity to L-Asparaginase. Therefore, the AS upregulation which is a consequence of amino acid deprivation by L-Asparaginase, is not the key-factor explaining resistance to L-Asparaginase in pediatric ALL.

In **chapter three** we studied mechanisms of L-Asparaginase resistance other than AS mRNA upregulation by determining several apoptotic features and both

serum and intracellular amino acid levels. Remarkably, the percentage of apoptotic cells and apoptotic read-out markers in leukemic cells did not change *in vivo* after administration of PEG-Asparaginase. Also *in vivo* no changes were observed in the levels of intracellular amino acids, including asparagine, aspartic acid, glutamine and glutamic acid, whereas these amino acids were affected in the serum of these patients. In contrast, *in vitro* exposure of patients' leukemic cells to L-Asparaginase induced apoptosis and affected the intracellular levels of amino acids. These data suggest that changes in apoptotic features and in intracellular amino acid levels can not be detected in freshly obtained patients' samples during treatment, since these affected cells are presumably quickly eliminated from the peripheral blood. Hence, only cells survive that do have an unchanged amino acid composition.

The intracellular levels of aspartic and glutamic acid were higher compared to normal cells. This might be related to a fast turnover of asparagine and glutamine in the proliferating leukemic blasts. The baseline intracellular levels of cystathionine were higher and taurine levels were lower in leukemic cells at diagnosis compared to normal cells. This may point to a low activity of γ-cystathionase in leukemic cells resulting in an abnormal methionine-cysteine pathway, that reduces the conversion of cystathionine into taurine.

In vivo responsiveness to PEG-Asparaginase was monitored by analyzing the decrease in leukemic cells during a therapeutic window with this drug before start of combination chemotherapy. We found that children with unfavorable immunophenotypic or genotypic characteristics (T-ALL and *BCR-ABL* positive ALL) were *in vivo* more resistant to PEG-Asparaginase than children with a favorable genotype (*TEL-AML1* positive and hyperdiploid ALL). We also showed that *in vitro* sensitivity to L-Asparaginase was related to a good clinical response to PEG-Asparaginase. The clinical response to PEG-Asparaginase as well as the *in vitro* sensitivity to L-Asparaginase are predictive for long-term clinical outcome in pediatric ALL. One additional dose of PEG-Asparaginase did not increase clinical toxicity with regard to the allergic reactions to next L-Asparaginase infusions nor with regard to changes in coagulation parameters or the incidence of thrombosis later on during therapy.

The pharmacokinetics of PEG-Asparaginase are not well characterized in cerebrospinal fluid (CSF). In **chapter four** we analyzed the kinetics of one dose of PEG-Asparaginase in the CSF. Despite the fact that a dose of 1000 IU/m^2 PEG-Asparaginase resulted in plasma activity levels > 100 IU/L and thus, as expected, plasma asparagine levels < 0.2 μM for more than 10 days, asparagine was still detectable in the CSF. Thus, PEG-Asparaginase at this dose could not fully deplete asparagine in the CSF.

PART II

The toxic effect of L-Asparaginase therapy is related to decreased protein synthesis. Important side effects of L-Asparaginase are disturbances in the coagulation system. In part II the results of studies focusing on these side effects are described.

Changes observed in coagulation parameters in patients treated with L-Asparaginase have not been consistent in different series of patients, which can be related to differences in age, genetic predisposition for hypercoagulability, co-administration of other chemotherapy and the product of L-Asparaginase used. In **chapter five** we describe a randomized study in childhood ALL, in which native *E. coli* L-Asparaginase (Crasnitin®, Bayer, Leverkusen, Germany) is compared to L-Asparaginase derived from *Erwinia chrysanthemi* (Erwinase®, Porton Products, London, United Kingdom). Initial treatment with prednisone during ALL induction gave rise to increased thrombin generation as was demonstrated by increased F1+2 (prothrombin fragment 1+2) and TAT (thrombin-antithrombin complex) levels. Addition of L-Asparaginase to the induction therapy lead to a decreased fibrinolytic potential due to decreased levels of alpha2-antiplasmin and plasminogen in children treated with *E. coli* L-Asparaginase but not in children treated with Erwinase. Thus, the use of prednisone in combination with *E. coli* L-Asparaginase (Crasnitin®, Bayer) but not Erwinase® led to an increased risk for thrombosis.

