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Asparagine levels in the cerebrospinal fluid of children with acute lymphoblastic leukemia treated with pegylated-asparaginase in the induction phase of the AIEOP-BFM ALL 2009 study

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ABSTRACT

Asparagine levels in cerebrospinal fluid and serum asparaginase activity were monitored in children with acute lymphoblastic leukemia treated with pegylated-asparaginase. The drug was given intravenously at a dose of 2,500 IU/m^2 on days 12 and 26. Serum and cerebrospinal fluid samples obtained on days 33 and 45 were analyzed centrally. Since physiological levels of asparagine in the cerebrospinal fluid of children and adolescents are 4-10 μmol/L, in this study asparagine depletion was considered complete when the concentration of asparagine was ≤0.2 μmol/L, i.e. below the lower limit of quantification of the assay used. Over 24 months 736 patients (AIEOP n=245, BFM n=491) and 903 cerebrospinal fluid samples (n=686 on day 33 and n=217 on day 45) were available for analysis. Data were analyzed separately for the AIEOP and BFM cohorts and yielded superimposable results. Independently of serum asparaginase activity levels, cerebrospinal fluid asparagine levels were significantly reduced during the investigated study phase but only 28% of analyzed samples showed complete asparagine depletion while relevant levels, ≥1 μmol/L, were still detectable in around 23% of them. Complete cerebrospinal fluid asparagine depletion was found in around 5-6% and 33-37% of samples at serum asparaginase activity levels <100 and ≥1,500 IU/L,

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respectively. In this study cerebrospinal fluid asparagine levels were reduced during pegylated-asparaginase treatment, but complete depletion was only observed in a minority of patients. No clear threshold of serum pegylated-asparaginase activity level resulting in complete cerebrospinal fluid asparagine depletion was identified. The consistency of the results found in the two independent data sets strengthen the observations of this study. Details of the treatment are available in the European Clinical Trials Database at *https://www.clinicaltrialsregister.eu/ctr-search/trial/2007-004270-43/IT*.

Introduction

Asparaginase is one of the major anticancer drugs used in the treatment of acute lymphoblastic leukemia (ALL). The enzyme reduces the levels of asparagine in serum by hydrolyzing it to aspartic acid and ammonia. Currently there are three commercially available asparaginase products.[1] The oldest one is the purified native enzyme extracted from *Escherichia coli*, subsequently also available in a polyethylene glycol conjugated form (PEG-asparaginase) commonly used as the first-line preparation in the treatment of children with ALL throughout Europe and USA. A third asparaginase product derived from *Erwinia chrysanthemi* (ERW-asparaginase) exists and, due to its structural differences with respect to the *E. coli* asparaginase products, has been primarily used as a second-line treatment in children with hypersensitivity reactions to the *E. coli* products.[2] Since leukemic cells need exogenous asparagine for their survival much more than the normal host cells do, the depletion of asparagine in serum serves as a surrogate for the anti-leukemic action of asparaginase, no matter which type of product is used. Due to this mechanism of action and to the pharmacodynamic ability of asparaginase products to reduce asparagine pools also in the cerebrospinal fluid (CSF), it has been questioned whether profound and prolonged asparagine depletion, as that determined in the serum, could be of relevance in preventing central nervous system (CNS) relapses.[3] Of course, it is exceedingly difficult to ascertain the role of a single drug in the prevention of ALL relapses, especially in an extramedullary compartment such as the CNS where relapses are quite rare. However, in a previous study, patients with higher CSF asparagine levels (>1 µmol/L) during asparaginase treatment were more likely to have isolated CNS relapse.[4]

Available studies reporting data on CSF asparagine depletion during asparaginase treatment have been mostly performed in limited cohorts of patients and using different asparaginase products, schedules and assays. In the past it has been consistently reported that profound and prolonged CSF asparagine depletion in children treated with standard induction chemotherapy treatment schedules[5–9] is achieved when native forms of asparaginase are used. To this end the conceptual question on how asparaginase products may determine asparagine depletion in the CSF remains unanswered. One possible explanation for the asparagine depletion observed in the CSF could lie in a continuous balance between the serum and CSF asparagine pools.[10,11] Another possible explanation is that, at peak levels, very small amounts of asparaginase products could cross the blood-brain barrier;[12] however, activity levels have never been directly measured in the CSF during the administration of native forms of asparaginase. Given that PEG-asparaginase has a far greater molecular weight than that of the native forms of asparaginase, it is conceivable that it is even more difficult for the pegylated form to cross the blood-brain barrier. Different results have been reported in patients treated with PEG-asparaginase wherein detectable CSF asparagine levels have been almost invariably reported thus suggesting that pharmacodynamic differences exist between the different asparaginase products.[4,13–15] We very recently demonstrated, even with the limitations of the experimental preclinical model adopted, that in the CSF of rats asparaginase activity levels could be measured, consistently even if transiently, only for non-pegylated formulations.[16]

In the international AIEOP-BFM ALL 2009 trial protocol (*https://www.clinicaltrialsregister.eu/ctr-search/trial/2007-004270-43/IT*), conducted by members of the *Associazione Italiana di Ematologia e Oncologia Pediatrica* (AIEOP) and Berlin-Frankfurt-Münster (BFM) group, children with newly diagnosed ALL have been treated with multiple antileukemic agents, including PEG-asparaginase as the first-line preparation. Since PEG-asparaginase has been used in the AIEOP-BFM ALL 2009 study protocol for the first time as a front-line asparaginase agent instead of the previously used native *E. coli* asparaginase product and since two different randomized studies, consisting of PEG-asparaginase-intensified schedules, are the most relevant treatment questions of the AIEOP-BFM ALL 2009 study, a tight therapeutic drug monitoring study of PEG-asparaginase treatment has been implemented to better understand the pharmacological phenomena underlying asparaginase treatment in this therapeutic context.

