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## The anti-asparagines antibodies correlate with L-asparagines activity and may affect clinical outcome of childhood acute lymphoblastic leukemia

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### Abstract

The primary aim of the study was to evaluate the importance of anti-asparaginase antibodies for L-asparaginase activity in children with standard and medium risk acute lymphoblastic leukemia (ALL). Forty-seven children with newly diagnosed ALL were included into the prospective study. Enzyme activity and the presence of anti-asparaginase antibodies (IgG and IgM class) were determined. Anti-asparaginase antibodies were identified in 13/47 (IgM class) and 10/47 (IgG class) patients in the induction and in 19/47 (IgM class) and 20/47 (IgG class) patients in the reinduction phase of treatment. The enzyme activity was lower in patients that were positive for anti-asparaginase antibodies, especially in reinduction phase (median 37 (20–180) vs 355 (141–499),  $p = 0.001$ ). An association between anti-asparaginase antibodies and the allergic reaction to the drug was found. Besides, the children who developed anti-asparaginase antibodies in the induction phase of treatment showed lower event-free survival as well as overall survival in comparison with children without antibodies. Since our study was carried out in a small number of patients, this observation is only speculative and needs to be confirmed by a further study on a larger sample size, with multivariable analysis. However, our data suggest that L-asparaginase activity together with anti-asparaginase antibodies measurements may become useful for effective therapy of ALL.

**Keywords:** *L-asparaginase, antibodies, allergy, acute lymphoblastic leukemia, children*

### Introduction

The L-asparaginase enzyme, an important agent in the treatment of acute lymphoblastic leukemia (ALL) and non-Hodgkin lymphoma (NHL), is not expressed in humans [1–4]. Thus, the use of L-asparaginase in the treatment of cancer may lead to the formation of specific antibodies to asparaginase. They are responsible for hypersensitivity reactions, which sometimes make the completion of the therapy impossible [2,5]. The even more important effect due to the presence of anti-asparaginase antibodies is the phenomenon of the so-called *silent inactivation*. It is an unexpected fast decrease in enzyme activity without any clinical symptoms of hypersensitivity [2,6].

In the previous publication we reported that the development of IgG anti-asparaginase antibodies is associated with the decreasing L-asparaginase activity during the treatment [7]. However, it was only the first, retrospective observation, concerning a small number of patients. The present study is planned as prospective to be performed in a larger group of patients and deals with the development and clinical importance of two classes of anti-asparaginase antibodies: IgM and IgG. Since the development of anti-asparaginase antibodies decreases the enzyme activity, it is likely that this phenomenon may also affect the clinical outcome in childhood ALL. Recently, the association between the formation of anti-asparaginase antibodies and adverse outcome in high-risk ALL was established [8]. Thus, our

additional goal was to evaluate the importance of anti-asparaginase antibodies in the outcome of childhood ALL in standard and medium risk groups of patients.

### Patients and study design

The investigation was in accordance with the principles of the Declaration of Helsinki, and the appropriate ethical committee approved the work and proposed study design.

At the beginning of the study, the power evaluation and the sample size estimation were done for prevalence of allergy and the decrease in enzyme activity. The estimated sample size in the prospective study, assuming 80% statistical power and  $\alpha = 0.05$ , as well as 25% prevalence of allergy and 30% antibody development in patients treated with L-asparaginase (average values were taken from literature [5,6,9,10] was  $n = 15$  for patients with allergy). The estimated sample size, assuming 80% statistical power and  $\alpha = 0.05$  as well as average decrease in enzyme activity among antibody positive individuals equaling 60 IU/l with standard deviation 156.7 IU/l (taken from the previous pilot study) was  $n = 77$ .

Since the average number of newly diagnosed children with standard and medium risk ALL at the Oncohematology Unit in Lodz is 15 and the estimated sample size for the survival analysis with antibodies development as a classified variable is relatively high ( $n = 300$ ), we did not consider this as a primary goal of the study.

