UPREGULATION OF ASPARAGINE SYNTHETASE EXPRESSION IS NOT LINKED TO THE CLINICAL RESPONSE TO L-ASPARAGINASE IN PEDIATRIC ACUTE LYMPHOBLASTIC LEUKEMIA

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Running title: The clinical value of asparagine synthetase upregulation in ALL
Abstract

L-Asparaginase is an effective drug for treatment of children with acute lymphoblastic leukemia. 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 re-synthesis of asparagine. In vitro, resistance to L-Asp has been associated with upregulation of AS mRNA expression. We monitored AS mRNA levels in leukemic cells before and during 5 days after 1000 IU/m² i.v. 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 four 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 upregulation was found between precursor B and T-ALL, nor between hyperdiploids, TEL/AML1 rearranged ALL or absence of genetic abnormalities. In 3 out of 12 T-ALL patients even a slight downregulation of AS mRNA expression upon L-Asp exposure was found. Concluding, although L-Asp exposure induces the expression of AS mRNA, the upregulated 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]. 25-60% of newly diagnosed ALL patients 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 upregulation of both mRNA, protein and activity of AS [5]. Resistance to L-Asp in cell lines is in vitro mediated by an upregulation of AS expression in response to asparagine depletion of culture medium [6, 7]. Whereas these cell line studies suggest that upregulation 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.

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 E.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 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. Eligible were children with ALL at initial diagnosis and presenting with white blood count (WBC) >10x10⁹/L. Similar to the study of 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. Several groups[12] showed that a plasma E.coli Asp activity > 100 IU/L leads to an asparagine depletion of < 0.2 µM in plasma. Muller demonstrated that one dose of 1000 IU/m² PEG-Asp resulted into > 100 U/L serum enzyme activity of L-Asp for three weeks [13]. In a previous study we confirmed that 1000 IU/m² PEG-Asp given as a therapeutic window at day −5 (i.e. 5 days before starting combined chemotherapy) results into >
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/m² PEG-Asp in a one hour infusion 5 days before starting the DCOG-ALL-9 combination chemotherapy treatment schedule. PEG-Asp, kindly provided by Medac (GmbH, 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 here after.

We decided to use the same definition for clinical response that is used for response to prednisone[16]: more than 1000 leukemic blasts per µl (1 x 10⁹/L) 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 < 1 x 10⁹/L of peripheral blood, as intermediate when leukemic cells were 1-10 x 10⁹/L, and as poor when leukemic cells were > 10 x 10⁹/L.

Between July 2000 and October 2002, 31 ALL patients were enrolled of which 25 children were diagnosed in the Erasmus MC-Sophia Children’s Hospital, Rotterdam, and 6 children in three other university hospitals in the Netherlands. Patients’ characteristics are shown in Table 1. 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 proB 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 (CD 19⁺, HLA-DR⁺) were classified into the following differentiation stages: proB-ALL cells were CD10⁻, cytoplasmic μ chain (cµ⁻) and surface immunoglobulin (sIg⁻); cALL were CD10+/cµ⁻/sIg⁻; preB were CD10+ or -/cµ+/sIg-. B-ALL cells characterized by CD10⁻/cµ⁻/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/guardians have given informed consent for these studies.

**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 to leukemic cells isolated out of bone marrow. Therefore, blood samples were collected during 5 consecutive days till 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 non-leukemic cells in the ALL samples were removed by
immunomagnetic beads as described by Kaspers et al [17]. All samples contained over 90% of 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 x 10^6 cells using Trizol reagent (Life Technologies) according to the manufacturer’s protocol, with minor modifications as reported previously [18]. The concentration of RNA was quantified spectrophotometrically and the quality was checked on agarose gels. Following a denaturation step of 5 minutes at 70°C, 1 µg of RNA was reversely transcribed into single stranded cDNA. The reverse transcription (RT) was performed in a total volume of 25µl 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°C for 30 minutes, 42°C for 15 minutes and 94°C for 5 minutes. The obtained cDNA was diluted to a final concentration of 8 ng/µl and stored at –80°C.

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 [18]. 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 GAPDH expression of the patient. The relative mRNA expression level of the target gene in each patient was calculated using the comparative cycle time (Ct) method [19].

Briefly, the target PCR Ct values, that is the cycle number at which emitted fluorescence exceeds the 10 x SD of baseline emissions as measured from cycles 3 to 12, is normalized by subtracting the GAPDH Ct value from the target PCR Ct value, which gives the ΔCt value. From this the ΔCt value, the relative expression level to GAPDH for AS is calculated using the following equation: relative mRNA expression = 2^{-ΔCt} 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 > 10 x 10^9/L were consecutively enrolled into the study. As is shown in Table 1, 31 children were eligible at the moment of analysis: 1 pro B, 9 common, 9 pre B and 12 T ALL patients.

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, five days after PEG-Asp was given. As can be seen in Figure 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 0.2 x 10^9/L at day 0. This was more than a 2-log decrease in leukemic cell burden. 21 (68%) children were PEG-Asp good responders (blast number < 1 x 10^9/L at day 0), 6 (19%) were intermediate responders (blasts 1- 10 x 10^9/L at day 0) and 4 (12%) children were poor responders (blasts > 10 x 10^9/L at day 0) (Figure 1).

