Leukemia (2008) 22, 1665–1679

www.nature.com/leu

ORIGINAL ARTICLE

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

IM Appel[1], KM Kazemier[1], J Boos[2], C Lanvers[2], J Huijmans[1], AJP Veerman[3,4], E van Wering[4], ML den Boer[1] and R Pieters[1]

[1]Department of Pediatric Oncology/Hematology, Erasmus MC/Sophia Children's Hospital, Erasmus University Medical Center, Rotterdam, The Netherlands; [2]Department of Pediatric Hematology/Oncology, University Children's Hospital Muenster, Muenster, Germany; [3]Department of Pediatrics, University Hospital, Vrije Universiteit, Amsterdam, The Netherlands and [4]Dutch Childhood Oncology Group, The Hague, The Netherlands

L-asparaginase is an effective drug for treatment of children with acute lymphoblastic leukemia (ALL). 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 pegylated (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 TELAML1 rearrangement, demonstrated a good clinical response compared to 8/17 T-ALL ($P=0.01$) and BCRABL-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, $P=0.004$; 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.
Leukemia (2008) **22,** 1665–1679; doi:10.1038/leu.2008.165; published online 26 June 2008
Keywords: PEG-asparaginase; intracellular effects; window response; drug resistance; ALL

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 unfavorable 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 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 upregulation of asparagine synthetase mRNA occurs in both sensitive and in resistant cases within 24 h 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 treatment was evaluated on the incidence of allergic reactions during treatment and changes in hemostasis.

Correspondence: Dr IM Appel, Department of Pediatric Oncology/Hematology, Erasmus MC/Sophia Children's Hospital, Dr Molewaterplein 60, Room Sp 2456, P.O. Box 2060, 3000 CB Rotterdam, The Netherlands. E-mail: i.m.appel@erasmusmc.nl
Received 2 March 2008; revised 15 May 2008; accepted 16 May 2008; published online 26 June 2008

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] Children with newly diagnosed ALL and presenting white blood count (WBC) $>10\times10^9$ per liter were eligible for the window study with PEG-asparaginase. Patients received a single dose of 1000 IU/m^2 PEG-asparaginase in an 1-h infusion 5 days before starting combination chemotherapy.[21] PEG-asparaginase (Oncaspar), kindly provided by Medac (GmbH, Hamburg, Germany), was used because of its lower immunogenicity than native (unpegylated) L-asparaginase,[22] which was important as native *E. coli* L-asparaginase (Paronal, four doses of 6000 IU/m^2 on days 29, 33, 36 and 40) was used as part of the regular combination chemotherapy given after the investigational window. After the PEG-asparaginase window, patients were divided into non-high-risk and high-risk criteria. Non-high-risk ALL was defined as WBC $<50\times10^9$ per liter, no mediastinal mass, absence of *t*(9;22), *t*(4;11) or other mixed lineage leukemia (MLL) rearrangements, no T-cell phenotype and no central nervous system or testicular involvement. All other leukemias were defined as high risk. All children received the combination induction therapy of dexamethasone, vincristine and intrathecal therapy during the first 4 weeks, and additional daunorubicin only in case of high-risk ALL.

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-DR$^+$) was classified into the following differentiation stages: proB-ALL cells were CD10$^-$, cytoplasmic μ chain$^-$ (cμ$^-$) and surface immunoglobulin$^-$ (sIg$^-$); c-ALL cells were CD10$^+$/cμ$^-$/sIg$^-$; pre-B cells were CD10$^{+\text{ or }-}$/cμ$^+$/sIg$^-$. B-ALL cells characterized by CD10$^-$/cμ$^-$/sIg$^+$ were excluded from the study. Cytogenetic analyses were performed by regular karyotyping and fluorescence *in situ* hybridization.[23]

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 1 h 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.[24] 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 prednison in ALL[25] 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 $<1\times10^9$ per liter of peripheral blood, as intermediate when leukemic cells were $1–10\times10^9$ per liter, and as poor when leukemic cells were $>10\times10^9$ per liter.

In vitro *cytotoxicity*

In vitro L-asparaginase cytotoxicity in leukemic cells taken at initial diagnosis (untreated) was determined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Cells were exposed to six different concentrations of L-asparaginase (Paronal; Christiaens BV, Breda, the Netherlands) ranging from 0.0032 to 10 IU/ml in duplicate for 4 days. Control cells were cultured without L-asparaginase. After 4 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 $\geqslant0.050$.[8]

In vitro sensitivity toward L-asparaginase was defined as LC_{50} $\leqslant0.033$ IU/ml, *in vitro* resistance toward L-asparaginase was defined as LC_{50} $\geqslant0.912$ IU/ml, and intermediate sensitive was defined as LC_{50} 0.033–0.912 IU/ml.[8]

As LC_{50} 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 performed by fluorescence-activated cell sorting 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. A total of 5×10^6 cells were harvested after 18, 24, 30 and 44 h 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 0, and twice a week from day 0 to 28. L-asparaginase activity was analyzed as described before.[26] After collection serum was isolated by centrifugation and stored immediately at −80 °C; serum levels of asparagine, aspartic acid, glutamine and glutamic acid were performed using high-performance liquid chromatography.[27] 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.[28]

Intracellular amino-acid levels

Intracellular levels of all essential and nonessential amino acids were measured by liquid column chromatography on a Biochrome 20 amino-acid analyzer with ninhydrin detection (Biochrome, Cambridge, UK). Blood samples were taken daily

from day −5 at diagnosis to day 0. At each time point 5×10^6 leukemic cells were lyzed in 100 µl of lysis buffer (150 ml NaCl, 30 mM Tris (pH 7.6), 10% glycerol, 1% Triton X-100, 1 mM phenylmethylsulphonylfluoride, 2 µg/ml aprotinin, 2 µg/ml leupeptine) for 15 min on ice. Cleared supernatants were stored at −80 °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 assay (Interchim Omnilabo, Rockford, Illinois, USA[29]).