Between January 2000 and December 2004, sixty-four children with newly diagnosed ALL were treated in the Oncohematology Unit. Fifty of them were grouped into the low and medium risk group according to the criteria of the BFM-ALL protocols [11]. In three children, the blood samples were not available to estimate the presence of anti-asparaginase antibody in the planned time-points.

Finally, forty-seven children (25 boys and 22 girls) aged 3–17 years (mean age 7.9) with newly diagnosed ALL were included in the study. The main initial white blood cells count in the study group was  $4.8 \times 10^9$  ( $0.8 - 20.5 \times 10^9$ ). At present in our study group, 38 children are free of disease with median period observation of thirty months. During the treatment seven children died, in four of them the disease relapse was the cause of death. The other children died because of severe infectious diseases. In two other children the disease relapsed; in one during the maintenance and in another one after the completion of therapy, both of them are alive after the second-line treatment and allogeneic bone marrow transplantation.

All children were treated according to the Polish Leukemia/Lymphoma Group Protocol based on BFM-ALL 95, and after the year 2002, according to the ALLIC Protocol. Children were assigned to the risk groups in compliance with the following criteria: initial white blood cell count, age at onset, and the presence of genetic rearrangements specific for poor-prognosis ALL. Only the children with standard or medium risk ALL were enrolled in the study. Children with high-risk ALL were excluded from the study.

There were two series of L-asparaginase in this protocol. Patients received eight doses of the drug (5000 IU/m<sup>2</sup>) every three days during induction (on days 12, 15, 18, 21, 24, 27, 30, 33) and four doses (10,000 IU/m<sup>2</sup>) on days 8, 11, 15, and 18 of the reinduction phase. The drug was diluted in 200–300 ml of 5% dextrose and then administered intravenously in 1-h infusion.

The treatment always began with the *E. coli* preparation of L-asparaginase (Asparaginase, Medac GmbH). During the reinduction phase of treatment, clinical signs of allergy to L-asparaginase occurred in 17 children. In one child the hypersensitivity reaction occurred after the last dose of the drug, so he did not receive any other preparation. In one child the enzyme was changed to Erwinia (Erwinia, Speywood), and in 15 children to PEG-asparaginase (Oncaspar, Medac GmbH). We did not use the same preparation of the enzyme after hypersensitivity event, even with antihistaminic treatment.

### Methods

#### *Asparaginase activity*

Blood samples were collected immediately before the next dose of L-asparaginase. They were centrifuged at 5000 rpm and the serum was stored in 4°C until use. Since we were not able to collect all samples from each time-point of L-asparaginase infusion, we established an average value of enzyme activity from all available samples for each individual separately for induction and reinduction phase. The mean number of measurements per patient in the first protocol was  $n = 5$  (in total 251 measurements) and in the second protocol was  $n = 3$  (in total 163).

The measurements of the enzyme activity were carried out no later than at the third day after collection. Serum samples were not frozen since L-asparaginase activity is stable at room temperature for approximately seven days [12].

The determination of enzyme activity was performed using the spectrophotometric method with the measurement of released ammonia after incubation of the blood sample with L-asparagine and color

reaction with Nessler's reagent, according to the method described by Boos et al. [10] and was precisely described in our previous report [7]. The reproducibility at 100 U/l value in our laboratory was  $100 \pm 18$  U/l. Lower level of quantification was 20 U/l. L-asparaginase activity was defined as low at the level below 100 IU/l identified in one patient at two measurements separately for both protocols.

#### *Anti-asparaginase antibodies*

The samples obtained on the last day of each L-asparaginase course (i.e., on day 33 in the induction phase and day 18 in the reinduction phase) were frozen in  $-20^{\circ}\text{C}$  and then used for determination of anti-asparaginase antibodies. In case of hypersensitivity reaction, the sample collected before the infusion of L-asparaginase was used for the antibodies assay.

