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## ASPARAGINE SYNTHETASE: A NEW POTENTIAL BIOMARKER IN OVARIAN CANCER

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### SUMMARY

L-Asparaginase (L-ASP) is an enzyme drug that has been an asset to leukemia treatment regimens for four decades. Variability in its clinical efficacy, however, has prompted the search for biomarkers capable of distinguishing responders from non-responders. In that regard, the NCI-60 cell line panel has served as a biomarker discovery platform and has led to the identification of a correlation between L-ASP efficacy and asparagine synthetase (ASNS) expression in cultured cells. The presence of that correlation in the ovarian subpanel of the NCI-60 has made a case for repositioning L-ASP to ovarian cancer. This review presents an overview of the biomarker development process, summarizes the efforts that have been invested thus far in developing ASNS as a biomarker for ovarian cancer treatment, highlights the role of RNAi and the limitations of the NCI-60 in that process, and addresses important considerations for next steps in the development of ASNS as a predictive biomarker.

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Ovarian cancer patients diagnosed at stage I or stage II have a 5-year survival rate greater than 80%. Unfortunately, however, more than 80% of patients present with stage III or IV disease, when the malignancy has spread beyond the ovaries and the 5-year survival is less than 20%. Treatment includes surgery, radiation therapy and/or chemotherapy, which is typically administered for six cycles at 3 to 4 weeks per cycle. Carboplatin is the standard first-line drug,<sup>1</sup> and hexamethylamine and topotecan are standard second-line agents. Taxanes (e.g., paclitaxel), mustards (e.g., melphalan) and anthracyclines (e.g., doxorubicin) are also common first-line or second-line options. Despite all of those compounds, however, intrinsic and acquired drug resistance contribute to high relapse and low survival rates. Nevertheless, the postgenomic guiding principal that “all cancers are different and should be treated as such” has motivated the identification of new ovarian cancer biomarkers.

“Biomarker” generally refers to a biological measurement of one of the following five types:<sup>2–6</sup> i) biomarkers of disease progression (referred to as “screening biomarkers” in healthy subjects and “diagnostic biomarkers” in symptomatic subjects), ii) prognostic biomarkers of outcome, iii) predictive biomarkers of therapeutic effect, iv) surrogate endpoints of therapeutic effect and v) biomarkers of toxicity or adverse events (also referred to as “safety biomarkers”). Since it is important that therapy for ovarian cancer be started in the earliest possible stage, the first biomarker category is presumably the most important. In that category, the most sensitive and specific biomarker (more properly, “biosignature”) for detecting ovarian cancer currently appears to be a panel of six serum proteins (leptin, prolactin, osteopontin, insulin-like growth factor II, macrophage inhibitory factor and CA125), which has been reported to identify ovarian cancer as early as stage I with a striking 99.3% positive predictive value and 99.2% negative predictive value.<sup>7</sup> The second biomarker category aims to predict outcome; examples include p53, p21, Ki-67, and *HOXA11* DNA methylation.<sup>8–11</sup> The third category is similar to the second but specifically aims to predict which patients will respond to a particular treatment, as is the case for the biomarker upon which this review is focused. The fourth biomarker category provides tools for monitoring treatment response and includes the “pharmacodynamic biomarker” subcategory. Two recent examples of surrogate markers include C-reactive protein, which has demonstrated potential as a surrogate for response to platinum therapy,<sup>12</sup> and cell-free DNA, which has demonstrated potential as a surrogate for response to antivascular therapy.<sup>13,14</sup> In the fifth biomarker category, lipocalin-2 (LCN2) upregulation has been identified as an early marker of cisplatin-induced nephrotoxicity.<sup>15</sup>

All five of the biomarker types have been invoked to curtail a substantial increase in attrition in phase II and III clinical trials—a point in the drug development process at which a significant monetary investment has been made.<sup>16</sup> Moreover, the spike in attrition rate has prompted the call for a “biomarker pipeline” that parallels the traditional drug discovery and development pipeline (Fig. 1). In addition to curtailing the attrition rate, a biomarker pipeline would also facilitate drug repositioning (i.e., finding new uses for old drugs).<sup>17</sup> The remainder of this article, in fact, discusses a biomarker-based rationale for repositioning the drug L-asparaginase.

## DISCOVERY OF A PREDICTIVE L-ASPARAGINASE EFFICACY BIOMARKER

L-Asparaginase (L-ASP), the only U.S. Food and Drug Administration (FDA)-approved enzyme drug for cancer, has been used in combination with traditional chemotherapy to treat acute lymphoblastic leukemia since the early 1970s. It acts by depleting circulating asparagine and glutamine (Fig. 2),<sup>18–22</sup> and the resulting nutrient deprivation leads to cell death via the amino acid response pathway.<sup>23–25</sup> The endogenous enzyme asparagine synthetase (ASNS), which catalyzes asparagine synthesis from aspartate and glutamine,<sup>26,27</sup> has long been believed to impart resistance to L-ASP. Hence, *ASNS* was a logical gene to investigate as an L-ASP efficacy biomarker. Toward that end, microarray-based molecular profiling of the NCI-60 cell line panel revealed a strong negative L-ASP/*ASNS* correlation in the leukemia subpanel of the NCI-60,<sup>28</sup> indicating that low L-ASP efficacy (measured as reduced cellular metabolism using a tetra-zolium-formazan conversion assay) was associated with high expression of *ASNS* mRNA and vice-versa.