The main findings of the above-mentioned therapeutic drug monitoring specifically related to CSF asparagine levels and asparaginase serum activity, analyzed in parallel after the administration of PEG-asparaginase in the induction phase of the AIEOP-BFM ALL 2009 study, are the focus of the present report.

Methods

Patients' eligibility and treatment schedule

Children ≥1 year and <18 years old diagnosed with ALL and eligible for the AIEOP-BFM ALL 2009 protocol were investigated in this study. PEG-asparaginase (Oncaspar®, Shire) was given during the induction phase (namely protocol IA) to children diagnosed and treated in the participating centers. The drug was given intravenously as a 2 h infusion at the dosage of 2,500 IU/m^2 with a maximum dose of 3,750 IU/m^2 on days 12 and 26. Details of the treatment are available in the European Clinical Trials Database at *https://www.clinicaltrialsregister.eu/ctr-search/trial/2007-004270-43/IT*. CSF asparagine levels were evaluated when lumbar punctures relevant for this part of the PEG-asparaginase study were scheduled in protocols IA and the subsequent consolidation phase protocol IB, i.e., on protocol days +33 and +45, respectively, which correspond to days 7 and 19 after the second PEG-asparaginase dose of protocol IA. Serum asparaginase activity levels were measured at the same two time points. Mainly because of the long half-life of PEG-asparaginase and the slow decay of the related activity levels, and in order to have a larger set of samples to be analyzed, data

analyses were focused on these two time points only but with the following adjustments: (i) CSF samples were considered analyzable when collected at a distance of 7±3 days and of 19±3 days after the second PEG-asparaginase dose (day +26) of protocol IA; (ii) the serum samples had to be collected the same day (±1) as the CSF samples. In the text, tables and figures of this report the CSF asparagine values obtained at day +33 ±3 and day +45 ±3 are simply referred to as day +33 and day +45.

Sample collection

Serum and CSF sample collection started on June 1st, 2010; CSF collection ended on December 31st, 2012. Samples were collected from patients treated according to the AIEOP-BFM ALL 2009 protocol in Italy (AIEOP), Germany (BFM-G), Austria (BFM-A), and the Czech Republic (CPH). CSF samples were immediately frozen at -80°C, shipped on dried ice and stocked at -80°C until amino acid analysis. Blood samples from a peripheral vein or central venous catheter were collected according to the treatment schedule. Serum was separated in 2 mL tubes and immediately frozen at -20°C until shipment. CSF asparagine levels and asparaginase serum activity were determined in the Laboratory of Cancer Pharmacology at the Department of Oncology of the "Mario Negri" Pharmacology Research Institute IRCCS (Milan, Italy) for the AIEOP samples, in the Clinical Pharmacology Laboratory of the Department of Pediatric Hematology and Oncology at the University Hospital of Muenster (Germany) for the BFM-G and CPH samples and in the Department of Pediatrics and Adolescent Medicine of the Medical University of Vienna (Austria) for the BFM-A CSF samples.

Determination of serum pegylated-asparaginase activity

PEG-asparaginase activity was evaluated with the commercially available enzymatic medac asparaginase activity test (MAAT) (medac GmbH, Hamburg, Germany) or with the L-aspartic β-hydroxamate (AHA) test.[17]

The medac asparaginase activity test

Briefly, the MAAT is an IVD-CE-certified test which is commercially available. It is a homogeneous microplate assay that analyses the catalytically active asparaginase in serum by detecting the amount of hydrolyzed substrate analogue of asparagine, quantified by photometric reading at 700 nm. The assay uses calibrators containing a native enzyme preparation from *E. coli* (ASP medac) and has a lower limit of quantification (LLOQ) of 30 U/L. All the values below the LLOQ were considered 0 U/L for the statistical analysis. The MAAT was used for the determination of asparaginase activity in the serum samples of AIEOP patients.

The L-aspartic β-hydroxamate test

AHA is the substrate for the quantification of native *E. coli*, pegylated *E. coli*, and *Erwinia chrysanthemii* asparaginase in human serum. Asparaginase hydrolyzes AHA to L-aspartic acid and hydroxylamine, which is determined at 710 nm after condensation with 8-hydroxyquinoline and oxidation to indooxine. The LLOQ is 5 U/L.[17] All the values below the LLOQ were considered 0 U/L for the statistical analysis. The AHA test was used for the determination of asparaginase activity in serum of CPH and BFM-G samples. Since the AHA test calibrates against known amounts of PEG-asparaginase in contrast to the MAAT, which uses native *E. coli* asparaginase as the calibrator, it considers the different substrate turnover rates of PEG-asparaginase compared to native *E. coli* asparaginase under the assay conditions. Thus, the PEG-asparaginase activity determined by the MAAT is a mean of 1.42 higher than that determined by the AHA test, as recently demonstrated.[18]

Determination of cerebrospinal fluid asparagine levels

CSF asparagine levels were measured using a high performance liquid chromatographic technique after derivatization with *o*-phthaldialdehyde as described by Turnell and Cooper[19] and already used in previous pharmacological studies performed by the AIEOP-BFM group.[15,20] The LLOQ was 0.2 μmol/L and all the analyzed data with results below this limit were considered 0 μmol/L for the statistical analysis. Since bloody CSF punctures might have altered the quantification of asparagine, either though the release of asparagine present in the erythrocytes or through possible contamination by asparaginase, CSF samples contaminated with blood were excluded from the analysis.

Informed consent

All patients and their parents or legal guardians signed appropriate informed consent for the biological study procedure encompassed in the AIEOP-BFM ALL 2009 study for the asparaginase therapeutic drug monitoring. Assent was given by patients according to ethical standards and national guidelines. Protocol studies were approved by each national and local review board, in accordance with the Declaration of Helsinki and national laws.

