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Update on the Use of L-Asparaginase in Infants and Adolescent Patients with Acute Lymphoblastic Leukemia

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ABSTRACT: Great improvements have been made in acute lymphoblastic leukemia (ALL) treatment in the past decades, especially due to the use of L-asparaginase (L-ASP). Despite the significant success rate, several side effects mainly caused by toxicity, asparaginase silent inactivation, and cellular resistance, encourage an open debate regarding the optimal dosage and formulation of L-ASP. Alternative sources of asparaginases have been constantly investigated in order to overcome hypersensitivity clinical toxicity. Additionally, genomic modulation as gene expression profiling, genetic polymorphisms, and epigenetic changes is also being investigated concerning their role in cellular resistance to L-ASP. Understanding the mechanisms that mediate the resistance to L-ASP treatment may bring new insights into ALL pathobiology and contribute to the development of more effective treatment strategies. In summary, this review presents an overview on L-ASP data and focuses on cellular mechanisms underlying resistance and alternative therapies for the use of asparaginase in childhood ALL treatment.

KEYWORDS: asparaginase, acute lymphoblastic leukemia, molecular resistance mechanisms, therapy updates

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Introduction

Acute lymphoblastic leukemia (ALL) is a hematological malignant disorder caused by excessive production of leukocytes and is the most common malignancy in children, representing 25–30% of all childhood malignancies. Great improvements have been made in ALL treatment, with successful long-term survival rates of approximately 80% over the past four decades.[1,2] Despite the significant success rate, the remaining 20% of patients still present treatment failure. Furthermore, surviving patients often present significant levels of toxicity, which warrants the need of new treatment strategies.[3]

ALL chemotherapy regimen consists of the following phases: remission-induction, consolidation, and continuation phase. Other treatments such as radiation therapy, steroids, and bone marrow or stem cell transplantations can also be included. Among the major drugs used during treatment phases are glucocorticoids, anthracyclines, vincristine and L-asparaginase (L-ASP), which has been used for a long time in ALL treatment.[4,5] Asparaginases are enzymes derived mainly from bacteria and the three enzymes that have been used are derived from *Escherichia coli* (*E. coli*-asparaginase), a pegylated form of native *E. coli*-asparaginase (PEG-asparaginase), and an *Erwinia chrysanthemi*-derived asparaginase (Erwinia-asparaginase).[6,7] A great improvement in patients overall survival was achieved by L-ASP administration, making it an essential drug in ALL treatment protocols[7–9]; however, several side effects, caused by L-ASP toxicity, encourage an open

debate among oncologists regarding the optimal dosage and formulation of L-ASP. Thus, this review presents an overview on L-ASP data and focuses on cellular mechanisms underlying resistance and the efficacy of different asparaginase formulations in childhood ALL treatment.

Traditional L-ASP Therapy and Current Alternatives

L-ASP enzyme is responsible for the conversion of L-asparagine to aspartic acid and ammonia. With the aid of L-asparagine synthetase (AS), normal cells can synthesize L-asparagine, whereas tumor cells are essentially dependent on extracellular pools of L-asparagine for cell proliferation and survival. The depletion of circulating pool of asparagine reflects asparaginase antitumor effect, by inhibiting DNA and protein synthesis and thus compromising tumor growth.[10]

Since its first description as an antitumor agent, the interest in L-ASP enzyme production has significantly increased. A wide range of microorganisms have presented L-ASP activity and among the major producers, bacteria, filamentous fungi, yeasts, and microbial sources from soil can be listed, although only asparaginase from *E. coli* and *E. chrysanthemi* have been produced on industrial scale.[11,12]

As initially mentioned, three asparaginase formulations are commercially available (*E. coli*-asparaginase, PEG-asparaginase, and Erwinia-asparaginase) and the drug activity and efficacy of each one of them can be influenced by drug structure, dosing schedule, and immunology reaction through antibody production.[13]

Several clinical studies support the use of L-ASP in ALL therapy and its use in remission-induction and intensification phases is critical in all pediatric ALL protocol. L-ASP treatment efficacy is closely related to ALL subtype and specific genetic abnormalities such as hyperdiploidia and *TEL-AML1* chromosomal rearrangement are the most sensitive, whereas high-risk ALL subtypes such as BCR-ABL positive and T-ALL are less susceptible.[14] High-dose use of L-ASP and prolonged intensification have been reported as critical for reduction of relapse and complete remission including high-risk patients such as lymphoblastic lymphomas and T-derived ALL.[4,15] Additionally, drug combinations using L-ASP along with corticosteroids (prednisolone and dexamethasone), and other chemotherapy agents as methotrexate, vincristine, and mercaptopurine, can potentiate L-ASP activity and consequently improve patient's outcome.[16,17]