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

Clinical toxicity

On the basis of clinical manifestations of diabetes mellitus, pancreatitis and hyperlipidemia, neurotoxicity and stroke laboratory parameters were monitored. Changes in coagulation and fibrinolysis were monitored throughout induction therapy. On days −5 and 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 performed with commercially available reagents and methods as described in detail previously.[30] Reference values were applied from the literature.[31–33]

Statistics

Differences between multiple groups were calculated using the Kruskal–Wallis test. The Mann–Whitney test was used when two 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 1.9–5.5 years). Event-free survival (EFS) defined as relapse-free survival was calculated according to Kaplan–Meier (± s.e.). Multiple regression was conducted using Cox proportional hazards regression models to assess prognostic factors for EFS.

Results

Patients

Between July 2000 and July 2004, 57 children with newly diagnosed ALL were enrolled in the PEG-asparaginase-window study (Table 1). As only patients with initial WBC $> 10 \times 10^9$ per liter were eligible for this study, more than half of these children were high-risk patients according to ALL-9 criteria.

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 34.5×10^9 per liter at day −5 to 0.18×10^9 per liter at day 0 (Figure 1). Out of 57, 35 (61%) 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 TELAML1/*t*(12;21)-positive ALL) were associated with a good clinical window response, whereas the poor prognostic genotype BCRABL/*t*(9;22) was associated with a poor clinical window response ($P = 0.04$) (Table 2). The 17 T-ALL patients had a significant poorer response to PEG-asparaginase compared to the 36 common/pre B cases ($P = 0.01$), BCRABL patients were not included.

Table 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 per liter) 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 B-lineage ALL	40
Hyperdiploid (>50 chromosomes)	11
TELAML1/*t*(12;21)	8
BCRABL/*t*(9;22)	2
MLL rearranged (11q23)	0
Normal (46 XX/XY)	8
Others	11

Abbreviations: ALL, acute lymphoblastic leukemia; MLL, mixed lineage leukemia; WBC, white blood count.

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 LC_{50} 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 2). In concordance with earlier studies,[34] T-ALL cells were significantly more *in vitro* resistant to L-asparaginase than precursor B-ALL cells (LC_{50} 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 132×10^9 per liter had a poor clinical response (WBC 25×10^9 per liter at day 0, absolute leukemic cell count 19.3×10^9 per liter), whereas her blasts were *in vitro* sensitive to L-asparaginase. She still is in continuous complete remission.

Apoptosis

Analysis of in vivo *induced apoptosis by PEG-asparaginase*. We analyzed 25 patients for different apoptotic parameters over time (Figure 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 to 24 patients. The median percentage of ALL cells showing phosphatidylserine externalization was 10.5% before starting treatment (day −5). These values did not significantly differ over time (Figure 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 over time (P = NS) (Figure 3b). The median percentage of cells with cleaved (and hence activated) caspase 3 at diagnosis was 2.7% and remained low (P = NS) (Figure 3c). The median percent of cells with PARP inactivation at diagnosis was 2.9% and remained low (P = NS) (Figure 3d).

These data show that the drop in WBC count and number of leukemic cells (Figure 1) seen after PEG-asparaginase administration was not simultaneously associated with an increase in

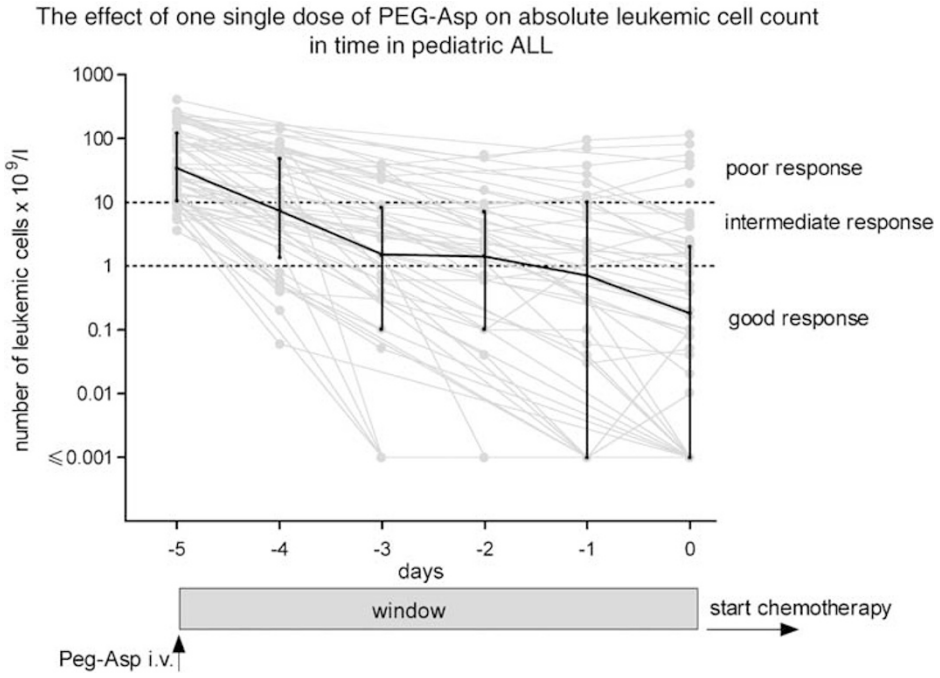

Figure 1 Clinical response to one dose of 1000 IU/m^2 PEG-asparaginase administered intravenously at day −5, that is 5 days upfront regular chemotherapy. A good clinical response to PEG-asparaginase was defined as $<1\times10^9$/l leukemic cells in the peripheral blood at day 0, an intermediate response was defined as 1–10×10^9/l, and a poor response as $>10\times10^9$/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. PEG, pegylated.