The baseline expression level of AS mRNA relative to GAPDH was median 0.24% (range 0.05% – 2.5%) in leukemic cells (>90% purity). This was in the range of healthy controls as described in our previous study [18]. The expression levels of AS in leukemic cells relative to GAPDH increased significantly median 3-fold from 0.24% (basal expression) to 0.73% (24 hours later) (p<0.00001), (Figure 2). During the following 4 days the expression of AS mRNA remained stable at the level of 24 hours (Figure 2).

The baseline expression level of AS mRNA did not differ between good and intermediate (p=0.614), good and poor (p=0.852) and intermediate and poor (p=1.0) responders respectively; nor did the upregulated AS levels after 24 hours of PEG-Asp differ between good and intermediate (p=0.614), good and poor (p=0.737) and intermediate and poor (p=0.914) responders respectively (Figure 3A and 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 4. The median levels of AS mRNA relative to GAPDH mRNA for c/preB ALL patients (0.21%) and for the T ALL patients (0.28%) did not significantly differ (p=0.376). One window-patient had a proB ALL for which the baseline expression of AS mRNA was three-fold higher than the other c/preB patients. To explore whether proB ALL is associated with a high AS mRNA expression we analyzed the AS expression of 23 infant proB ALL cases. Infants with proB ALL had median 0.15% (range 0.07-1.43) AS mRNA expression levels which was not significantly different from the baseline AS expression values in non-infants with c/preB or T-ALL (Figure 4A). The c/preB ALL group had a median baseline AS expression level of 0.21% that rose significantly to median 0.72% one day later (median 3.99 fold individual upregulation, p=0.0004). Patients with T-ALL demonstrated a significant increase from basal 0.28% to 0.68% one day later (median 1.94 fold individual upregulation, p=0.012). T-ALL patients tended to have a lower individual upregulation of AS mRNA compared with the c/pre B-ALL children, but this was not statistically different (p=0.107) (Figure 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 T-ALL cases of whom 2 were clinically good responders.
The baseline nor 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 since 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 by exposing cells to sublethal concentrations of this drug [20]. Resistant cells upregulated AS expression and activity by 60-fold. It is well known that AS plays a crucial role in maintaining amino acid homeostasis in cells [21]. A rapid transcriptional control of the AS gene occurs following deprivation of any single essential amino acid [6, 22]. In 1997 Hutson et al [6] demonstrated that depletion of the intracellular asparagine pool by L-Asp was sufficient to activate \textit{in vitro} AS expression in human leukemic cell lines. The increase in AS mRNA expression also resulted in a simultaneous upregulation of AS protein levels and AS enzyme activity [6, 7]. The direct correlation between mRNA, protein and activity levels was confirmed by Irino et al [23]. The activity of AS was inversely related to the sensitivity to L-Asp in human leukemia cell lines [6, 7]. These studies suggest that the expression of the AS gene is linked to resistance to L-Asp. However in addition to the fact that these studies did not deal with primary patient samples but cell lines, Wagner and Boos [24] argued that the test conditions in Hutson’s experiments were not comparable with \textit{in vivo} situations, where various products and substrates, like a.o. aspartate, glutamate, glutamine and ammonia, are all part of metabolic pathways and equilibrium conditions [5].

In 1969 Haskell studied \textit{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 E.coli L-Asp 200 IU/kg 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, AML, CML and CLL). Haskell suggested that L-Asp resistance was related to the capacity of leukemic cells to upregulate 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.

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 ASP or PEG-Asp is the same if the serum enzyme activity of L-Asp is higher than 100 U/L [13]. We studied whether baseline levels or upregulated levels of AS mRNA expression in leukemic cells after an \textit{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 [18]. Upregulation 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 1 and 2), it is unlikely that only leukemic cells resistant to PEG-Asp with intrinsic higher AS expression levels were left over on day –4. Both baseline and L-Asp induced AS mRNA expression levels did not differ between patients with good, intermediate or poor response (Figure 3). So, L-Asp induced upregulation of AS mRNA is not related to early \textit{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 due to the fact that 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 \textit{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/preB ALL patients (Figure 4). Remarkable was the finding that 3 out of 12 children with T-ALL even demonstrated a slight AS mRNA downregulation, which would in vitro even point to sensitivity for L-Asp. Hyperdiploidy and the TEL/AML1 fusion are related to favourable outcome in childhood ALL[26, 29] and are both in vitro sensitive to L-Asp [18, 30, 31]. In a previous study in TEL/AML1 positive ALL, Stams et al [18] 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 upregulation 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.