Older children seem to be at higher risk for thrombo-embolic events during L-Asparaginase therapy than younger children. However, till date no studies on age-related changes in coagulation parameters due to L-Asparaginase have been performed. In **chapter six** we report on the effect of age on changes in coagulation and fibrinolysis in children with newly diagnosed ALL during induction therapy, which were treated according to the DCOG-ALL-9 treatment schedule. The effects of a four-week period of dexamethasone resulted in a hypercoagulable state due to upregulation of all coagulation parameters except fibrinogen. Thereafter, during tapering of dexamethasone and during administration of L-Asparaginase it appears that larger effects on the levels of coagulation proteins were found in older children. All anticoagulant proteins (AT, protein C and protein S) declined significantly, the more so in children aged 11 - 16 years. The fibrinolytic potential was decreased most in the oldest group of children and recovery to normal levels of alpha2-antiplasmin and plasminogen took longer in older children compared to younger children. In conclusion, the sum of changes in coagulation proteins induced by L-Asparaginase contributes to the increased risk for thrombosis in children between 11-16 years of age.

In addition to the clinical and biological effects of one *in vivo* systemic dose of PEG-Asparaginase as described in **chapter four,** we also measured the effects of monotherapy with one dose of PEG-Asparaginase on the coagulation system in this chapter. At diagnosis data pointed to enhanced thrombin generation. Consumption coagulopathy was monitored in 17 patients. Giordano *et al.* suggested that T ALL had higher TAT (thrombin-antithrombin complex) levels than precursor B at diagnosis. We could not confirm this: both precursor B and T ALL patients demonstrated equally increased levels at diagnosis. Uszinsky *et al.* suggested that about 70% of children

with ALL had high TAT levels at diagnosis related to the disease. We analyzed 38 children at diagnosis and found 35 (92%) patients with increased TAT levels at diagnosis.

A profound decrease in nearly all coagulation factors was observed five days after PEG-Asparaginase administration, with a decrease in thrombin generation and in fibrinolytic potential. No clinical thrombo-embolic complications were seen in this period.

L-Asparaginase is mostly used in combination with other drugs, like corticosteroids. **Chapter seven** reports on changes in coagulation parameters in two different combination chemotherapy schedules in a group of high-risk ALL patients. During induction therapy according to the DCOG-ALL-9 protocol dexamethasone was administered daily for 4 weeks followed by 2 weeks of tapering during which native L-Asparaginase was given twice a week; during intensification one weekly dose of the same native L-Asparaginase was combined with one week dexamethasone every three weeks. During induction the same observations were made as described in chapter six, i.e. patients had an increased risk for thrombosis during the induction phase of combination chemotherapy. However, during intensification the levels of the coagulation proteins did not significantly change. Only fibrinogen changed significantly upon administration of dexamethasone during 7 days. One week of dexamethasone therapy every 3 weeks combined with weekly infusions of Paronal® caused much less hypercoagulability compared to weekly Paronal® after a prolonged period of dexamethasone and tapering dexamethasone to zero. We concluded that concomitant treatment of L-Asparaginase and dexamethasone interferes with the balance between bleeding and thrombosis during L-Asparaginase therapy in childhood ALL.

PART III

Finally, our findings presented in this thesis are put in perspective and possible directions for the future are discussed in **chapter 8**.

SAMENVATTING

Algemeen

Acute lymfatische leukemie (ALL) is de meest voorkomende vorm van kanker op de kinderleeftijd. In Nederland wordt elk jaar bij 120 kinderen deze diagnose gesteld. De behandeling bestaat uit chemotherapie gedurende twee jaar. De kans op genezing is de laatste decennia aanzienlijk toegenomen. Met behulp van combinatiechemotherapie geneest 80 - 85%. Toch geneest dus nog een aanzienlijk percentage niet. Hierdoor blijft recidief-ALL van alle vormen van kinderkanker de belangrijkste doodsoorzaak. Risicofactoren voor het terugkomen van de ziekte zijn: de leeftijd, het aantal witte bloedcellen (leukemiecellen) bij diagnose, het immunophenotype (het differentiatie-stadium van de leukemiecel), het genotype (de aanwezigheid van bepaalde chromosomale afwijkingen in de leukemiecel) en de eerste reactie op de ingestelde behandeling. Het verschil in behandelingsresultaten van alle subtypes van ALL wordt voor een deel toegeschreven aan de gevoeligheid of ongevoeligheid van leukemiecellen voor de medicijnen.