Table 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*
	2		*38*				*17*	*57*
No. of patients	*MLL germ line*	*MLL rearranged*	*TELAML1*	*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

Abbreviations: ALL, acute lymphoblastic leukemia; MLL, mixed lineage leukemia; PEG, pegylated.
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 $<1\times10^9$ per liter of peripheral blood, as intermediate, when leukemic cells were 1–10×10^9 per liter, and as poor when leukemic cells were $>10\times10^9$ per liter.

in vivo apoptotic markers. None of the apoptotic markers was linked to immunophenotype or genotype.

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 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 ($P<0.001$) (Figures 4a and b). 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 4a). The level of serum aspartic acid (Figure 4b) increased after 1 h 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 days −3 and 0. Compared to the level measured at diagnosis before 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).

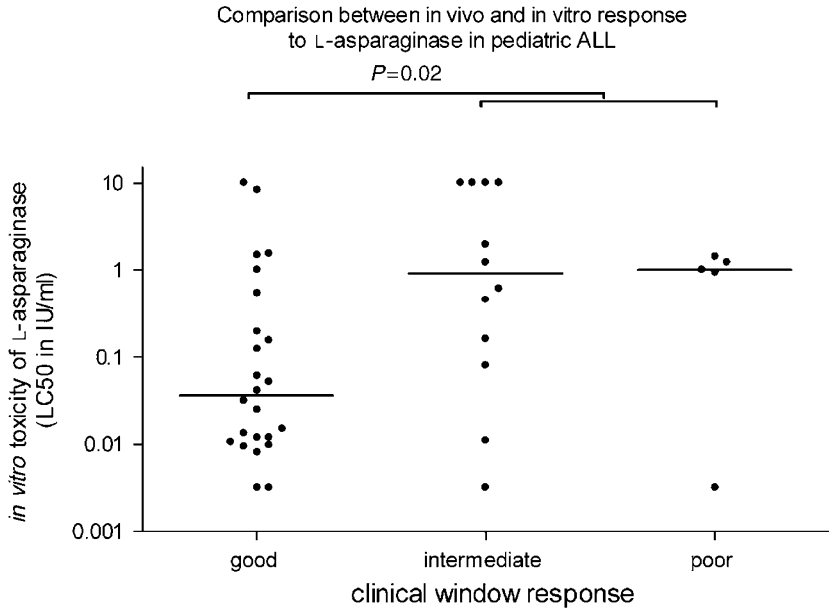

Figure 2 Comparison between *in vivo* and *in vitro* response to L-asparaginase. In 41 children *in vitro* toxicity of L-asparaginase (LC50 in IU/ml) was measured and related to the *in vivo* response to 1000 IU/m^2 PEG-Asparaginase given upfront regular chemotherapy. Good responders (leukemic cells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

Most glutamine levels stayed beyond the upper limit of detection (>250 μM) after PEG-asparaginase treatment (Figure 4b). However, glutamic acid levels significantly increased from median 87.6 μM (p25th–75th: 57.8–124 μM) before treatment to 310 μM (p25th–75th: 178–396 μM) 1 h after therapy ($P<0.001$) and to 239 μM (p25th–75th: 125–306 μM) at day–3 ($P<0.001$) and 159 μM (p25th–75th: 127–300 μM) at day 0 ($P<0.001$) (Figure 4b).

The serum peak levels of PEG-asparaginase did not differ between clinical response groups. No correlation with age, immunophenotype or WBC 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 (25th–75th 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; 25th–75th percentile: 347–568 μg/ml) ($P<0.0001$). The amino-acid levels were therefore expressed as μmol/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). Aspartic acid ($P<0.05$), glutamic acid ($P<0.05$) and cystathionine ($P<0.01$) were twofold 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 over time (Table 3), as is also shown in Figure 5a for asparagine, aspartic acid, glutamine and glutamic acid. The intracellular amino-acid levels at diagnosis and the *in vivo* response to PEG-asparaginase did not correlate (Figure 6; Table 4). Results were borderline significant for leucine, but not significant if adjusted for multiple testing. One remarkable difference demonstrated valine, which was two times higher in nonresponding patients.

As amino-acid levels did not change over time, the relation between clinical response and amino-acid levels over 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 4).

In vitro *L-asparaginase exposure*. In contrast to the lack of *in vivo* intracellular depletion of asparagine and glutamine (Figure 5a), significant intracellular depletion was detected after *in vitro* exposure to L-asparaginase. Leukemic blasts of six 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 h after exposure. Significant intracellular asparagine depletion was observed with 0.1 IU/ml and 10 IU/ml of L-asparaginase at all time points tested ($P<0.05$) (Figure 5b). Only after exposure to 10 IU/ml of L-asparaginase, intracellular glutamine levels declined significantly within 3 h ($P<0.001$), whereas no significant rise in glutamic acid could be observed (Figure 5b).