The determination of anti-asparaginase IgG and IgM antibodies was performed using ELISA assay in our own modification as described previously [7]. Briefly, ELISA plates were coated with antigen (Asparaginase, Medac GmbH) and incubated. Patient plasma was serially added to plates and incubated; thereafter it was developed with horseradish peroxidase-conjugated rabbit anti-human IgG and IgM monoclonal antibodies (DAKO, Denmark). Then, the substrate solution was added to each well, individual wells were read using ELISA reader at a wavelength of 450 nm and the results were given as optical density (OD). The quantitative method was based on the examination of the presence of anti-asparaginase antibodies in the serum collected from thirty-one healthy, untreated volunteers. A count higher than two standard deviations of OD was considered as positive. The similar assay was performed for IgM anti-asparaginase antibodies.

#### **Statistical analysis**

Since all quantitative variables did not have a normal distribution, the differences in continuous variables were determined with the non-parametric Mann-Whitney's test and the data were always presented as median (lower and upper quartile). The categorized variables were analyzed using the  $\chi^2$  test with Yates' modification or the Fisher's exact test. The survival data was determined as a cumulative proportion of

event-free survival (EFS) and of overall survival (OS) of the patients. The event was classified as the occurrence of relapse or death in remission. The progression of the disease was not considered as an endpoint because all treated individuals achieved complete remission. The EFS-time was calculated from the date of diagnosis to the first event or to the date of the last follow-up. Probabilities of EFS (pEFS) and OS (pOS) were estimated using the Kaplan–Meier method. The comparison of the survival time between groups was performed by applying the Cox *F*-test. For all analyses,  $p < 0.05$  were considered as significant. No correction for multiple comparisons was done.

#### **Results**

##### *Induction phase*

We did not observe any hypersensitivity reactions during the induction phase of treatment. All children completed the L-asparaginase course with the same preparation of the drug. On the 33rd day of induction, anti-asparaginase antibodies were identified in 13/47 (IgM class) and 10/47 (IgG class) patients. The low enzyme activity ( $< 100$  IU/l) was observed in 7/47 children. Only three patients had concomitantly anti-asparaginase antibodies and low enzyme activity at the last day of induction, so we did not identify any correlation between the presence of anti-asparaginase antibodies and the enzyme activity in the induction phase. However, even in this phase of treatment the trend towards the lower enzyme activity in children with positive antibodies was noted (see Table I,  $p = 0.1$ ).

Despite the small number of children studied, we made quite an interesting observation. In children who developed IgG anti-asparaginase antibodies, a lower event-free survival (EFS) as well as overall survival (OS) were noted when compared with children without antibodies (EFS 0.57 vs 0.79,  $p < 0.02$  and OS 0.57 vs 0.89,  $p < 0.01$ , respectively). Similar observation was found for IgM antibodies (Figure 1).

##### *Reinduction phase*

In the reinduction phase of the protocol, clinically manifested hypersensitivity to L-asparaginase

Table I. Median L-asparaginase activity in the studied children with and without anti-asparaginase antibodies in both phases of treatment.

| L-ASP activity                                     | IgM (–)       | IgM (+)      | <i>p</i> | IgG (–)       | IgG (+)      | <i>p</i> |
|----------------------------------------------------|---------------|--------------|----------|---------------|--------------|----------|
| Median (lower and upper quartiles) in induction    | 220 (153–341) | 145 (56–250) | 0.1      | 207 (136–354) | 150 (47–216) | 0.1      |
| Median (lower and upper quartiles) in re-induction | 317 (117–440) | 37 (20–219)  | 0.02     | 355 (141–499) | 37 (20–180)  | 0.001    |

occurred in 17 of 47 children. The symptoms of hypersensitivity to L-asparaginase such as skin rash, abdominal pain, headache, dyspnea or bronchospasm were classified as mild to moderate according to the WHO criteria of drug toxicity. An anaphylactic shock with decreased blood pressure was observed in two patients. An association between anti-asparaginase antibodies and a clinically evident allergic reaction to the drug was also found. Asparaginase activity was significantly lower in children who presented a hypersensitivity reaction when compared with children with no allergic reaction (the median L-asparaginase

activity 36 IU/l (0–36) vs 330 IU/l (160–548),  $p=0.0005$ ) (Figure 2). The low L-asparaginase activity was found in 73% of patients with positive anti-asparaginase antibodies, and only in 22% of negative patients ( $p=0.004$ ).