L-Asparaginase

De behandeling van ALL is vastgelegd in protocollen. Elk type leukemie wordt tegenwoordig volgens een eigen protocol behandeld.

De inductietherapie van een behandelingsprotocol voor ALL is erop gericht om in 4 tot 6 weken een complete morfologische remissie te bewerkstelligen. L-Asparaginase neemt naast vincristine, een corticosteroid en soms een anthracycline, een zeer belangrijke plaats in bij de inductiebehandeling. Na de inductie volgt een behandeling gericht op het voorkomen van uitbreiden van de leukemie naar de hersenen, vervolgens een re-inductie- en/of een intensificatie fase. Uiteindelijk volgt een onderhoudsbehandeling, waardoor de totale behandeling 2 jaar duurt. Toevoegen van L-Asparaginase aan de inductie-, re-inductie- en aan een eventuele intensificatie fase is in de behandeling van grote waarde gebleken. Een meer intensieve behandeling met L-Asparaginase verbeterde de uiteindelijke kans op genezing.

In **hoofdstuk 1** beschrijven we de ontwikkeling van L-Asparaginase, via een bij toeval ontdekt geneesmiddel tot een onmisbaar product in de behandeling van ALL bij kinderen. Elk geneesmiddel zal zich op een bepaalde manier in het menselijk lichaam gedragen. Pharmacokinetiek is het bestuderen van de opname, de verdeling, het metabolisme en de uitscheiding van medicijnen in het menselijk lichaam. Pharmacodynamiek beschrijft de relatie tussen de pharmacokinetiek van bepaalde medicijnen en hun pharmacologische effect op de ziekte. Verschillende medicijnen, dus ook de verschillende vormen van L-Asparaginase, zullen een verschil in pharmacodynamiek vertonen. De gevoeligheid (de sensitiviteit) en de ongevoeligheid (de resistentie) voor medicijnen kunnen door verschillen in pharmacodynamiek worden bepaald. Resistentie tegen geneesmiddelen tegen leukemie is een belangrijke oorzaak van therapiefalen. Daarom is een beter begrip noodzakelijk van het mechanisme dat eventuele resistentie tegen L-Asparaginase veroorzaakt.

DEEL I

Pharmacokinetiek en pharmacodynamiek van L-Asparaginase

Deel I van dit proefschrift is gericht op de pharmacokinetiek en de pharmacodynamiek van L-Asparaginase. L-Asparaginase is een enzym dat asparagine omzet in aspartaat en ammoniak. Dit leidt tot een tekort aan het aminozuur asparagine waardoor er minder asparagine beschikbaar komt voor de aanmaak van eiwitten. Waarschijnlijk beschikken juist leukemiecellen niet over het enzym asparagine-synthetase dat de vorming van asparagine weer stimuleert, om dit tekort aan asparagine op te vangen. Het tekort aan asparagine zal leiden tot afsterven van de leukemiecellen. Laboratorium studies op gekweekte cellijnen (*in vitro*-studies) wezen erop dat leukemische cellijnen die ongevoelig waren voor L-Asparaginase wel asparagine-synthetase konden produceren. Hierdoor werd dus de antileukemische werking van L-Asparaginase tenietgedaan. Bij leukemiecellen van patiënten was hier tot op heden geen onderzoek naar gedaan.