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

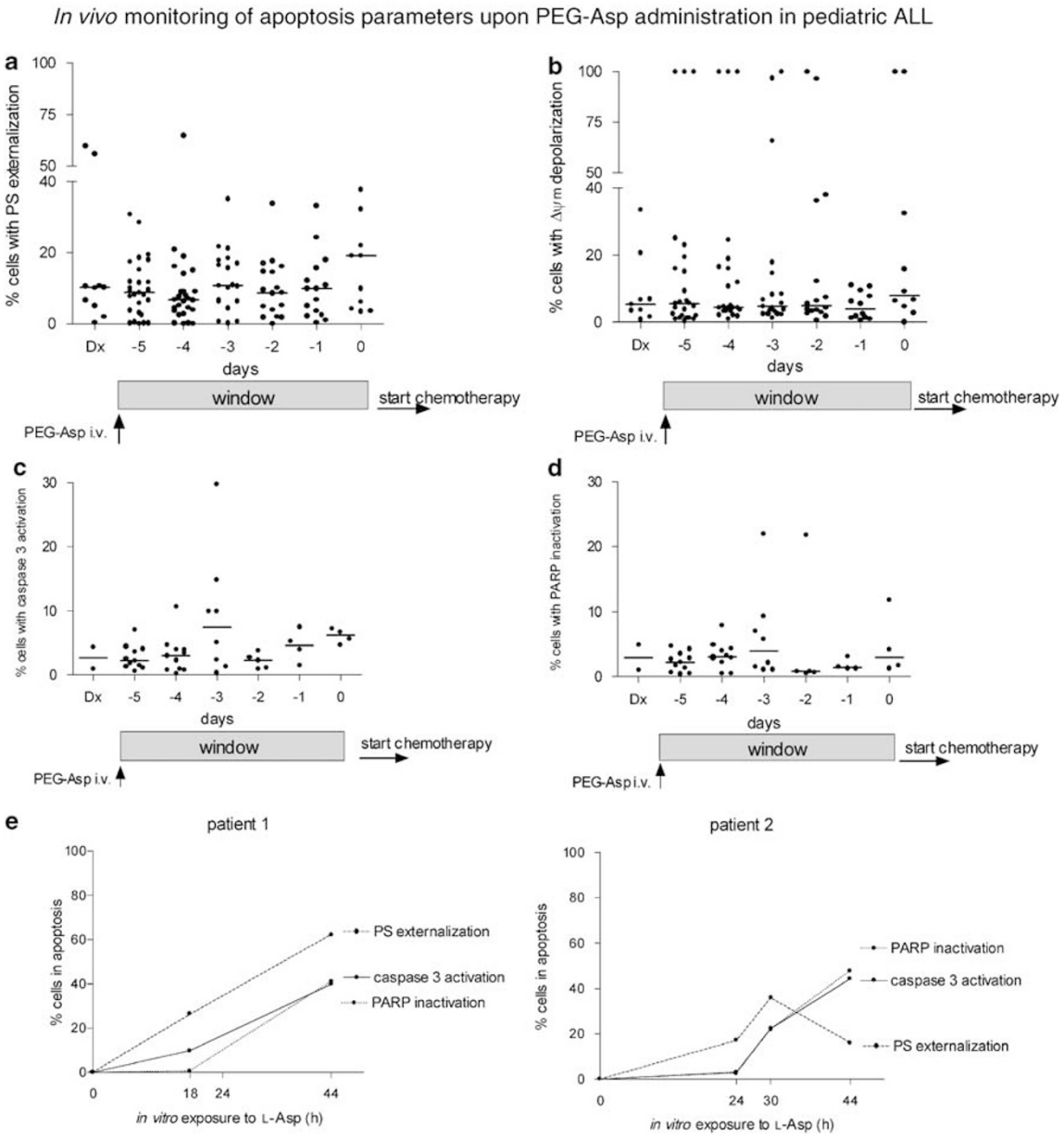

Figure 3 *In vivo* induced apoptosis registered before and during a five day period after the administration of 1000 IU/m^2 PEG-asparaginase upfront regular chemotherapy. Percent of cells with PS externalization (**a**), with $\Delta\psi$m depolarization (**b**), caspase 3 activation (**c**) and PARP inactivation (**d**) are shown. The effect of *in vitro* exposure to 10 IU/ml L-asparaginase in time is given in (**e**) for two patients. PARP, Poly-ADP-ribose-polymerase; PEG, pegylated; PS, phosphatidylserine.

the 30 high-risk patients were scheduled to receive nine 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 doses during induction.

Of 30 high-risk patients, 1 relapsed within 2 months after diagnosis and changed protocol before the intensification phase. Of the remaining 29, 15 (52%) children demonstrated an allergic reaction. Moreover, 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-asparaginase window.

Changes in hemostasis. At diagnosis data point to enhanced thrombin generation. Consumption coagulopathy was monitored in 17 patients (Table 5).

On day 0, 5 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 28 and at the end of induction on day 43 (after four doses of Paronal) demonstrated the same pattern as for patients who did not receive PEG-asparaginase window treatment, as published recently.[35]