Statistical analysis

Descriptive analyses include the distribution of patients' characteristics and dot plots on CSF asparagine concentration and PEG-asparaginase serum activity. Box plots and scatter plots were used to describe continuous values, with the Wilcoxon test to compare medians. Data are presented separately on the original scale according to the type of enzymatic test used, which was MAAT for AIEOP samples and AHA for all other samples, collectively identified as BFM samples.

Results

Between June 2010 and December 2012 1,764 patients were unselectively enrolled in Italian, German, Czech, and Austrian centers adopting the AIEOP-BFM ALL 2009 study protocol. Overall, 736 patients were included in the present study, 245 of whom belonged to the AIEOP cohort and 491 to the BFM cohort. Their main biological and clinical characteristics are presented in Table 1. The distribution of these characteristics is superimposable to that of the entire cohort of 1,764 patients enrolled in the study in the same period (*data not shown*).

The total number of CSF samples collected in the two groups was 903. Overall, 903 CSF samples were collected on days 33 and/or 45, of which 314 in the AIEOP cohort and 589 in the BFM cohort.

Asparagine levels in cerebrospinal fluid

Of the 903 CSF samples analyzed for asparagine levels, 686 (AIEOP n=230 and BFM n=456) were collected on protocol day +33 and 217 (AIEOP n=84 and BFM n=133) on protocol day +45. The distribution of different CSF asparagine levels detected at the CSF punctures (on days +33 and +45) is presented in Table 2 and Figure 1 (A and B for the AIEOP and BFM cohorts, respectively). Given that the physiological concentration of asparagine in the CSF of children and adolescents ranges between 4 and 10 μmol/L, the CSF asparagine levels found in this study were overall quite consistently reduced at both time points, as depicted in Figure 1. Independently of the levels of asparaginase activity, CSF asparagine levels were significantly reduced during the investigated study phase but

only 28% of analyzed samples showed complete asparagine depletion (i.e. below the LLOQ) while relevant levels (≥1 μmol/L) were still detectable in around 23% of them. In particular, asparagine levels ≥1 μmol/L were detected at days +33 and +45 in 16.9% and 34.6% (AIEOP, Table 2A) and in 18.9% and 41.4% (BFM, Table 2B) of analyzed CSF samples, respectively. Median levels were significantly higher at day +45 than at day +33 (AIEOP, $P<0.001$; BFM, $P<0.001$).

Asparaginase activity in serum and correlation with cerebrospinal fluid asparagine levels

Overall there were 753 serum samples (AIEOP n=271 and BFM n=482) corresponding to the available CSF samples of which 574 (AIEOP n=198 and BFM n=376) were collected on day +33 and 179 (AIEOP n=73 and BFM n=106) on day +45. The mean PEG-asparaginase activity levels measured in these serum samples were 1,839 (±685) IU/L and 314 (±266) IU/L at days +33 and +45 in AIEOP samples (MAAT) and 1,226 (±470) IU/L and 222 (±141) IU/L in BFM samples (AHA test), respectively. PEG-asparaginase activity <100 IU/L was found in 1.9% of serum samples (2.5% in AIEOP and 1.6% in BFM) taken at day +33 and in 19.6% of serum samples (17.8% in AIEOP and 20.8% in BFM) taken at day +45 (Table 5A,B)

As shown in Table 3A (for the AIEOP cohort) and 3B (for the BFM cohort), at serum asparaginase activity levels <100 IU/L, 100% and 89.3% of the respective patients had CSF asparagine levels >0.2 μmol/L, while this rate decreased to approximately 70% at asparaginase activity ≥100 IU/L. Figures 2 and 3 (subdivided in A and B for the AIEOP and BFM cohorts, respectively) show the CSF asparagine levels at days +33 and +45 in relationship to the asparaginase activity detected at the same time points. At serum asparaginase activity levels lower than 100 IU/L the median CSF asparagine concentration was higher than that in the cohorts with serum activity above 100 IU/L and below 500 IU/L ($P<0.003$ for AIEOP and $P<0.002$ for BFM) and that with an overall activity level above 100 IU/L ($P<0.001$). When asparaginase activity and CSF asparagine levels were determined using progressively higher activity level intervals, even at high asparaginase serum levels of ≥1,500 IU/L, CSF asparagine levels below the LLOQ were found in roughly one-third of the samples (Table 3A and B), indicating that the asparagine level in the CSF was above the LLOQ even at higher levels of asparaginase activity in serum.

Table 1. Main biological and clinical characteristics at the onset of acute lymphoblastic leukemia in 736 patients enrolled in the AIEOP-BFM ALL 2009 protocol with at least one cerebrospinal fluid sample. Data are reported separately for the cohorts of patients belonging to the *Associazione Italiana di Ematologia e Oncologia Pediatrica* (AIEOP) and Berlin-Frankfurt-Münster (BFM) groups.

	AIEOP		BFM	
	N	%	N	%
Number of patients	245	100	491	100
Gender				
Male	149	60.8	291	59.3
Female	96	39.2	200	40.7
Age				
1-5 years	145	59.2	272	55.4
6-9 years	40	16.3	96	19.6
10-14 years	43	17.6	78	15.9
15-17 years	17	6.9	45	9.2
WBC (x 10⁹/L)				
<20	164	66.9	315	64.2
20-100	60	24.5	112	22.8
≥100	21	8.6	64	13.0
Final risk group				
T non HR	18	7.3	45	9.2
BCP SR	67	27.4	164	33.4
BCP MR	100	40.8	172	35.0
HR	60	24.5	110	22.4
CNS status				
CNS 1/2	234	95.5	426	86.8
CNS 3	3	1.2	16	3.3
Not known	8	3.3	49	10.0
Immunophenotype				
BCP	214	87.3	414	84.5
T	31	12.7	76	15.5
Not known			1	
Genetics				
TEL-AML positive	41	16.7	96	19.6
MLL-AF-4 positive	1	0.4	2	0.4
CSF sample available				
At day +33 only	161	65.7	358	72.9
At day +45 only	15	6.1	35	7.1
At both days	69	28.2	98	20.0

AIEOP: *Associazione Italiana di Ematologia e Oncologia Pediatrica*; BFM: Berlin-Frankfurt-Münster. WBC: white blood cells; HR: high risk; BCP: B-cell precursor; SR: standard risk; MR: medium risk; CNS: central nervous system; CSF: cerebrospinal fluid.