In **hoofdstuk 2** beschrijven wij onze studie naar de concentraties mRNA van asparagine-synthetase bij diagnose van de leukemie, dus voordat de behandeling is begonnen, en na behandeling met 1 gift PEG-Asparaginase. Wij onderzochten of de veranderingen in de expressie van het mRNA van asparagine-synthetase gekoppeld waren aan de klinische respons op PEG-Asparaginase. De asparagine-synthetase expressie was bij diagnose even hoog in ALL-cellen als in gezonde witte bloedcellen. Bij alle kinderen met ALL maten wij binnen 24 uur na de behandeling met PEG-Asparaginase een duidelijke 'opregulatie' van het asparagine-synthetase. Deze toegenomen concentraties bleven in daarop volgende dagen gelijk. Opvallend was dat de mate van opregulatie niet gecorreleerd was aan een slechtere klinische reactie op PEG-Asparaginase. Bovendien verschilde de expressie van asparagine-synthetase bij diagnose en na behandeling met PEG-Asparaginase niet tussen T-ALL cellen die *in vitro* (buiten het lichaam getest) voor L-Asparaginase relatief ongevoelig zijn en de voor L-Asparaginase gevoelige voorloper B-ALL-cellen. Kinderen met de chromosomale afwijking *TEL-AML1* of met hyperdiploïdie (meer dan 50 chromosomen) in hun leukemiecellen vertoonden geen lagere waardes van de asparagine-synthetase expressie, die hun hogere gevoeligheid voor L-Asparaginase zou hebben kunnen verklaren. Kennelijk is opregulatie van asparagine-synthetase, die optreedt als reactie op het tekort aan asparagine dat door L-Asparaginase wordt veroorzaakt, dus *in vivo,* niet een belangrijke oorzaak van resistentie tegen het middel L-Asparaginase.

In **hoofdstuk 3** zochten we naar andere mechanismen die resistentie tegen L-Asparaginase zouden kunnen verklaren. We keken naar het fenomeen apoptose (gereguleerde celdood) en we onderzochten het effect van L-Asparaginase op de aminozuren in de leukemiecellen en in het bloed. Vreemd genoeg veranderden de apoptose-parameters die wij testten niet wanneer de kinderen waren behandeld met PEG-Asparaginase, terwijl er klinisch toch een duidelijke afname was van het aantal leukemiecellen. Ook de concentraties van aminozuren in de leukemiecellen bleven hetzelfde, in tegenstelling tot de waardes in het bloed van de patiënten. Als we daarentegen dezelfde leukemiecellen buiten het lichaam (*in vitro)* bloot stelden aan

L-Asparaginase konden we wel apoptose meten en ook veranderingen in de intracellulaire aminozuren registreren. Dit betekent dus dat we veranderingen in apoptose en in de concentraties van aminozuren in de leukemie cellen *in vivo* niet kunnen vastleggen. Kennelijk worden leukemiecellen die aangepakt zijn door L-Asparaginase onmiddellijk uit de bloedcirculatie verwijderd. Alleen cellen met een normale aminozuursamenstelling kunnen overleven.

Bij diagnose waren de concentraties van aspartaat en glutamaat in de leukemiecellen hoger dan in gezonde witte bloedcellen. Dit kan misschien verklaard worden door het feit dat leukemiecellen zich snel delen en daardoor een hoge turnover van asparagine en glutamine in aspartaat en glutamaat hebben. Ook het cystathionine-gehalte was bij diagnose hoger, terwijl taurine juist een lagere concentratie had ten opzichte van normale cellen. Dit wijst waarschijnlijk op een lage activiteit van het enzym γ-cystathionase in leukemiecellen, waardoor minder cystathionine in taurine kan worden omgezet.

De *in vivo* respons van de leukemie voor PEG-Asparaginase konden we bepalen aan de hand van de afname van het aantal leukemische cellen tijdens de 5 dagen tussen de gift PEG-Asparaginase en de start van de verdere chemotherapie. Kinderen met een ongunstig immunophenotype of genotype (zoals T-cel-leukemie en Philadelphia chromosoom positieve leukemie) waren *in vivo* meer resistent voor PEG-Asparaginase dan kinderen met een gunstig genotype (zoals *TEL-AML1* positieve leukemie of hyperdiploïdie). Bovendien vonden we dat die leukemie die *in vitro* gevoelig was voor L-Asparaginase ook een goede klinische respons *in vivo* op PEG-Asparaginase vertoonde. De klinische respons op PEG-Asparaginase bleek net zoals de *in vitro* gevoeligheid voor L-Asparaginase de overlevingskans op lange termijn te kunnen voorspellen.