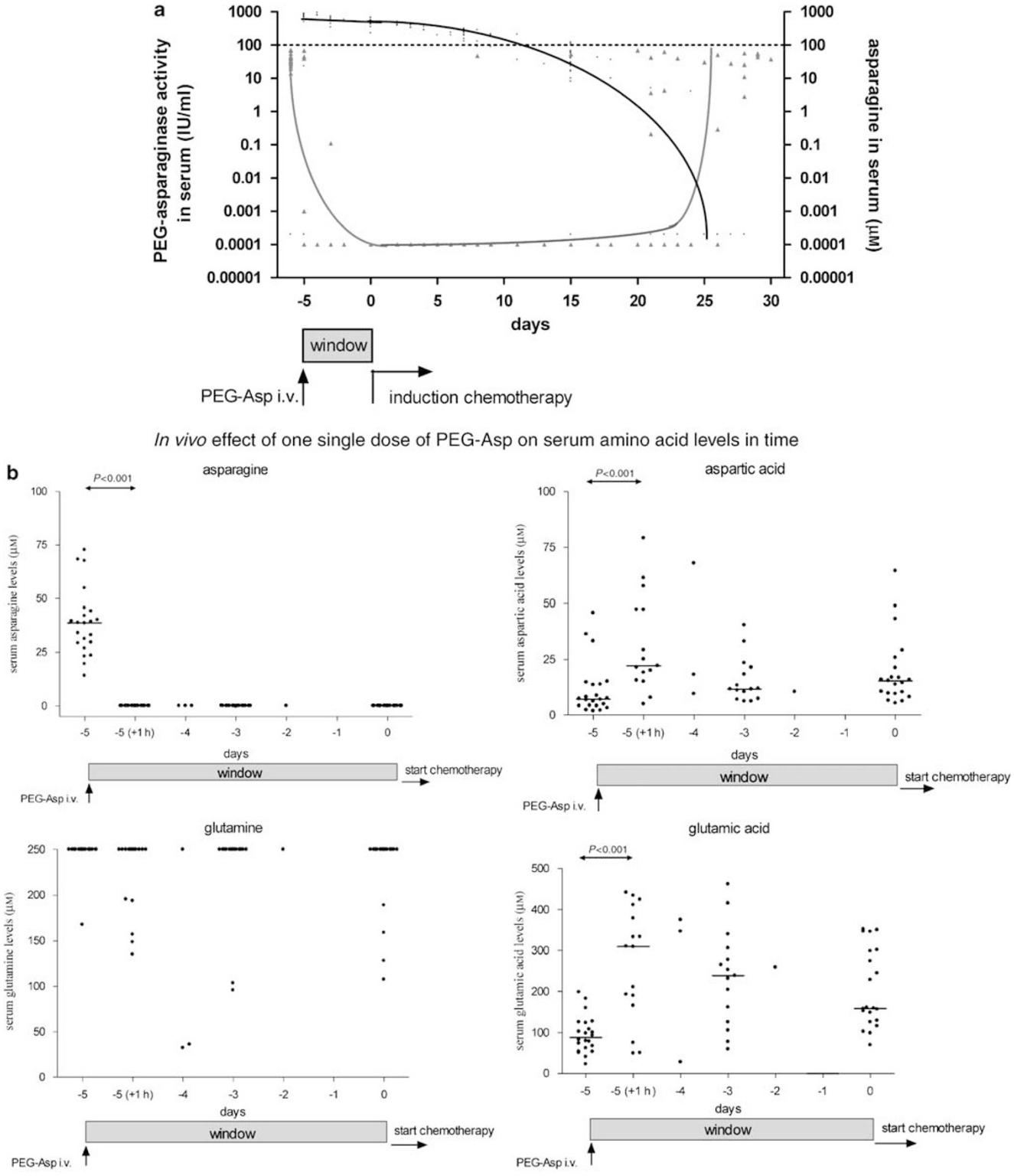

Figure 4 (**a**) The effect of 1000 IU/m^2 PEG-asparaginase administered i.v. 5 days upfront regular chemotherapy on serum asparagine levels in time. The levels of PEG-Asparaginase (left *Y*-axis in IU/ml) are given in black, the levels of asparagine (right *Y*-axis in μM) in gray; 0.0001 μM is the detection limit of asparagine, the upper dotted line is the reference L-asparaginase activity level of 100 IU/ml. (**b**) Serum amino-acid levels of 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 h after the administration of PEG-asparaginase are indicated by $P<0.001$. i.v., intravenously; PEG, pegylated; ALL, acute lymphoblastic leukemia.

Outcome and prognostic factors

Survival analysis of *in vivo* response to PEG-asparaginase showed that clinically good window responders had a more favorable outcome than clinically poor window responders (3-year EFS ± s.e.: 91 ± 6% for good, 81 ± 10% for intermediate and 33 ± 19% for poor responders (median follow-up 4.1 years;

Table 3 Effect of one single dose of PEG-asparaginase on intracellular amino-acid levels in newly diagnosed ALL

Window days	*ALL patients*						*Normal controls*
	−5 (before asparaginase)	*−4*	*−3*	*−2*	*−1*	*0*	
Essential amino acids	μmol/mg, median (p25–p75)	μmol/mg, median (p25–p75)	μmol/mg, median (p25–p75)	μmol/mg, median (p25–p75)	μmol/mg, median (p25–p75)	μmol/mg, median (p25–p75)	μmol/mg, median (p25–p75)
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)
Nonessential 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)

Abbreviations: ALL, acute lymphoblastic leukemia; PEG, pegylated.
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 nine 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: $^1P \leqslant 0.05$, $^2P \leqslant 0.01$ and $^3P < 0.001$ for the comparison of patient data with normal controls.

Figure 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 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 *in 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 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 6). The *P*-value of the trend analysis for all three groups was 0.16.

Of the other variables including age, sex, WBC, immunophenotype and genotype, only WBC ($>$ or $< 50 \times 10^9$ per liter) at diagnosis was significantly related to outcome in the Cox univariate analysis ($P = 0.02$) (Table 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 ($P = 0.042$) (χ^2-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 1). Children with unfavorable characteristics like T-ALL or BCRABL/*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.[34] For BCRABL/*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.

Parameters of apoptosis

Apoptotic markers at diagnosis did not predict the clinical response. We, like others[36] 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.[37]