Table 2. Distribution of asparagine levels in cerebrospinal fluid, at each sampling point (days +33 and +45) in the (A) *Associazione Italiana di Ematologia e Oncologia Pediatrica* (AIEOP) and (B) Berlin-Frankfurt-Münster (BFM) cohorts.

A

	Day +33		Day +45	
	N	%	N	%
N. of samples with CSF asparagine levels (μM)	230		84	
<LLOQ	77	33.5	20	23.8
>0.2 ≤0.5	74	32.2	18	21.4
>0.5 ≤1	40	17.4	17	20.2
>1 ≤4	33	14.3	24	28.6
>4	6	2.6	56.0	
Mean* (SD)	0.61 (1.2)		1.12 (1.6)	
Median* (IQR)	0.30 (0-0.76)		0.58 (0.22-1.43)	

B

	Day +33		Day +45	
	N	%	N	%
N. of samples with CSF asparagine levels (μM)	456		133	
<LLOQ	133	29.2	26	19.6
>0.2 ≤0.5	138	30.3	30	22.6
>0.5 ≤1	99	21.7	22	16.5
>1 ≤4	76	16.7	52	39.1
>4	10	2.2	3	2.3
Mean* (SD)	0.83(2.4)		0.9 (0.9)	
Median* (IQR)	0.4 (0-0.85)		0.75 (0.3-1.3)	

(*) samples with values below the lower limit of quantification are assigned a value of 0. CSF: cerebrospinal fluid; LLOQ: lower limit of quantification; SD: standard deviation; IQR: interquartile range.

Of note, the data on the relationship between serum asparaginase activity and CSF asparagine levels obtained in the two cohorts of patients were consistent within subgroups defined by clinical and biological characteristics, such as sex, age, white blood cell count at diagnosis and immunophenotype (Table 4).

Table 5 shows asparagine levels in CSF according to whether the asparaginase activity in the serum was less than 100 IU/L or above this level at days +33 and +45 in (A) AIEOP and (B) BFM cohorts. The asparagine levels in CSF samples were significantly higher when serum asparaginase activity was below 100 IU/L than when it was higher than 100 IU/L in both cohorts (AIEOP and BFM).

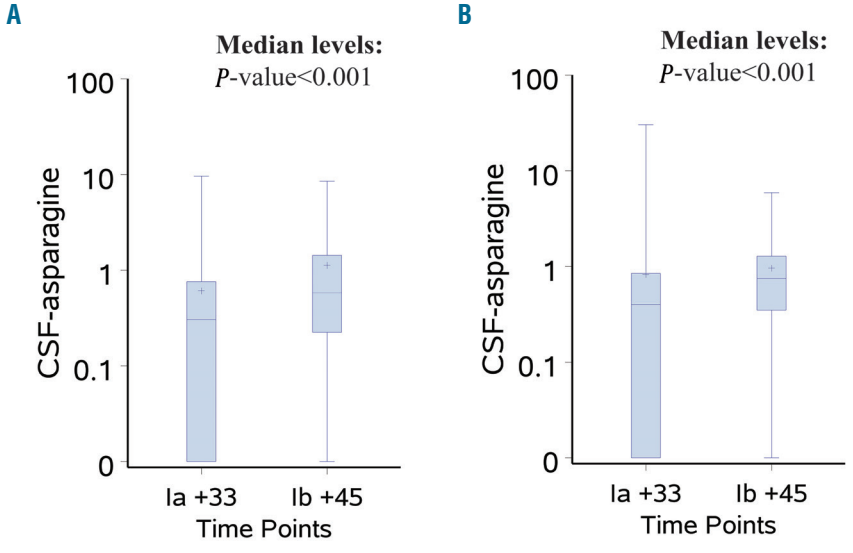

Figure 1. Asparagine levels in the cerebrospinal fluid during the induction phase. Box plots of asparagine levels (μmol/L) of cerebrospinal (CSF) punctures scheduled on days +33 and +45 in the (A) *Associazione Italiana di Ematologia e Oncologia Pediatrica* (AIEOP) and (B) Berlin-Frankfurt-Münster (BFM) cohorts.

Table 3. Asparagine levels in cerebrospinal fluid punctures scheduled on days +33 and +45 sorted according to respective serum asparaginase activity levels in the (A) *Associazione Italiana di Ematologia e Oncologia Pediatrica* (AIEOP) and (B) Berlin-Frankfurt-Münster (BFM) cohorts.