Deze ene extra gift PEG-Asparaginase zorgde later in de behandeling niet voor extra toxiciteit. Het aantal allergische reacties op L-Asparaginase bij volgende giften van dit medicijn was niet verhoogd. Ook traden er niet meer complicaties, zoals bloedingen of tromboses, op door veranderingen in de stolling die veroorzaakt kunnen worden door L-Asparaginase.

De pharmacokinetiek van PEG-Asparaginase in de hersenvloeistof (liquor) is niet goed bekend. In **hoofdstuk 4** onderzochten we de kinetiek van 1 dosis PEG-Asparaginase in de liquor. Met 1 gift PEG-Asparaginase bereikten we, zoals verwacht, gedurende 10 dagen een L-Asparaginase concentratie in het bloed van meer dan 100 IU/L en hiermee een volledige depletie van asparagine (< 0.2 μM). Toch konden we in de liquor nog steeds asparagine aantonen. Deze dosering PEG-Asparaginase leidde dus niet tot een volledige depletie van asparagine in de hersenvloeistof.

DEEL II

L-Asparaginase en de bloedstolling

De effectiviteit van L-Asparaginase berust op het teweeg brengen van een compleet tekort aan asparagine in bloed en dus in de leukemiecellen. Dit tekort aan asparagine is echter ook de oorzaak van bijwerkingen van L-Asparaginase. Een

verminderde eiwitaanmaak door een tekort aan asparagine in de lever veroorzaakt stoornissen in de aanmaak van stollingseiwitten. In deel II richten we ons op deze bijwerking van L-Asparaginase.

De veranderingen die door L-Asparaginase in de bloedstolling ontstaan zijn afhankelijk van, de leeftijd van de patiënt, de aanleg voor trombose, andere tegelijkertijd toegediende medicatie en het soort L-Asparaginase dat wordt voorgeschreven. In **hoofdstuk 5** beschrijven wij een gerandomiseerde studie, waarin kinderen met ALL volgens een zelfde protocol werden behandeld, met als enige verschil Crasnitin® (L-Asparaginase geproduceerd uit een *E. coli*-stam) in de ene groep en Erwinase® (een L-Asparaginase gemaakt van *Erwinia chrysanthemi*) in de andere groep. De eerste fase van de inductiebehandeling met o.a. prednison leidde tot toegenomen stolselaanmaak (thrombinegeneratie). Het toevoegen van L-Asparaginase aan de behandeling veroorzaakte alleen in de groep behandeld met Crasnitin® een forse afname van de stollingseiwitten die nodig zijn om een stolsel weer af te breken, de fibrinolyse. Een toegenomen thrombinegeneratie tezamen met een afgenomen potentie voor fibrinolyse leidt tot een verhoogd risico op trombose. De oorzaak van het feit dat dit alleen gebeurde in de groep die met prednison en Crasnitin® was behandeld en niet in de groep die met prednison en Erwinase® was behandeld, bleek te liggen in verschillen in de pharmacokinetiek van deze twee L-Asparaginases.

Uit ervaring weten we dat oudere kinderen vaker een trombose krijgen tijdens de inductiebehandeling van ALL dan jongere kinderen. Tot nu toe was er geen onderzoek gedaan naar het effect van L-Asparaginase op stollingseiwitten bij kinderen van verschillende leeftijdscategorieën. In **hoofdstuk 6** hebben we gekeken of de leeftijd van kinderen met ALL van invloed was op het effect van de L-Asparaginase-behandeling op de stolselaanmaak en de stolselafbraak. Bij alle kinderen leidde 4 weken behandeling in de inductie met dexamethason tot een zeer actieve thrombinegeneratie doordat bijna alle stollingsfactoren werden opgejaagd door corticosteroïden. Tijdens het afbouwen van de dexamethason en het tegelijkertijd starten van de behandeling met L-Asparaginase daalden alle stollingsparameters fors, door een verminderde eiwitaanmaak bij het tekort aan asparagine dat L-Asparaginase had bewerkstelligd. De grootste daling in de stollingseiwitten die tegen trombose beschermen en van de eiwitten die een gemaakt stolsel moeten afbreken, trad op bij kinderen ouder dan 11 jaar. Bovendien duurde ook het herstel naar normale waardes langer bij de oudere kinderen dan bij jongere. Deze leeftijdsafhankelijke verschillen in door steroïden en L-Asparaginase veroorzaakte veranderingen in de bloedstolling verklaren voor een deel het verhoogde risico op trombose tijdens de inductiebehandeling van ALL bij de oudere kinderen.