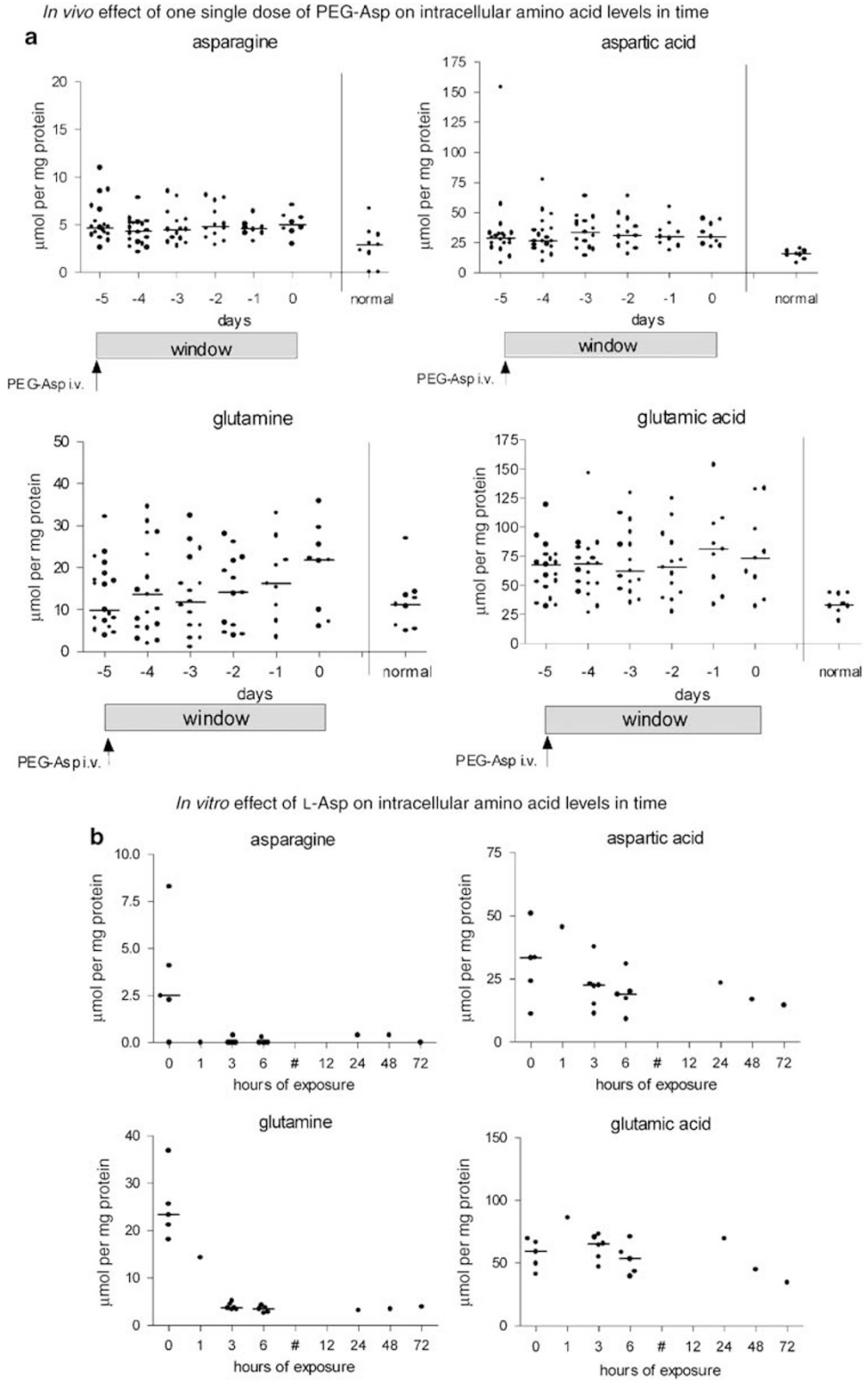

Figure 5 The effect of L-asparaginase on intracellular amino-acid levels of asparagine, aspartic acid, glutamine and glutamic acid in leukemic cells of children with newly diagnosed ALL. (**a**) *In vivo* effect of one dose of 1000 IU/m^2 PEG-asparaginase measured in leukemic cells of 19 children with ALL compared to the levels found in peripheral blood cells of nine healthy control cases. Data are given as µmol/mg of intracellular protein. (**b**) 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 six 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. ALL, acute lymphoblastic leukemia; PEG, pegylated.

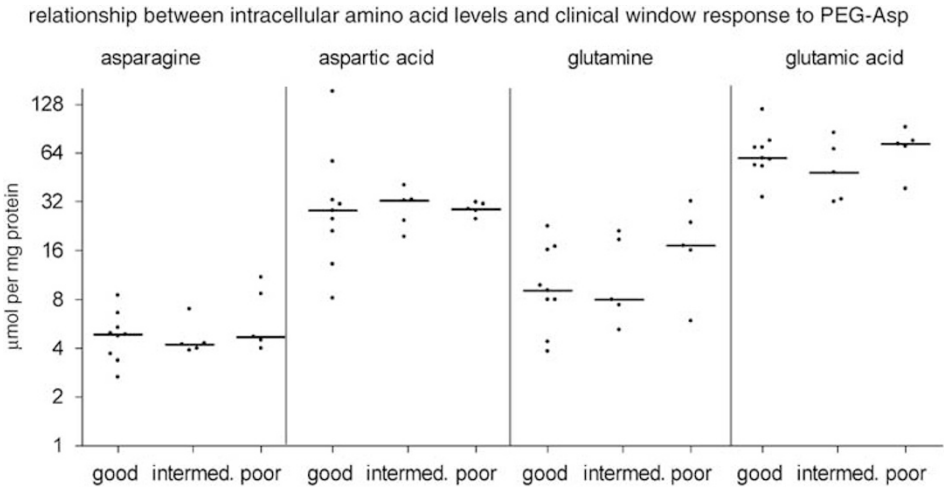

Figure 6 Intracellular amino-acid levels (in µmol/mg of intracellular protein) of asparagine, aspartic acid, glutamine and glutamic acid at diagnosis of 19 children with ALL, related to their clinical response to one dose of 1000 IU/m^2 PEG-asparaginase 5 days later, as defined in figure 1. ALL, acute lymphoblastic leukemia; PEG, pegylated.

Table 4 Intracellular amino-acid levels at diagnosis related to *in vitro* and *in vivo* sensitivity

Essential amino acids	In vitro *ASP resistant*	In vitro *ASP sensitive*	In vivo *poor responders*	In vivo *intermediate/ good responders*
Medians (µmol/mg)				
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)
Nonessential amino acids				
Medians (µmol/mg)				
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)

Abbreviations: ASP, asparaginase; PEG, pegylated.
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.

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.[38] 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 nonresponding 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

Table 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 four Paronal infusions

Asparaginase Dose	*Day -5 PEG-ASP 1000 IU/m²*	*Day 0*	*Day 29 Paronal 6000 IU/m²*	*Day 33 Paronal 6000 IU/m²*	*Day 36 Paronal 6000 IU/m²*	*Day 40 Paronal 6000 IU/m²*	*Day 43*
Screening (s)							
APTT (30–42 s)	32 (27–37)						
PT (11.7–16 s)	13.9 (12.4–15.6)						
Thrombin time (22–26 s)	27 (25.0–30.5)						
Procoagulants							
Fibrinogen (1.6–4.3 g/l)	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)
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							
F 1+2 pmol/l (69–229 pmol/l)	576 (259–1067)	365 (211–588)	201[1] (130–290)	230 (167–293)	267 (178–344)	427 (189–623)	243 (161–362)
TAT (1.5–4.1 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)
Fibrinolysis							
α-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(80–450 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
D-dimer (0.1–0.55 mg/l)	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)

Abbreviations: ALL, acute lymphoblastic leukemia; PEG, pegylated; PEG-ASP, pegylated asparaginase.
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 IU/ml or as indicated otherwise. Normal reference ranges are 0.80–1.30 IU/ml, or as indicated between brackets. Data are given as medians with interquartile ranges. Superscripts refer to *P*-values: [1] = $P \leqslant 0.05$, [2] = $P < 0.01$ and [3] = $P < 0.001$ related to the previous measurement.