The number of children with positive anti-asparaginase antibodies was higher than in the induction phase. Anti-asparaginase antibodies in IgM and IgG classes were found in 19/47 patients and in 20/47, respectively. In reinduction, anti-asparaginase antibodies were associated with the enzyme activity (Figure 3). The lower L-asparaginase

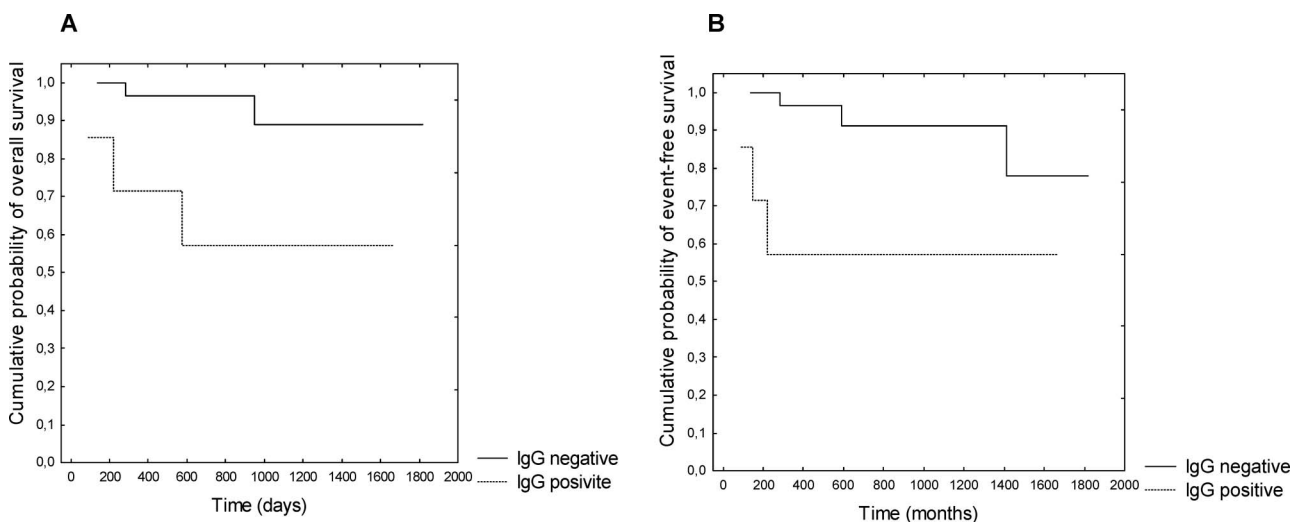


Figure 1. The influence of the anti-asparaginase IgG antibodies in induction on the overall survival (A) and event free survival (B) in the study group.