In **hoofdstuk 4** analyseerden we het effect van 1 dosis PEG-Asparaginase als monotherapie op de bloedstolling. Bij diagnose van ALL vertoonden de kinderen een geactiveerde stolling, zowel een toegenomen thrombinegeneratie als ook tekenen van verhoogde stolselafbraak. Dit is een bekend gegeven bij kwaadaardige aandoeningen. Deze afwijkingen bleken in onze studie niet gecorreleerd aan een bepaald type leukemie, zoals anderen wel eens beschreven hadden. De concentratie

van bijna alle stollingseiwitten nam fors af na 1 gift PEG-Asparaginase. Dit leidde bij geen van de kinderen tot een klinische complicatie.

L-Asparaginase wordt meestal met andere middelen gecombineerd in de behandeling van ALL. Vooral de combinatie met corticosteroïden lijkt van invloed op het effect van L-Asparaginase op de stolling. In **hoofdstuk 7** beschrijven we het effect van 1 soort L-Asparaginase in relatie tot twee verschillende schema's van de corticosteroïd medicatie. Kinderen met ALL werden tussen 1997 en 2004 in de inductie met de combinatie dexamethason en L-Asparaginase behandeld en tevens in een fase van intensificatie. In de inductie bestaat de behandeling onder andere uit 4 weken dexamethason en daarna 4 giften L-Asparaginase in 2 weken, terwijl in de intensificatie steeds 1 week dexamethason per 3 weken wordt gecombineerd met negen wekelijkse giften L-Asparaginase. De stollingswaarden veranderden aanzienlijk in de inductie zoals beschreven in hoofdstuk 6. Echter tijdens de intensificatie veranderden de stollingsuitslagen niet noemenswaardig. Er was tijdens de fase van intensificatie, gelet op de stollingsparameters, geen verhoogd risico op trombose. Het effect van gelijktijdige behandeling van corticosteroïden en L-Asparaginase op de stolling is dus afhankelijk van het precieze toedieningschema van deze combinatie.

DEEL III

Conclusie

We kunnen stellen dat L-Asparaginase steeds belangrijker wordt in de behandeling van ALL bij kinderen. Intensief gebruik is van groot belang voor een maximale overlevingskans van kinderen met ALL. De balans tussen maximale effectiviteit en minimale toxiciteit verdient veel aandacht. In **hoofdstuk 8** analyseren we in een algemene discussie alle verkregen data in relatie tot beschikbare kennis uit de literatuur. Hieruit volgen een aantal aanbevelingen voor de toekomst, zoals het meten van de L-Asparaginase-activiteit om op individuele basis de dosering aan te passen om een algehele asparaginedepletie te bewerkstelligen. De klinische respons op L-Asparaginase voorspelt de overlevingskans en kan in de toekomst gebruikt worden in de keus voor bepaalde behandelstrategieën. Studies zullen moeten uitwijzen of het gebruik van bloedverdunners bij oudere kinderen leidt tot minder tromboses tijdens de inductiebehandeling van ALL.

Curriculum vitae

Inge M. Appel, born 6 november 1950 in Groningen, The Netherlands

1971-1977	Medical School, Erasmus University Rotterdam
1977	Medical Qualifing examination
1978-1981	Pediatric Residency, Zuiderziekenhuis Rotterdam (Head Prof. dr. C.J. de Groot).
1981-1982	Pediatric Residency, Sophia Childrens Hospital, Erasmus University Rotterdam (Head Prof. dr. H.K.A. Visser).
1982-present	Staffmember, department of Pediatrics, subdivision Haematology/ Oncology, Sophia Childrens Hospital Rotterdam.
2000-2007	part-time research fellow as a pediatric oncologist on the oncology research laboratory Erasmus Medical Centre Rotterdam (head: Prof. dr. R. Pieters, biologist Dr. M.L. den Boer).