A

	PEG-asparaginase activity in serum (IU/L)											
	0 <100		≥100 <500		≥500 <1000		≥1000 <1500		≥1500		Total	
	N	%	N	%	N	%	N	%	N	%	N	%
N. of samples with CSF and serum data	18	6.6	50	18.5	19	7.0	40	14.7	144	53.1	271	
CSF ASN Concentration												
<LLOQ	0	0.0	10	20.0	8	42.1	12	30.0	48	33.3	78	28.8
>0.2 ≤0.5	2	11.1	10	20.0	7	36.8	10	22.5	52	36.1	81	29.9
>0.5 ≤1	4	22.2	10	20.0	3	15.8	9	22.5	26	18.1	52	19.2
>1 ≤4	6	33.3	17	34.0	1	5.3	9	22.5	18	12.5	51	18.8
>4	6	33.3	3	6.0	0	0.0	0	0.0	0	0.0	9	3.3
Mean* (SD)	3.36 (3.1)		1.12 (1.4)		0.35 (0.5)		0.59 (0.6)		0.41 (0.5)		0.75 (1.3)	
Median* (IQR)	2.08 (0.57-6.35)		0.63 (0.28-1.44)		0.25 (0-0.4)		0.39 (0-0.89)		0.29 (0-0.65)		0.36 (0-0.87)	

B

	PEG-asparaginase activity in serum (IU/L)											
	0 <100		≥100 <500		≥500 <1000		≥1000 <1500		≥1500		Total	
	N	%	N	%	N	%	N	%	N	%	N	%
N. of samples with CSF and serum data	28	5.8	90	18.7	116	24.1	142	29.4	106	22.0	482	
CSF asparagina concentration												
<LLOQ	3	10.7	21	23.3	31	26.7	42	29.6	39	36.8	136	28.2
>0.2 ≤0.5	3	10.7	27	30.0	38	32.8	46	32.4	35	33.0	149	30.9
>0.5 ≤1	8	28.6	15	16.7	28	24.1	33	23.2	18	17.0	102	21.2
>1 ≤4	12	42.9	27	30.0	18	15.5	21	14.8	13	12.3	91	18.9
>4	2	7.1	0	0.0	1	0.9	0	0.0	1	0.9	4	0.8
Mean* (SD)	1.50 (1.52)		0.67 (0.59)		0.70 (2.03)		0.49 (0.48)		0.43 (0.59)		0.62 (1.17)	
Median* (IQR)	1.02 (0.56-2.12)		0.45 (0.22-1.10)		0.40 (0-0.83)		0.40 (0-0.80)		0.30 (0.01-0.65)		0.40 (0.01-0.88)	

(*) samples with values below the lower limit of quantification are assigned a value of 0. PEG: pegylated; CSF: cerebrospinal fluid; LLOQ: lower limit of quantification; SD: standard deviation; IQR: interquartile range.

Discussion

Survival rates obtained with chemotherapy schedules applied over the last three decades in childhood ALL have increased progressively and currently approach 90%.[3]

Asparaginase has been shown to play a key role in obtaining such excellent results and for this reason the drug has been invariably included, since its introduction in clinical practice, in the polychemotherapy schedules designed for childhood ALL. The enzyme exerts its antileukemic activity by depleting the systemic pools of asparagine, an amino acid essential for the rapid proliferation of malignant lymphoblasts.[21–23]

It is well known that the activity of any asparaginase product may be pharmacologically monitored by measuring serum asparaginase activity levels, which reflect the enzyme's ability to deplete circulating asparagine pools and are considered a surrogate marker of its clinical efficacy.[1] There is currently a wide agreement that activity levels of at least 0.1 IU/mL (i.e. 100 IU/L) should be achieved and maintained during the whole planned asparaginase treatment to ensure maximal asparagine depletion in serum (<0.2 μmol/L) and maximal therapeutic efficacy.[9,21] Whether a similarly profound and prolonged asparagine depletion in the CSF is needed to prevent CNS relapses is not known; however, there are reports associating the two phenomena.[4]

The physiological concentration of asparagine in human CSF varies depending on the age of the patient. In children and adolescents its concentration ranges between 4 and 10 μmol/L.[24] Although these values are lower than those found in serum (about 40-80 μmol/L), it has been reported that these levels ensure the growth of leukemic blasts.[25] For this reason, in principle, it is reasonable to study asparagine depletion in CSF to evaluate its clinical relevance better and prospectively.

The main purpose of the study reported here was to evaluate the level of asparagine depletion in the CSF and

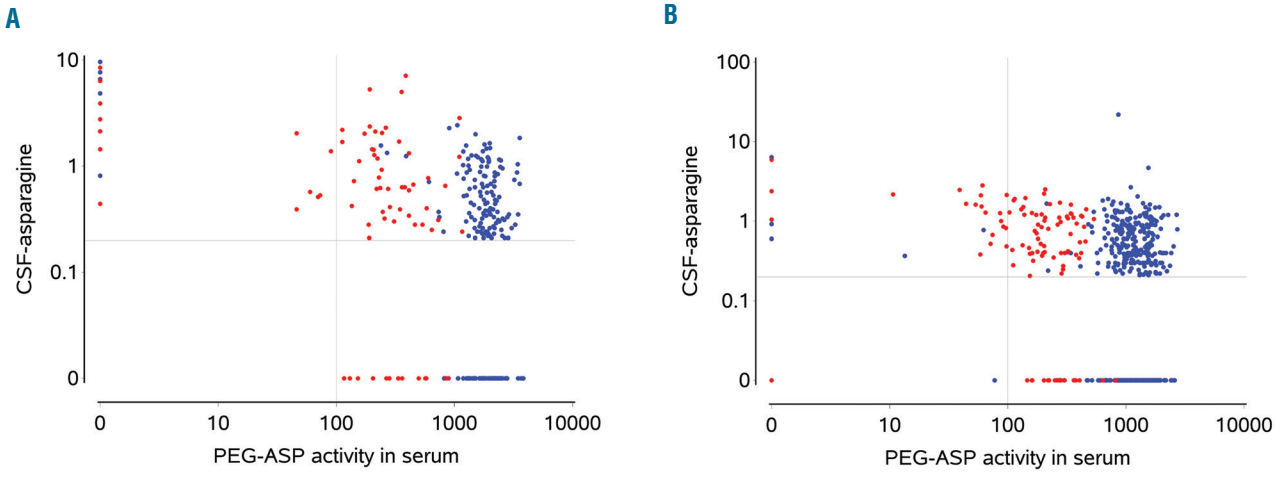

Figure 2. Effect of pegylated-asparaginase activity on asparagine level in the cerebrospinal fluid. Distribution of asparagine levels (μmol/L) detected in cerebrospinal (CSF) samples collected during puncture on days +33 (blue dots) and +45 (red dots) *versus* pegylated asparaginase (PEG-ASP) activity levels (IU/L) detected in serum at the same sampling day (±1) in the (A) *Associazione Italiana di Ematologia e Oncologia Pediatrica* (AIEOP) and (B) Berlin-Frankfurt-Münster (BFM) cohorts.