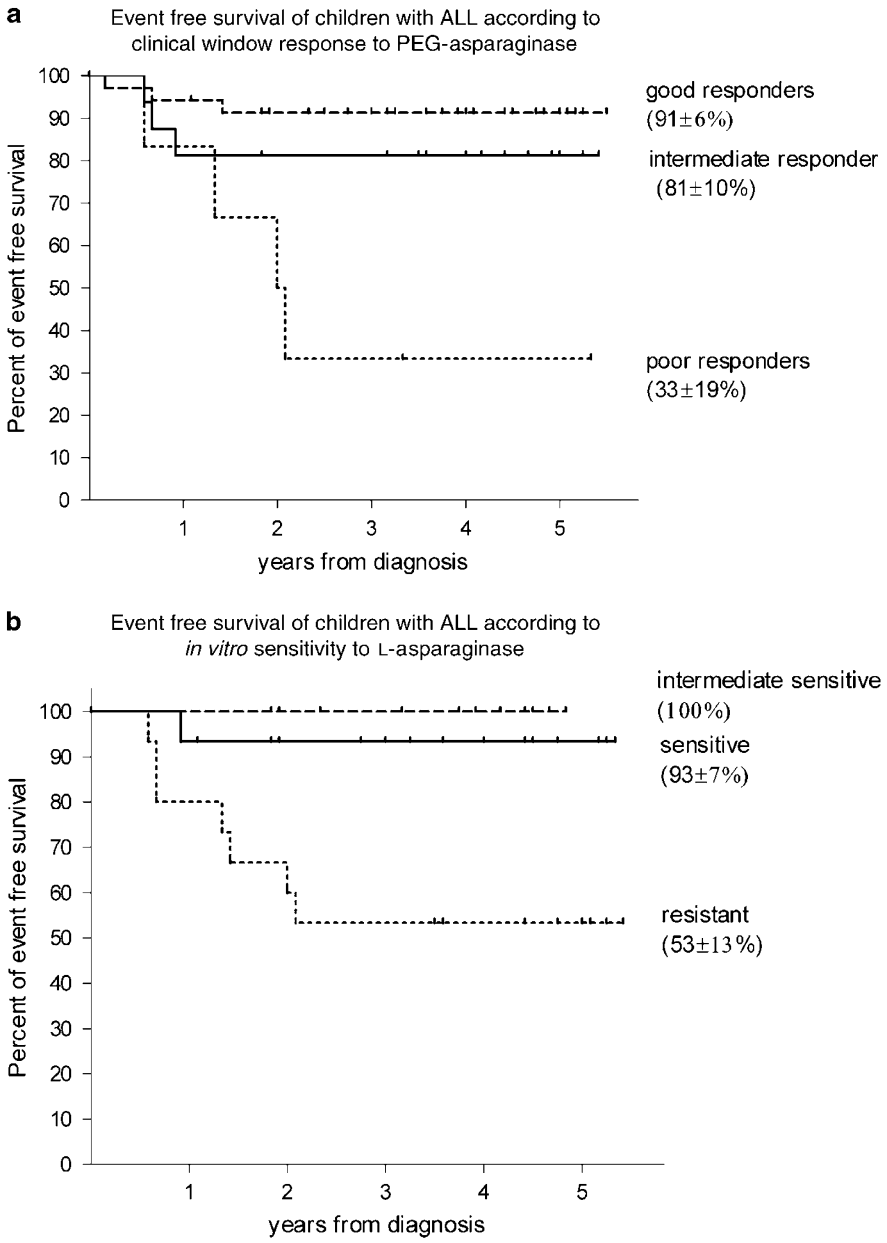

Figure 7 (**a**) Relationship between event free-survival (EFS) and clinical response to the PEG-asparaginase window: 35 good responders, 16 intermediate and six poor responders. The prognosis differed between good and intermediate responders compared to poor responders ($P=0.004$). (**b**) Relationship between EFS and *in vitro* sensitivity to L-asparaginase. Fifteen children were sensitive, 11 intermediate sensitive and 15 were resistant to L-asparaginase. The prognosis differed between the sensitive and intermediate sensitive group compared to the group resistant to L-asparaginase ($P<0.01$). PEG, pegylated.

cannot 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,[39,40] whereas literature on *in vivo* measurements is lacking.

Iwamoto *et al*.[41] recently described the protective effect of mesenchymal cells in L-asparaginase cytotoxicity. They postulated that the interaction between ALL cells and the micro-environment 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). At present protection of ALL cells from L-asparaginase toxicity by mesenchymal cells is part of an ongoing debate.

Another possible explanation for the discrepancy of cellular asparagine levels *in vivo* and *in vitro* comes from the Italian group of Bussolati.[42,43] *In vitro* L-asparaginase rapidly hydrolyzes asparagine and subsequently glutamine if asparagine is depleted.[44] As 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

Table 6 Univariate analyses not stratified for treatment arm

	P-*value*	*Hazard ratio*	*95% CI*	
Age[a]	0.918	0.9	0.2	4.3
Sex	0.214	0.4	0.1	1.8
WBC[b]	0.022	6.1	1.3	28.7
Immunophenotype[c]	0.344	1.9	0.5	7.0
Genotype[d]	0.115	5.3	0.7	41.7
Clinical response to PEG-asparaginase[e]	0.004	6.4	1.8	22.9
L-asparaginase sensitivity (*in vitro*)[f]	0.014	3.7	1.3	10.7