Professional membership

Dutch Society of Paediatricians, section Haematology and Oncology
Dutch Society of Thrombosis and Haemostasis
Member of the Dutch working group on hemoglobinopathies
Secretary of the Dutch working group ANS-SIKK
(Adviescommissie Neonatale Screening Sikkelcelziekte)
Dutch Childhood Oncology Group (DCOG)
President of the Lymfoma Committee of the DCLSG until 2002
EICNHL (European Intergroup on Childhood NHL) until 2002
Chairman of the protocol committee of NHL94 and ALCL99
Member of disease committee on bone marrow failure syndromes of the DCOG
International Society for Pediatric Oncology (SIOP)
European Society of Haematology/Oncologie
American Society of Pediatric Haematology/Oncology

Dankwoord

Jarenlang had ik al actief en met veel plezier in de kinderoncologie gewerkt in het academische milieu van het Sophia Kinderziekenhuis, toen moest het er toch nog van komen:

Rob Pieters werd tot hoogleraar kinderoncologie in het Sophia Kinderziekenhuis benoemd met in zijn kielzog een groep jonge enthousiaste nieuwe kinderoncologen! Dit moest voor mij het moment worden om ruim baan te maken voor aanstormend talent. Ik stelde voor om de zorg van de benigne hematologie en de hemostase op mij te nemen, en op mijn vraag of ik dat niet zou kunnen combineren met enig onderzoek, sprak Rob:

"dan moet je ook promoveren…"

Maar voordien waren daar eerst Dr. George van Zanen en zeker ook Dr. Karel Hählen. De liefde voor kinderen met een ernstige ziekte zoals kanker en het zorgvuldig toepassen van protocollaire geneeskunde heb ik van hen geleerd.

Met Prof. Dr. Rob Pieters als promotor werd ik rustig maar nadrukkelijk geïntroduceerd in de wereld van wetenschappelijk denken. Logisch redeneren en precies formuleren bleken niet eenvoudig. Gelukkig heb jij de onderzoekslijnen helder voor de geest, waardoor de positie van de kinderoncologie in Rotterdam op een zeer hoog plan is getild. Ik voel me vereerd dat ik met dit proefschrift een klein steentje heb kunnen bijdragen.

Natuurlijk ben je als clinicus èn aio èn 50-plusser een vreemde eend in de bijt. Vooral het net geïnstalleerde research-laboratorium op de 15^{e} moest hier even aan wennen! Het is prima gegaan. Dr. Monique den Boer, hoofd van het research-laboratorium kinderoncologie, wees mij de meest geroutineerde analistes toe en hield zelf de touwtjes van het onderzoek strak in handen. Als geen ander ben jij, Monique, in staat geweest om de onderzoeksresultaten en de voor mij zo lastige statistiek in goed Engels te formuleren. Dank voor alle aandacht die je hieraan hebt besteed.

De overige leden van de kleine commissie naast Rob Pieters en Monique den Boer, Prof. dr. Pieter Sonneveld, Prof. dr. Huib Pols en Dr. Frank Leebeek wil ik bedanken voor hun bereidheid het manuscript op snelle wijze kritisch te beoordelen.

Het echte werk is gedaan aan de bench: Nathalie Reniers beet het spits af en leerde mij niet alleen de beginselen van de moleculaire biologie, maar ook vele finesses van de computer. Hoewel je uit Rotterdam bent vertrokken, zien we elkaar gelukkig nog steeds. Karin Kazemier mocht het werk afmaken. Ongelooflijk dat iemand zoveel dingen tegelijk kon doen. Altijd drie stappen verder met je gedachtes dan ik, maar ook altijd even behulpzaam in weer recapituleren.

Beneden op het laboratorium speciële hematologie werd ondertussen minstens zo hard gewerkt. Rolinda Stigter en Carla van Kessel hebben tussen de bedrijven door met een voor analistes kenmerkende precisie alle stollingstesten uitgevoerd. Nog steeds voeren we zeer verhelderende discussies over de interpretatie van technieken en hun uitkomsten.

Met al die stollingsuitslagen ging ik naar Wim Hop, de statisticus. Uren zaten we samen in dat kleine kamertje aan de kop van de faculteit (dus een prachtig uitzicht). Het belang van ANOVA en SAS PROC MIXED is goed tot mij doorgedrongen!