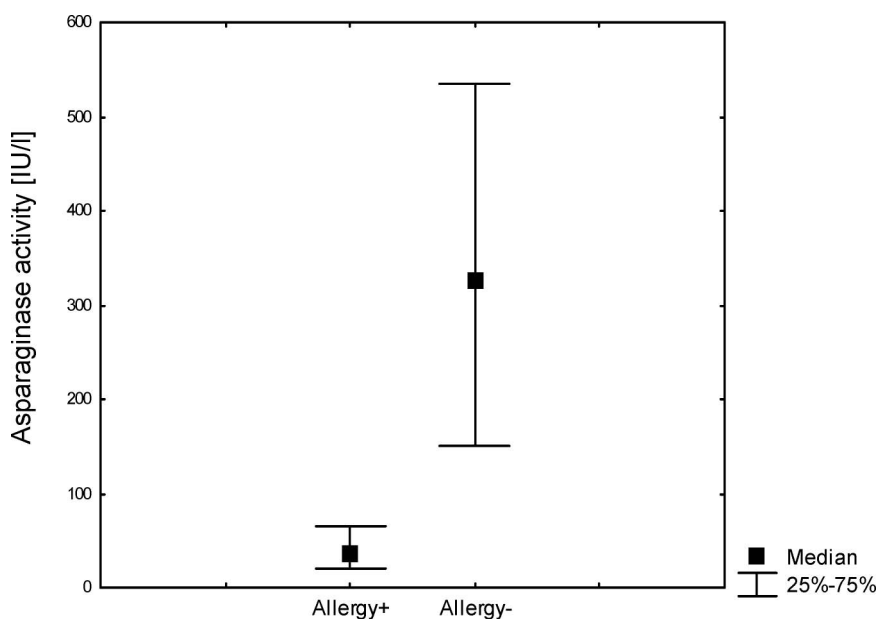


Figure 2. The association between the clinically manifested hypersensitivity reaction to L-asparaginase in reinduction and serum enzyme activity in the reinduction.

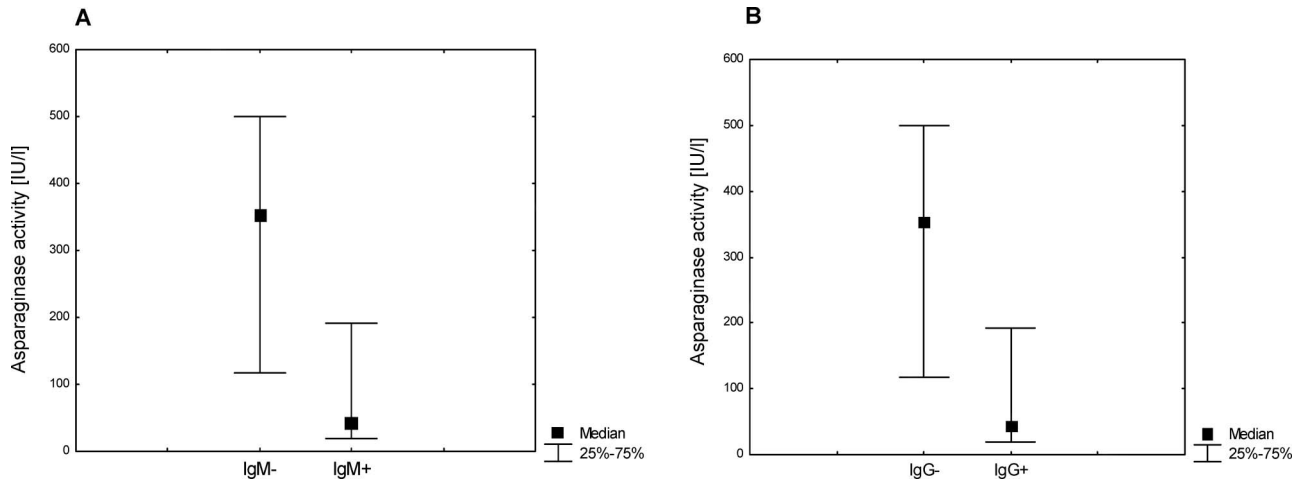


Figure 3. The association between the presence of anti-asparaginase antibodies (IgM class – A and IgG class – B) and serum enzyme activity in the reinduction.

activity was associated with the presence of IgM antibodies (317 (117–440) vs 37 (20–219) IU/l,  $p = 0.02$ ) and IgG antibodies (355 (141–499) vs 37 (20–180) IU/l,  $p = 0.001$ ).

In the reinduction phase, no association between the presence of anti-asparaginase antibodies with the cumulative proportion of the event-free survival and of the overall survival was found.