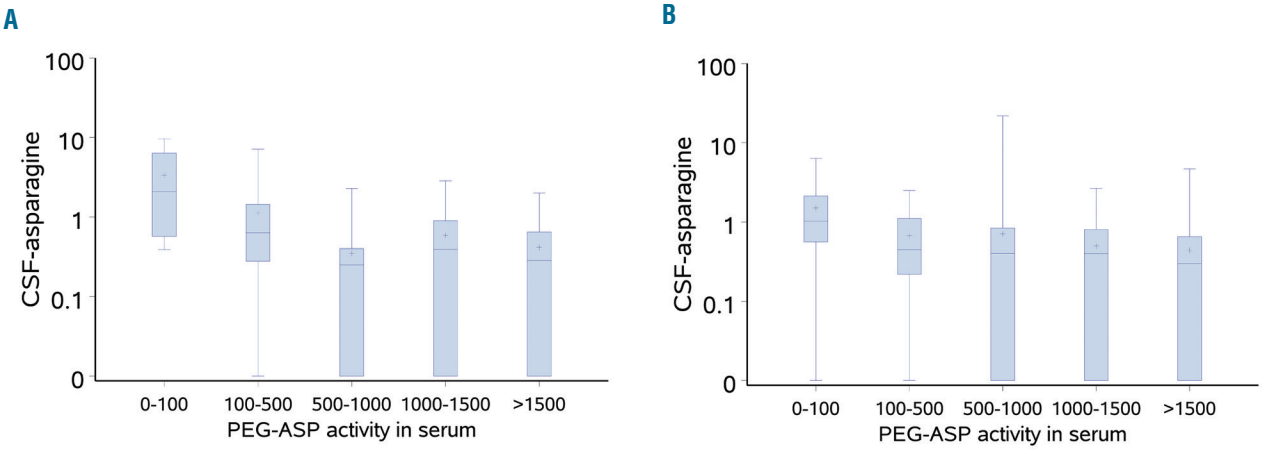

Figure 3. Cerebrospinal fluid asparagine concentration at different levels of pegylated-asparaginase activity. Box plots of asparagine cerebrospinal fluid (CSF) concentrations (μmol/L) *versus* categorized pegylated-asparaginase (PEG-ASP) activity levels (IU/L) in serum collected at the same sampling points (±1 day) in the (A) *Associazione Italiana di Ematologia e Oncologia Pediatrica* (AIEOP) and (B) Berlin-Frankfurt-Münster (BFM) cohorts.

therefore the pharmacodynamic effect exerted by PEG-asparaginase on the CNS of children with ALL. The interest in this aspect derives from various studies showing that different degrees of asparagine depletion in the CSF may depend not only on the asparaginase product used[4–9] but, apparently, also on the levels of asparaginase activity achieved in serum.[26–28]

In this study the administration of PEG-asparaginase in the induction phase of the ALL treatment adopted was associated with a significant, even if widely variable, reduction of CSF asparagine below the physiological levels. However, complete asparagine depletion was observed overall in only about 28% of the analyzed CSF samples. Considerable CSF asparagine levels, greater than 1 μmol/L, and thus higher than the LLOQ of the assay used, which is considered the threshold of complete asparagine depletion, were detected overall in 23% of the analyzed CSF samples, with this latter figure becoming 17-19% and 35-41% for samples taken 7 and 19 days, respectively, after PEG-asparaginase administration (Table 2A,B).

The findings of the large therapeutic drug monitoring program regarding asparaginase activity levels in serum conducted in the frame of the AIEOP-BFM ALL 2009 study and here reported regard exclusively the subset of patients studied to evaluate asparagine depletion in the CSF. These data show that after a dose of 2,500 IU/m^2, PEG-asparaginase activity levels much higher than 100 IU/L are achieved in induction in the vast majority of patients and are maintained both 7 and 14 days following a standard administration schedule including PEG-asparaginase, thus fully confirming the prolonged half-life of this product (Table 5A,B).

In the serum samples collected along with CSF samples, PEG-asparaginase activity less than 100 IU/L was only detected in around 5-6% of samples, when the samples were taken ≤19 days after administration of PEG-asparaginase (Tables 3A,B and 5A,B). We can, therefore, assume

Table 4. Asparagine levels in cerebrospinal fluid sorted by asparaginase activity levels in serum, at scheduled cerebrospinal fluid punctures (days +33 and +45) in the (A) *Associazione Italiana di Ematologia e Oncologia Pediatrica* (AIEOP) and (B) Berlin-Frankfurt-Münster (BFM) cohorts.