Despite the successful role of the use of L-ASP in childhood ALL treatment, its use is limited and constantly re-evaluated due to serious side effects mainly caused by toxicity. Interestingly, most of the observed side effects arise from a second substrate specificity of asparaginase, which can also deplete the concentration of glutamine due to its structural similarity.[18,19] Among the side effects provoked by this glutaminase side activity of L-ASP are pancreatitis, hemostasis abnormalities, thrombotic and neurological complications, and hypersensitivity reactions (eg, clinical allergy) due to antibody production. Usually, children are more tolerant to L-ASP-induced side effects, whereas adolescents and young adults are more sensitive and often develop significant morbidity.[20–22]

Innumerous studies have reported that delivery of concomitant vincristine and prednisone and shorter time intervals between L-ASP doses reduces the probability of hypersensitivity reactions[23]; however, concomitant therapy with anthracyclines and/or steroids may increase the risk of pancreatitis.[4] To overcome toxicity and severe side effects, different asparaginase formulations have been constantly modulated, and the best approach to deal with such an administration schedule has been the main focus of clinical investigation studies in the last decades. For instance, the risk for pancreatitis or thromboembolism seems to be similar among different L-ASP preparations.[4,24] Aspects of different asparaginases formulations are listed in Table 1.

One of the major concerns during L-ASP treatment is the development of drug resistance mechanisms which are mainly derived through antibodies production in response to L-ASP, since all asparaginase sources are from a variety of microorganisms.[1,7] Yet, unnecessary doses are also a challenge to be defeated. Repeated administration of L-ASP leads to the development of specific antibodies and hypersensitivity reactions.[25,26] For example, in case of allergic reactions to native *E. coli*-asparaginase, patients are usually switched to either PEG-asparaginase or Erwinia-asparaginase,[27,28] although similar incidence rates of hypersensitive reactions have been reported for both native L-ASP and PEG-asparaginase. Reactions to Erwinia-asparaginase, however, may be less frequent.[24]

In particular cases, hypersensitivity does not have an evident clinical presentation but can still result in inhibition of asparaginase function, leading to a condition known as "silent inactivation."[28–30] Recent studies showed that children with silent inactivation of native *E. coli*-asparaginase are more prone to poor outcomes as they were not benefited by immediately switch to alternative asparaginase agents.[31]

Additionally, native *E. coli*-asparaginase used in induction can lead to PEG-asparaginase silent inactivation. As reported by Tong and colleagues, a high incidence of PEG-asparaginase inactivation (22% clinical allergy and 8% silent inactivation) in the intensification phase has been observed because of antibody development against native *E. coli*-asparaginase used in induction.[32] This implies that PEG-asparaginase should be used upfront during induction course instead of native *E. coli*-asparaginase since this approach has been shown to result in less antibody production. If antibody titers are low, PEG-asparaginase may still provide adequate activity levels[29]; for example, a dose of 2500 IU/m^2 given weekly has been shown to provide therapeutic levels in relapsed ALL patients who have previously received *E. coli*-asparaginase.[33]

It is interesting to point out that cross-reactivity between antibodies against native *E. coli* L-ASP and its pegylated form

Table 1. Aspects of different types of asparaginases used in acute lymphoblastic leukemia treatment.

COMMON SIDE EFFECTS*	ASPARAGINASE TYPE	DOSE-ADMINISTRATION ROUTE	CONCOMITANT DRUG ADMINISTRATION	COMMENTS	CITATIONS
Pancreatitis 1–18% [61,62,24]	*E. coli* asparaginase	6000–10000 IU/m^2-IM	Prednisolone Dexamethasone Vincristine Methotrexate**	15–20% patients treated with *E. coli* will develop hypersensitivity Antibodies production more commonly observed when compared with PEG-asp	61, 65, 68
Liver dysfunction most patients[63,64] Hypersensitivity 10–30%[65]	PEG- asparaginase	2500 IU/ m^2-IM and IV	Prednisolone/ Dexamethasone	Longer half-life (app. 6 days) and slower clearance Lower immunogenicity due to the covalent conjugation to PEG Option for ALL relapsed patients	61, 67, 69, 70
CNS complications 0–33%[24,62] Hyperglycemia 11–19%[63,66]	*Erwinia* asparaginase	10000–30000 IU/m^2-IM and IV	Prednisolone/ Dexamethasone	Optimal administration route is still inconsistent Short half-life; higher dose and increased dosing frequency required Should be used for the second or third-line treatment for patients with hypersensitivity to *E. Coli* ASP	65, 71, 27, 9