## Discussion

The L-asparaginase enzyme is present in many plants and animals, but not in humans. The molecule of L-asparaginase is characterized by high molecular weight and a complicated protein structure. Thus, L-asparaginase is highly immunogenic in treated patients, which results in a specific humoral response. The production of anti-asparaginase antibodies is common in children with ALL [9,10] and has been found to be higher in children with clinical signs of hypersensitivity [10,14]. It was established that the measurement of anti-asparaginase antibodies might play a role in predicting future hypersensitivity to the drug [10].

In the present study, we observed that the presence of anti-asparaginase antibodies was more common during the second course with L-asparaginase; however, they were present even in the induction phase. After 8 doses of the drug in the induction phase of treatment, we did not find a clear significant relationship between the development of anti-asparaginase antibodies and clinically obvious allergic reactions during the later treatment. During reinduction of treatment we found the association between the presence of anti-asparaginase antibodies and clinical signs of the allergy. Thus, the occurrence of the allergy is associated with antibody formation, but

the predictive role of anti-asparaginase antibodies measurements in induction phase for future hypersensitivity is still inconclusive.

The literature data have also suggested that the hypersensitivity is associated with the decrease of L-ASP activity. In one report serum asparaginase activity was undetectable in four of five patients within one week after anaphylactic reaction [10]. Another study of 154 children observed that neither the hypersensitivity reactions nor the presence of anti-asparaginase antibody influenced the clinical outcome of ALL children [15].

Our observations indicate that the occurrence of a hypersensitivity reaction is not important for the outcome in children with ALL. The clinically manifested hypersensitivity reactions against L-asparaginase were observed in 1/6 (16%) of the patients who relapsed later, and in 16/43 (39%) patients who did not relapse. Probably, the use of alternative L-asparaginase preparation after the occurrence of hypersensitivity prevented the immunological inactivation of the enzyme. Larson et al. have established that the immunological inactivation or destruction of *E. coli* asparaginase in patients who become hypersensitive is less important if they are secondarily treated with Erwinia [16].

The presence of anti-asparaginase antibodies has been suggested to affect serum enzyme activity [6,14]. Our study consistently revealed that in the induction phase children positive for anti-asparaginase antibodies had the lower L-asparaginase activity than the children without antibodies; however, differences were not significant. Furthermore, in the reinduction phase, during the second exposure to the enzyme, the clear association between the presence of anti-asparaginase antibodies and serum drug activity was found similarly for both classes of immunoglobulins.

The clinical importance of the anti-asparaginase antibody is still not unequivocally stated. It was established that the formation of anti-asparaginase antibodies might alter the pharmacokinetics of Erwinia-asparaginase, but only during the second period of treatment with this drug [17]. The same authors indicated that the development of anti-Erwinia antibodies was unrelated to the risk of relapse [18]. In other study the association between anti-asparaginase antibodies and the increased risk of relapse was found [5]. Since we found slightly different prevalence of IgM and IgG antibodies among ALL patients treated with L-asparaginase further longitudinal studies are needed to define which class of immunoglobulins has stronger predictive value for lower L-asparaginase activity in both induction and reinduction phase.

Recently, Panosyan et al. observed in a large cohort study that the formation of anti-asparaginase antibodies can be associated with the undetectable L-asparaginase activity and may correlate with adverse outcome in high-risk ALL [8]. In our study we analyzed only the children with standard or medium risk ALL. The children who developed anti-asparaginase antibodies in the induction phase of treatment showed lower event-free survival, as well as overall survival in comparison with children without antibodies. This observation might indicate the clinical importance of the development of anti-asparaginase antibodies, especially in the induction phase. Since our study was carried out on a small number of patients, this observation is only speculative and needs to be confirmed by a further study on a larger sample size, with multivariable analysis. However, low L-asparaginase activity in patients positive for anti-asparaginase found in our study is consistent with the effect of presence of these antibodies on survival probability. Thus, our data suggest that L-asparaginase activity together with anti-asparaginase antibodies measurements may become useful for effective therapy of ALL.

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