AS mRNA upregulation 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, 21]. 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, i.e. 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 upregulation 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 upregulation of AS mRNA in childhood ALL cells occurs within 24 hours after in vivo exposure to PEG-Asp, but these upregulated levels are not associated with an early (poor) response to PEG-Asp in this small group of children.
Table 1. Characteristics of 31 patients treated with one dose of PEG-Asp upfront the DCOG-ALL9 study

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
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<tbody>
<tr>
<td>Total</td>
<td>31</td>
</tr>
<tr>
<td>Male / Female</td>
<td>17 / 14</td>
</tr>
<tr>
<td>Age</td>
<td>median 4.2 years (range 1.2-13.1)</td>
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<tr>
<td>WBC at diagnosis</td>
<td>median 47 x 10^9/l (range 11.4-417)</td>
</tr>
<tr>
<td>Immunophenotype</td>
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</tr>
<tr>
<td>pro B ALL</td>
<td>1</td>
</tr>
<tr>
<td>common ALL</td>
<td>9</td>
</tr>
<tr>
<td>pre B ALL</td>
<td>9</td>
</tr>
<tr>
<td>T ALL</td>
<td>12</td>
</tr>
<tr>
<td>Cytogenetic characteristics</td>
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</tr>
<tr>
<td>Hyperdiploid</td>
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</tr>
<tr>
<td>TEL-AML1 fusion</td>
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</tr>
<tr>
<td>BCR-ABL fusion</td>
<td>1*</td>
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<tr>
<td>MLL gene rearranged</td>
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<td>others</td>
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<tr>
<td>CNS involvement **</td>
<td>(yes/no)</td>
</tr>
<tr>
<td></td>
<td>0/31</td>
</tr>
</tbody>
</table>

* one patient had both a hyperdiploidy and a BCR-ABL fusion

** CNS involvement defined as >5 cells/μl with blasts in the cerebro spinal fluid
Figure 1

Clinical response to PEG-Asp in ALL

number of leukemic cells in peripheral blood (x 10^9/L)

-5 -4 -3 -2 -1 0

days after initial Dx

window

1000 IU/m^2 PEG-Asp i.v.

induction CT

poor responders

intermediate responders

good responders
Figure 2

In vivo upregulation of asparagine synthetase mRNA expression

Table 2. Asparagine synthetase (AS) mRNA expression values (medians (p25-p75)) in time

<table>
<thead>
<tr>
<th>days</th>
<th>-5</th>
<th>-4</th>
<th>-3</th>
<th>-2</th>
<th>-1</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median expression of AS compared to GAPDH (%) (p25-p75)</td>
<td>0.26 (0.16-0.46)</td>
<td>0.75 (0.45-1.4)</td>
<td>0.75 (0.52-1.18)</td>
<td>0.87 (0.47-1.17)</td>
<td>1.05 (0.48-1.05)</td>
<td>0.99 (0.53-1.8)</td>
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<tr>
<td>Wilcoxon signed rank test compared between successive days</td>
<td>p&lt;0.0001</td>
<td>p=0.4</td>
<td>p=0.4</td>
<td>p=0.06</td>
<td>p=0.4</td>
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<tr>
<td>Wilcoxon signed rank test; day-5 compared to d-4, d-3, d-2, d-1, d 0</td>
<td>p&lt;0.0001</td>
<td>p=0.0014</td>
<td>p&lt;0.0001</td>
<td>p=0.001</td>
<td>p=0.002</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3 A

Baseline asparagine synthetase mRNA expression and clinical window response

Figure 3 B

PEG-Asp induced changes in asparagine synthetase mRNA expression and clinical window response
Figure 4 A

Baseline asparagine synthetase mRNA expression and immunophenotype

Figure 4 B

PEG-Asp induced changes in asparagine synthetase mRNA expression and immunophenotype
Legends

Table 1
Characteristics of 31 patients treated with one dose of pegylated L-asparaginase (PEG-Asp) (1000 IU/m² i.v.) upfront the DCOG (Dutch Childhood Oncology Group) ALL-9 combination chemotherapy.

Table 2
Median (p25-p75) expression values of ASmRNA compared to 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.

Figure 1
Clinical response to pegylated L-asparaginase (PEG-Asp) in pediatric acute lymphoblastic leukemia (ALL). Clinical response to 1000 IU/m² i.v. 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/75th percentile.

A good clinical response is defined by < 1 x 10⁹/blasts/L at day 0, an intermediate response by 1 - 10 x 10⁹/blasts/L on day 0, and poor response by > 10 x 10⁹/blasts/L at day 0. Dotted lines indicate the cut-off values for these clinical responses.

Figure 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/m² i.v. given at day –5).

* = 3-fold increase in AS mRNA from day-5 to day-4 (p<0.00001).
**Figure 3**

Relationship between asparagine synthetase (AS) mRNA expression induced by pegylated L-asparaginase (PEG-Asp) and clinical response.

(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 1.

**Figure 4**

Relationship between immunophenotype and asparagine synthetase (AS) mRNA expression

(A) Baseline AS mRNA expression levels, (B) pegylated L-asparaginase (PEG-Asp) induced changes in AS expression levels in acute lymphoblastic leukemia (ALL) cells. Dots represent individual expression values, solid lines represent the median expression value per group.
References


Upregulation of asparagine synthetase expression is not linked to the clinical response to L-asparaginase in pediatric acute lymphoblastic leukemia

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