Notes: *Common side effects observed in all L-ASP formulations. **The improvement observed with L-ASP was not significant.
Abbreviations: IM, intramuscular; IV, intravenous; PEG, polyethylene glycol; ALL, Acute lymphoblastic leukemia; *E. Coli, Escherichia coli.*

can frequently occur, but does not affect Erwinia-derived enzyme.[30] Thus, switching to Erwinia-asparaginase in case of allergy or silent inactivation of PEG-asparaginase can be an alternative to achieve effective asparaginase levels.[32]

In clinical practice, however, the relevance of asparaginase antibodies seems to be limited, hampered by low specificity of the tests currently available to antibody detection, and thus, monitoring the serum asparaginase activity levels is a suitable strategy.[30,34] Silent inactivation detection is critically important to prevent useless continuation of an inactive asparaginase product, which may lead to worse event-free survival (EFS) as shown by Panosyan et al. (2004) and Vrooman et al. (2013).[31,35] However, it has been recently shown that among patients initially treated with PEG-asparaginase on frontline protocols (which subsequently relapsed), silent inactivation does not seem to be a significant clinical issue. Nevertheless, drug monitoring is the only way to detect cases of silent inactivation of asparaginase agents and ensure adequate drug levels and toxicity management.[30]

Despite *E. coli* and Erwinia-derived L-ASP known side effects, they are still preferred due to their considerable efficacy, economic production, and ease of process modification, optimization, and purification. However, new formulations and approaches to optimize this enzyme administration have constantly been proposed. For instance, several asparaginase formulations from different fungi strains have been reported as the search for alternative asparaginases in eukaryotic microorganisms could provide less toxic enzymes.

Extracellular L-ASP produced by *Aspergillus terreus* (strain PC–1.7.A) and *Bacillus licheniformis* has been purified and presented promising antitumor effects in several cancer cell lines along with low glutaminase activity and no cytotoxicity effect against normal human cells.[36,37] Although bacteria-derived asparaginase are relatively more stable than corresponding enzymes derived from plants or animals,[12] these alternative sources have also been investigated. In plants, L-ASP enzymes are required to catalyze the release of ammonia from asparagine (which is the main nitrogen-relocation molecule in these organisms), and are presented in significant amount in a variety of plant species. For example, *Withania somnifera*, a traditionally Indian medicinal plant, is an alternative source of L-ASP with high specificity and potential success for future large-scale production.[38,39]

In another innovative attempt to optimize asparaginase activity, L-ASP encapsulated within erythrocytes (GRASPA®) has been used to enhance asparaginase half-life. A phase I/II study has reported long-term serum asparagine depletion, good tolerance, and lower administration doses, as one single injection of 150 IU/kg of GRASPA® provided similar activity as eight injections at 10,000 IU/m^2 of native *E. coli*-asparaginase. This study, conducted in both children and adults in refractory ALL, also showed a significant reduction of allergic reactions and coagulation disorders, supporting the safety profile of GRASPA®.[40]

Altogether, the findings previously described emphasize the urge for individualized dose schedule as well as careful enzyme activity monitoring in all patients undergoing repeated courses of L-ASP treatment. Additionally, new alternative asparaginase sources could certainly optimize the drug administration and lead to a better outcome during ALL treatment.

Mechanisms Underlying the Cellular Response to L-ASP

The main effects induced by L-ASP on *in vitro* leukemia cells involve suppression of protein synthesis, G1 cell cycle arrest, and apoptosis induction. However, the exact events that lead to cell death following L-ASP treatment are unknown.[41–43] In order to understand the mechanism underlying sensitivity or resistance observed in clinical practice, several works have studied the response induced by L-ASP in clinical samples and *in vitro* models of leukemia. This information is important since the prognosis for patients with ALL is closely related to the cellular resistance to chemotherapeutic agents.[44,45]

One of the mechanisms of L-ASP resistance could be associated with AS expression, which can directly modulate asparagine synthesis. Different studies attempted to investigate the expression levels of this enzyme before and after L-ASP treatment to address the potential role of AS expression in asparaginase treatment resistance. Cell line studies showed that L-ASP-sensitive leukemic cells have low intracellular AS activity and are dependent on the availability of extracellular asparagine.[43] Andrulis et al. demonstrated that complete asparagine depletion *in vitro* results in an amino acid-dependent upregulation of mRNA, protein, and activity of AS.[46] 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.[41,47] 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.[41] Recent studies 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, but not in TEL/AML1-positive children.[48,49]

Appel et al. reported that 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. Moreover, it is noteworthy that L-ASP-induced upregulation of AS mRNA is not related to early *in vivo* blast reduction in childhood ALL and thus is not predictive for the short-term clinical response to L-ASP.[50]

Recently, gene expression profiling revealed that L-ASP-resistant ALL cells overexpressed several ribosomal protein-encoding genes as well as initiation factors.[45] 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.[51] This study emphasizes the fact that leukemic cell lines and primary samples from leukemia patients are different from each other and cell line data cannot be totally extrapolated to primary patients' cells.