Abbreviations: ALL, acute lymphoblastic leukemia; PEG, pegylated; WBC, white blood count.
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 the outcome.
[a]<or>10 years.
[b]<or>50×10^9 per liter.
[c]T versus precursor B-ALL.
[d]Hyperdiploidy and TEL AML1 compared to the others.
[e]Good and intermediate clinical responders compared to poor responders to PEG-asparaginase.
[f]*In vitro* sensitive and intermediate sensitive compared to resistant to L-asparaginase.

detectable in leukemic blasts after *in vivo* exposure to PEG-asparaginase. Not only Boos *et al.*[44] but also Fine *et al.*[45] 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.[46] It is known that malignant cells are more dependent on an exogenous source of asparagine and glutamine than normal cells.[47]

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.[48] 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.*[49] 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 L-asparaginase-resistant tumor cells.[50,51] Asparagine is required for the transamination of glyoxylate resulting in glycine and serine depletion, which could have severe consequences for purine biosynthesis.[49] However, we did not find evidence to support these hypotheses as 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.[52] In our group valine levels before L-asparaginase treatment were two 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 as Silverman *et al.* observed 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 published recently in children treated with the same protocol without the PEG-asparaginase window.[35] Disturbances in hemostasis due to amino-acid depletion are probably a local effect, that is, in the liver only.[53,54]

Outcome and prognostic factors

Both the clinical response to PEG-asparaginase window and the *in vitro* sensitivity to L-asparaginase are predictive for outcome. The prognostic relevance of the *in vitro* sensitivity has been previously shown.[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 TELAML1 positivity and hyperdiploidy show a good clinical response to PEG-asparaginase and children with the prognostic unfavorable factors T-cell immunophenotype or BCRABL/*t*(9;22) 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 cannot 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-acid 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.

Authorship

IMA designed and analyzed experiments and wrote the paper; KMK performed the research on the laboratory and analyzed the

data; JB contributed vital tools for experiments on serum L-asparaginase activity and serum amino-acid analyses; CL performed experiments and contributed vital tools for experiments on serum L-asparaginase activity and serum amino-acid analyses; JH contributed vital tools for experiments on intracellular amino-acid analyses; AJPV is chairman of the DCOG-ALL-9 protocol; EvW contributed vital tools on behalf of the DCOG; MLdB designed and analyzed experiments and contributed to writing the paper; RP designed and analyzed experiments and contributed to writing the paper.

References

1 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.
2 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.
3 Pieters R, Loonen AH, Huismans DR, Broekema GJ, Dirven MW, Heyenbrok MW *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, 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.
7 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.
8 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.
9 Asselin BL, Kreissman S, Coppola DJ, Bernal SD, Leavitt PR, Gelber RD *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. -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, 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.
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, 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.
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, 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. *J Clin Oncol* 1996; **14**: 911–918.
21 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.
22 Yoshimoto T, Nishimura H, Saito Y, Sakurai K, Kamisaki Y, Wada H *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.
23 Slater RM, Smeets DF, Hagemeijer A, De Jong B, Beverstock CG, Geraedts JP *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.
24 Kaspers GJ, Veerman AJ, Pieters R, Broekema GJ, Huismans DR, Kazemier KM *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.
25 Schrappe M, Reiter A, Zimmermann M, Harbott J, Ludwig WD, Henze G *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.
26 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.
27 Lenda K, Svenneby G. Rapid high-performance liquid chromatographic determination of amino acids in synaptosomal extracts. *J Chromatogr* 1980; **198**: 516–519.
28 Appel IM, Pinheiro JP, den Boer ML, Lanvers C, Reniers NC, Boos J *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.
29 Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD *et al.* Measurement of protein using bicinchoninic acid. *Anal Biochem* 1985; **150**: 76–85.
30 Appel IM, Hop WC, Pieters R. Changes in hypercoagulability by asparaginase: a randomized study between two asparaginases. *Blood Coagul Fibrinolysis* 2006; **17**: 139–146.
31 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.
32 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.
33 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.
34 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.
35 Appel IM, Hop WCJ, van Kessel-Bakvis C, Stigter R, Pieters R. Age-related changes by asparaginase on coagulation and fibrinolysis in childhood acute lymphoblastic leukemia. *Thromb Haemost August* 2008.

36 Stahnke K, Fulda S, Friesen C, Strauss G, Debatin KM. Activation of apoptosis pathways in peripheral blood lymphocytes by *in vivo* chemotherapy. *Blood* 2001; **98**: 3066–3073.
37 Durrieu F, Belloc F, Lacoste L, Dumain P, Chabrol J, Dachary-Prigent J *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.
38 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.
39 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.
40 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.
41 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.
42 Rotoli BM, Uggeri J, Dall'Asta V, Visigalli R, Barilli A, Gatti R *et al*. Inhibition of glutamine synthetase triggers apoptosis in asparaginase-resistant cells. *Cell Physiol Biochem* 2005; **15**: 281–292.
43 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; **60**: 751–758.
44 Wagner A, Boos J. Unphysiological effects contributing to asparaginase toxicity *in vitro*. *Am J Physiol* 1998; **274** (4 Part 1): C1185–C1186.
45 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.
46 Newsholme EA, Crabtree B, Ardawi MS. Glutamine metabolism in lymphocytes: its biochemical, physiological and clinical importance. *Q J Exp Physiol* 1985; **70**: 473–489.
47 Iiboshi Y, Papst PJ, Hunger SP, Terada N. -asparaginase inhibits the rapamycin-targeted signaling pathway. *Biochem Biophys Res Commun* 1999; **260**: 534–539.
48 Wakayama K, Besa EC, Baskin SI. Changes in intracellular taurine content of human leukemic cells. *Nagoya J Med Sci* 1983; **45**: 89–96.
49 Chakrabarti R, Schuster SM. -asparaginase: perspectives on the mechanism of action and resistance. *Int J Ped Hem/Oncol* 1997; **4**: 597–611.
50 Ryan WL, Sornson HC. Glycine inhibition of asparaginase. *Science* 1970; **167**: 1512–1513.
51 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.
52 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.
53 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.
54 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.