Als derde laboratorium dat participeerde in dit onderzoek noem ik het laboratorium van de Klinische Genetica (sectie Chemische Basisdiagnostiek - Metabole) waar Wistaria onder leiding van Dr. Jan Huijmans alle intracellulaire aminozuurmetingen heeft uitgevoerd: onverstoorbaar, en altijd even vriendelijk.

Vòòr meten komt een goed protocol schrijven, patiënten en ouders inlichten en vooral materiaal voor laboratorium onderzoek verzamelen: wie konden dat beter coördineren en uitvoeren dan Eline Visser en Inekee van der Vaart. Eigenlijk zijn zij de motor van ons research instituut. Geweldig dat ook ik van jullie diensten gebruik heb mogen maken.

Altijd even behulpzaam waren ook Jeanine Arnolds en Jacqueline Dito van ons secretariaat. 'Even' helpen bijvoorbeeld met elektronisch een artikel "submitten": alles wil geleerd zijn.

De 'finishing touch' is gebeurd door Margo Terlouw, een oude bekende van het specieel hematologisch laboratorium waar zij vroeger als analiste werkte. De precisie van toen komt nu fantastisch van pas bij het redigeren van proefschriften. Geweldig bedankt voor je leuke ideeën en je vlotte uitwerking.

Dank voor de samenwerking met de SKION, waardoor de window met PEG-Asparaginase in de behandelstrategie van ALL-9 kon worden opgenomen.

Het laboratorium van Prof. dr. Joachim Boos en Dr. Claudia Lanvers in Münster was vooral in het begin nauw betrokken bij zowel de planning van de PEG-Asparaginase window, als ook bij de technische uitvoering: 'Ich bedanke mich für die schöne Zusammenarbeit mit Ihnen'.

Wetenschappelijk leren denken is eigenlijk het belangrijkste doel van onderzoek doen. Dr. Jules Meijerink vind ik zo'n echte wetenschapper. Erg kritisch, maar ook zeer fair. Dank voor je geduld en begrip in menig gesprek over jou passie: de wetenschap.

Wetenschap kost tijd, erg veel tijd zelfs. Toch heb ik een groot deel van het onderzoek in werktijd kunnen doen, mede dankzij o.a. Auke Beishuizen en later Marjon Cnossen. De hematologie moest wel doordraaien: zij deden de poli's en consulten, ik hoefde alleen maar mee te denken. Ook Desirée Bezemer, Andrieca de Vries en Inge van der Sluis droegen hun steentje bij tijdens hun stage hematologie binnen hun fellowship kinderoncologie. Alle kinderoncologen toonden zeer warme belangstelling. Vooral kamergenote Friederieke Hakvoort heeft het misschien moeten ontgelden wanneer er weer iets mis ging met bijvoorbeeld de computer, op de valreep mijn excuses. Het ga jullie goed in Italië!

Al die andere collegae en lieve mensen van de laboratoria, de polikliniek, de afdelingen moeten zich realiseren dat het schrijven van een proefschrift deels een eenzame exercitie is, maar het kan nooit zonder teamwerk. De afdeling kinderoncologie/hematologie van het Sophia Kinderziekenhuis is een prachtig

team; ik ben er trots op al zolang lid van dit team te zijn en ben trouwens niet van plan weg te gaan!

En tenslotte de allerbelangrijkste groep: ik wil nadrukkelijk de ouders en hun kinderen bedanken die ermee akkoord gingen om deel te nemen aan het onderzoek. De zorgen rond de behandeling van een kind met leukemie zijn enorm, toch konden en kunnen ouders en kinderen in die moeilijke fase het opbrengen om in wetenschappelijk onderzoek te participeren. Hopelijk kunnen andere kinderen van de resultaten van dit onderzoek profiteren.

Het thuisfront heeft waarschijnlijk bij het afronden van het boekje toch wel wat te lijden gehad. Lieve Rolf, nog even en we hebben het rijk alleen! Alle kinders prima geparkeerd, daar kunnen we trots op zijn. Er komt steeds meer tijd voor ons samen. Hans, Frank en Carolien zijn allen volwassen met eigen toekomstplannen. Ik geniet van jullie.

Het grootste probleem van het afgelopen jaar was mijn tuin: serieus verwaarloosd! Dat is het eerstvolgende project na de promotie.

Inge Appel