A

	Asparagine levels in CSF (μmol/L)					
	Serum PEG-ASP activity 0 <100 IU/L		Serum PEG-ASP activity ≥100 IU/L		Total	
	Mean (SD)	Median (IQR)	Mean (SD)	Median (IQR)	Mean (SD)	Median (IQR)
Gender						
Male	4.0 (3.5)	3.0 (0.8-7.7)	0.7 (0.8)	0.4 (0.2-0.9)	0.9 (1.4)	0.4 (0.2-1.0)
Female	2.6 (2.6)	1.7 (0.5-4.6)	0.4 (0.8)	0.2 (0-0.6)	0.6 (1.2)	0.3 (0-0.7)
Age at diagnosis						
1-9 years	3.3 (3.0)	2.1 (0.6-6.4)	0.6 (0.8)	0.4 (0-0.8)	0.8 (1.3)	0.4 (0-1.0)
10-17 years	3.4 (4.4)	1.4 (0.4-8.5)	0.5 (0.8)	0.3 (0-0.7)	0.7 (1.3)	0.3 (0-0.8)
WBC count at diagnosis						
<20 x 10^6/L	3.5 (3.0)	2.8 (0.5-6.4)	0.5 (0.8)	0.3 (0-0.7)	0.7 (1.2)	0.4 (0-0.8)
≥20 x 10^6/L	3.1 (3.5)	1.4 (0.6-7.7)	0.6 (0.9)	0.4 (0-1.0)	0.8 (1.5)	0.4 (0-1.0)
Immunophenotype						
B lineage	2.7 (2.5)	2.0 (0.5-4.8)	0.6 (0.8)	0.3 (0-0.8)	0.7 (1.1)	0.4 (0-0.8)
T ALL	6.5 (4.5)	8.5 (1.3-9.6)	0.6 (0.7)	0.3 (0-0.9)	1.2 (2.2)	0.4 (0-1.4)

B

	Asparagine levels in CSF (μmol/L)					
	Serum PEG-ASP activity 0 <100 IU/L		Serum PEG-ASP activity ≥100		Total	
	Mean (SD)	Median (IQR)	Mean (SD)	Median (IQR)	Mean (SD)	Median (IQR)
Gender						
Male	1.7 (1.8)	1.3 (0.5-2.2)	0.5 (0.6)	0.4 (0.0-0.8)	0.6 (0.8)	0.4 (0.0-0.9)
Female	1.1 (0.5)	0.9 (0.7-1.2)	0.6 (1.6)	0.4 (0.0-0.8)	0.6 (1.5)	0.4 (0.0-0.9)
Age at diagnosis						
1-9 years	1.7 (1.7)	1.3 (0.6-2.2)	0.6 (1.3)	0.4 (0.0-0.9)	0.7 (1.3)	0.4 (0.0-0.9)
10-17 years	0.8 (0.6)	0.9 (0.4-1.1)	0.5 (0.5)	0.4 (0.0-0.7)	0.5 (0.5)	0.4 (0.0-0.8)
WBC count at diagnosis						
<20 x 10^6/L	1.8 (1.8)	1.3 (0.7-2.1)	0.6 (1.4)	0.4 (0.0-0.8)	0.7 (1.4)	0.4 (0.0-0.9)
≥20 x 10^6/L	1.0 (0.8)	1.0 (0.4-1.4)	0.5 (0.5)	0.4 (0.0-0.8)	0.6 (0.6)	0.4 (0.0-0.9)
Immunophenotype						
B lineage	1.4 (1.3)	1.0 (0.5-2.1)	0.6 (1.2)	0.4 (0.0-0.8)	0.6 (1.2)	0.4 (0.0-0.9)
T ALL	1.9 (2.2)	1.3 (0.9-1.6)	0.5 (0.5)	0.4 (0.0-0.8)	0.6 (0.8)	0.4 (0.0-0.9)

CSF: cerebrospinal fluid; PEG-ASP: pegylated asparaginase; SD: standard deviation; IQR: interquartile range; WBC: white blood cell; ALL: acute lymphoblastic leukemia.

that serum asparagine levels of patients enrolled in this pharmacological study were continuously depleted for at least 14 days after each PEG-asparaginase dose in the majority of the patients.

In our study when CSF asparagine levels were evaluated in relationship to serum asparaginase activity levels, a considerable number of samples was found not to have levels below the LLOQ. In patients with serum asparaginase activity levels below 100 IU/L (which is considered insufficient to consistently obtain complete asparagine depletion in serum) only 6.5% of the corresponding CSF samples had levels below the LLOQ. When the level of asparaginase activity was 100 IU/L or higher, 70% of the samples had asparagine levels higher than 0.2 μmol/L (i.e. higher than the LLOQ). Furthermore, at serum asparaginase activity levels of 100 IU/L or higher – including samples with activity levels greater than 1500 IU/L - only about one third of the corresponding CSF samples had asparagine levels below the LLOQ.

Some studies have been conducted in the past on aspects related to CSF asparagine depletion along with administration of different asparaginase products. Dibenedetto *et al.* evaluated CSF asparagine levels 3 days after the administration of the fourth dose of ERW-asparaginase (given at a dosage of 10,000 IU/m^2 intramuscularly every 72 h) and found them to be below the LLOQ (≤0.2 μmol/L) in 75% of treated children. Despite the small number of cases analyzed in that experience and based on the fact that asparaginase is believed not to be able to cross the barrier between blood and CSF, it was concluded that this phenomenon was the reflection of the asparagine depletion observed in serum.[7] On the other hand, Ahlke *et al.* showed that 2,500 IU/m^2 un-pegylated *E. coli* asparaginase led to complete depletion of CSF asparagine 2 or 3 days after application. Median trough plasma activity levels in this dose-group were 106 IU/L (26-349 IU/L).[29] Among the findings of our study, we observed that even at asparaginase activity levels greater than 1500 IU/L CSF asparagine levels below the LLOQ were found in roughly 30 to 40% of the samples (Table 3).