In primary patients' samples, the exposure to L-ASP altered the expression of a number of genes related to protein synthesis (ie, tRNA synthetases and amino acid transporters). However, no genes discriminative for L-ASP resistance in patient samples were found. These data point to a consistent coordinated response to amino acid starvation, which occurs regardless of the level of resistance to L-ASP in patients' cells. Therefore, asparagine synthetase upregulation may be a consequence of amino acid deprivation by L-ASP, but it is not the limiting key factor explaining resistance to L-ASP in pediatric ALL. It is important to highlight, however, that the studies conducted so far only presented results on AS expression and failed to investigate the AS activity, thus limiting the data to support the hypothesis that AS expression could mediate L-ASP resistance.

Other investigation approaches have focused on identifying different mechanisms of L-ASP resistance. AS gene polymorphism has been described as one of the genomic determinants of asparaginase sensitivity among variations in *ATF5*, *ASS1* genes as well as gene variants from aspartate metabolism pathway. Pastorczak et al. suggested that a 14-bp tandem repeat sequence located in the first intron of AS gene may act as a transcriptional enhancer element; carriers of more than two repeats (>R2) may exhibit a higher expression of AS.[52] Additionally, Rousseau and coworkers reported that ALL pediatric patients, who were homozygous for double repeat (R2) of the first intron tandem repeat sequence of AS gene, had reduced EFS.[53] On the other hand, Pastorczak et al. revealed that R3 carriers with a poor response at day 15 had an increased risk of events.[52] Based on the results of these studies, it is likely that genetic variability in the AS gene may influence the clinical outcome of children with ALL; nevertheless, these data need to be confirmed by further investigations in other populations and different treatment protocols.

Although most studies investigating the L-ASP resistance have focused on ALL cells, new information has been emerging from the role of stromal cells in the synthesis of AS. The stromal cells, which form the microenvironment where leukemic cells grow, are basically formed by bone marrow-derived mesenchymal cells (MSCs). AS expression levels in MSCs from ALL patients were on average 20 times higher than those in leukemia cells. Moreover, MSCs protected ALL cells from asparaginase cytotoxicity in co-culture experiments. This protective effect correlated with levels of AS expression.[54] Laranjeira et al. showed that stromal cells induced the IGFBP7 expression by ALL cells.[55] IGFBP7, in an insulin/IGF-dependent manner, enhanced AS expression and asparagine secretion by BMSCs, thus providing a stromal-dependent mechanism by which IGFBP7 protects ALL from L-ASP treatment. Recently, Dimitriou et al. found

that values of the AS mRNA of MSCs seem to reach a peak at diagnosis, and tend to decline with treatment.[56] Besides the MSCs, a study has showed the function of adipocytes in the leukemia microenvironment to protect leukemia cells during L-ASP treatment.[57] Altogether, these results provide a new basis for understanding asparaginase resistance in ALL and indicate that the niche in the bone marrow have a pivotal importance in the ALL cells resistance to L-ASP.

Besides genomic modulation and alterations, epigenetic changes are also investigated concerning its role in resistance to L-ASP. MicroRNAs regulate the activity of protein-coding genes including those involved in hematopoietic cancers. Schotte et al. analyzed the expression levels of 397 miRNA by stem-looped RT-qPCR miRNA assays.[58] The results demonstrated that different genetic subtypes of ALL and drug-resistant cases have unique miRNA expression profiles and selected miRNA was associated with the clinical outcome of ALL patients. But only the miR-454 was expressed at a 1.9-fold lower level in L-ASP-resistant cases.

MicroRNA-196b (miR-196b) is highly expressed in Mixed-Lineage Leukemia (MLL)-rearranged ALL.[59] It has previously been shown that both MLL-rearranged and T-ALL pediatric ALL cases are more resistant to prednisolone and L-ASP.[60] However, Schotte et al. did not find evidence that miR-196b contributes to resistance to these drugs since patients with high miR-196b expression were not more resistant to both drugs than patients with low miR-196b expression levels.[61]

Final Considerations

Despite the progress made so far, more studies are needed to find whether the molecular data can be further associated with clinical response to L-ASP. Moreover, understanding the mechanisms underlying resistance to L-ASP treatment may bring new insights into ALL tumor biology and contribute to the development of more effective treatment strategies, such as individualized dose schedule as well as alternative asparaginase drug combinations and sources.

Author Contributions

Contributed to the writing of the manuscript: AFA, KSB, and VSS. Jointly developed the structure and arguments for the paper: AFA, KSB, and VSS. Made critical revisions and approved the final version: VSS. All authors reviewed and approved of the final manuscript.

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