In the following years, two additional studies investigated this phenomenon in patients treated with asparaginase. The first, conducted in 1996 by Gentili *et al.*,[5] evaluated 44 patients with newly diagnosed ALL treated in the induction phase of a BFM-oriented protocol with 10,000 IU/m^2 of ERW-asparaginase every 3 days. The analysis of CSF and serum asparagine levels, measured on average 3 days after each dose, revealed CSF asparagine levels similar to those reported in the previously reported study by Dibenedetto *et al.*[7] The second study, performed by Rizzari *et al.*,[6] compared the ability of ERW-asparaginase and native *E. coli* asparaginase to deplete asparagine in the CSF. In all the 62 patients treated in the induction phase with either intravenous or intramuscular ERW-asparaginase or native *E. coli* asparaginase (10,000 IU/m^2 every 3 days), asparagine levels in both the serum and CSF remained below the LLOQ (≤0.2 μmol/L) even if asparaginase activity levels were higher in the group treated with *E. coli* asparaginase than in that treated with ERW-asparaginase. Similar results were found in a study by Woo *et al.*[8]

A different trend has been found in studies performed so far in patients treated with PEG-asparaginase. Vieira Pinheiro *et al.*[30] studied patients treated with PEG-asparaginase within the German Cooperative Acute Lymphoblastic Leukemia (COALL) study and Rizzari *et al.*[15] patients treated with the same product within the AIEOP ALL 2000 study. Overall, both studies showed that CSF asparagine levels in patients treated with PEG-asparaginase were undetectable (i.e., below the detection limit) only in a fraction of patients, no matter if serum asparaginase activity levels were much higher than 100 IU/L. Additional studies reported by the Nordic Society of Pediatric Hematology and Oncology and even more recently by the Dutch Childhood Leukemia Study Group (DCLSG) confirmed these observations.[14,27,31,32]

Based on the most updated scientific evidence it is not possible to provide a clear and incontrovertible explanation on how asparaginase products may achieve the observed asparagine depletion in the CSF. It has been hypothesized that the asparagine depletion observed in the CSF could result from a continuous balance between the serum and CSF asparagine pools.[10,11] Another possible explanation can be inferred from the specific physico-chemical properties of the native asparaginase products compared to the PEG-asparaginase product. It is conceivable that native asparaginase formulations, given their lower molecular weight and steric size, might have some capacity to penetrate, even in very low amounts, into the CSF thus providing local asparaginase activity.

Table 5. Asparagine levels in cerebrospinal fluid by levels of asparaginase activity in serum, at the cerebrospinal fluid sampling points (days +33 and +45) in the (A) *Associazione Italiana di Ematologia e Oncologia Pediatrica* (AIEOP) and (B) Berlin-Frankfurt-Münster (BFM) cohorts.

A

	PEG-asparaginase activity in serum (IU/L)					
	0 <100		≥100		Total	
	N	%	N	%	N	%
N. of samples with CSF and serum data	18	6.6	253	93.4	271	
CSF asparagine levels at day +33						
N.	5		193		198	
Mean* (SD) (μmol/L)	5.9 (3.3)		0.4 (0.5)		0.6 (1.1)	
Median* (μmol/L)	6.59		0.31		0.32	
IQR (μmol/L)	4.85-7.65		0-0.71		0-0.76	
CSF asparagine levels at day +45						
N.	13		60		73	
Mean* (SD) (μmol/L)	2.4 (2.5)		1.0 (1.3)		1.2 (1.7)	
Median* (μmol/L)	1.44		0.60		0.61	
IQR (μmol/L)	0.53-2.76		0.22-1.30		0.3-1.4	

B

	PEG-asparaginase activity in serum (IU/L)					
	0 <100		≥10		Total	
	N	%	N	%	N	%
N. of samples with CSF and serum data	28	5.8	454	94.2	482	
CSF asparagine levels at day +33						
N.	6		370		376	
Mean* (SD) (μmol/L)	1.50 (2.39)		0.54 (1.22)		0.56 (1.25)	
Median* (μmol/L)	0.68		0.38		0.38	
IQR (μmol/L)	0.37-0.91		0.01-0.79		001-0.79	
CSF asparagine levels at day +45						
N.	22		84		106	
Mean* (SD) (μmol/L)	1.50 (1.27)		0.69 (0.60)		0.86 (0.84)	
Median* (μmol/L)	1.27		0.49		0.68	
IQR (μmol/L)	0.67-2.13		0.23-1.12		0.32-1.23	

PEG: pegylated; CSF: cerebrospinal fluid; SD: standard deviation; IQR: interquartile range.

Nevertheless, it has been postulated that this activity never exceeds 0.2% of that present in serum.[11] Conversely, this may not be possible for PEG-asparaginase, mainly because of its tertiary structure.[16,32,33] However, so far there is no clear proof that any asparaginase product determines any degree of CSF asparagine depletion in humans by directly penetrating the CSF. To contribute to this issue a preclinical study was recently conducted to evaluate whether the three commercially available asparaginase formulations have different abilities to enter the CSF and reduce local asparagine levels. Even with the limitations of the model used in that preclinical experience, the enzymatic activity measured in CSF demonstrated that asparaginase products, in particular both the native forms derived from *Erwinia chrysanthemi* and *E. coli*, may transiently penetrate the CNS when administered at high doses, whereas the PEG-asparaginase product does not, most probably because of the differences in molecular weight.[16,34,35]

To conclude, the findings of the therapeutic drug monitoring performed in our study and reported here indicate that: (i) the administration of PEG-asparaginase was able to cause a broad reduction of physiological CSF asparagine levels (normally 4-10 μmol/L) but complete asparagine depletion was observed overall in only about 28% of the analyzed CSF samples; (ii) CSF asparagine levels greater than 1 μmol/L (thus higher than the LLOQ of the assay adopted) were detectable in 23% of the analyzed samples; (iii) at serum asparaginase activity levels less than 100 IU/L only 6.5% of the CSF samples had asparagine levels below the LLOQ; and (iv) at serum asparaginase activity levels of 100 IU/L and higher, up to 1,500 IU/L and beyond, CSF asparagine levels were lower than the LLOQ in only about 33-37% of the samples. Thus, a further increase of the PEG-asparaginase dose would not help to obtain complete CSF asparagine depletion.

The consistent results found in the two independent data sets presented here strengthen the observations inferred from this study.

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