The NCI-60, originally assembled to screen for anticancer agents,<sup>29–31</sup> has been profiled more extensively at the molecular level than any other set of cells in existence.<sup>32</sup> The NCI-60 therefore made it possible to ask, “In addition to leukemia, do any additional cancer cell types exhibit the L-ASP/ASNS correlation?” Interestingly, the ovarian subpanel did,<sup>28</sup> suggesting the possibility that L-ASP might be used to treat ovarian cancer and that its efficacy might be predicted by ASNS expression. The *P* value for the ovarian correlation, unfortunately, was not statistically significant after Bonferroni correction for multiple comparisons. Nevertheless, the correlation was later corroborated by additional mRNA expression microarray platforms<sup>33,34</sup> and was also extended to the DNA level by comparative genomic hybridization.<sup>35</sup> The correlation (uncorrected for multiple comparisons) was statistically significant with all of these platforms. Mechanistically, concordance between mRNA and DNA level correlations suggests that ASNS mRNA expression is strongly influenced by 7q copy number. Hence, a multi-pronged approach to biomarker discovery provided early rationale for repositioning L-ASP to possible application against a low-ASNS subset of ovarian cancer.

The observation of a statistically significant L-ASP/ASNS correlation warranted proceeding to the next phase of the biomarker development process—testing for causality (Fig. 1). Technically speaking, causality testing is not a formal requirement for the development of a biomarker, since a non-causally linked biomarker could in theory exhibit strong predictive power. Nevertheless, causality testing indicates a more direct association and, therefore, provides a stronger rationale for further development of a biomarker. Accordingly, the L-ASP/ASNS correlation was next tested for causality using RNA interference (RNAi). Small-interfering RNA (siRNA)-mediated silencing of ASNS in three ovarian lines caused 3- to over 500-fold potentiation of L-ASP activity, indicating that the correlation was indeed causal.<sup>33</sup> Furthermore, the L-ASP/ASNS causal link was also found to be independent of multidrug resistance as shown by experiments in a doxorubicin (Adriamycin)-selected, multidrug-resistant cell line, OVCAR-8/ADR.<sup>33</sup> Hence, multidrug-resistant ovarian cancers with low ASNS expression might be sensitive to L-ASP. That robustness is a promising characteristic of the L-ASP/ASNS relationship considering the prominence of multidrug resistance in ovarian cancer and that clinical biomarker evaluation would probably have to be conducted, at least initially, in patients who have failed front-line therapy. These findings warranted proceeding to the next step of the biomarker development process—assay development.

## ASNS ASSAY DEVELOPMENT

The assay development phase should ideally be conducted in conjunction with (continued) biological validation efforts. In that regard, since the NCI-60 panel contains only six independent ovarian cancer lines, a variety of ASNS assays were next tested in a larger set of 19 ovarian lines. In that set, the relationship between ASNS mRNA expression L-ASP efficacy did not appear as strong as it had for the NCI-60 ovarian lines. However, ASNS protein did show a persuasive association with efficacy. Using rabbit polyclonal anti-ASNS antibodies and an immunoassay (both developed for the purpose), we found that ASNS protein expression is a moderately strong predictor of L-ASP efficacy (Pearson  $r = -0.65$ ,

one-tailed  $P = 0.0014$ ).<sup>36</sup> In other words, the data suggest, but by no means prove, that ASNS protein is a better indicator of L-ASP efficacy in vitro than is *ASNS* mRNA.

## PROJECTING TO THE CLINIC

Can we really expect L-ASP to benefit ovarian cancer on the basis of an in vitro correlation with ASNS expression? Single gene predictors of drug efficacy are indeed rare, and establishing the reliability of such relationships requires thorough validation.<sup>37</sup> Several lines of evidence provide a rationale for continued development of ASNS as a predictive biomarker of L-ASP efficacy. First, the L-ASP/ASNS correlation of  $r = -0.65$  in a diverse set of ovarian cancer lines is moderately strong.<sup>36</sup> Second, the correlation is causal.<sup>33</sup> Third, the causal link is unaffected by the development of multidrug resistance.<sup>33</sup> Fourth, the L-ASP/ASNS relationship overrides numerous markers that predict poor chemotherapeutic outcome, including p53 mutation, *HER2* overexpression, epidermal growth factor overexpression, CD10 negativity, platinum resistance and taxane resistance (as discussed in <sup>33</sup>). Fifth, the correlation between L-ASP efficacy and *ASNS* mRNA expression in the NCI-60 is much stronger than the correlation between some FDA-approved targeted therapies and their FDA-approved clinical biomarkers (as discussed in <sup>36</sup>). Taken together, those findings have prompted planning for a clinical trial at the National Cancer Institute to evaluate L-ASP efficacy in ovarian cancers using ASNS protein expression as a predictive biomarker.

## CONCLUSIONS

Since drug resistance significantly reduces the number of available therapeutic options for ovarian cancer, adding a potential new drug to the arsenal would represent a significant advance, especially if that new drug did not exhibit cross-resistance with existing therapies. L-ASP is one such agent that could prove useful for the treatment of a low-ASNS subset of ovarian cancers.

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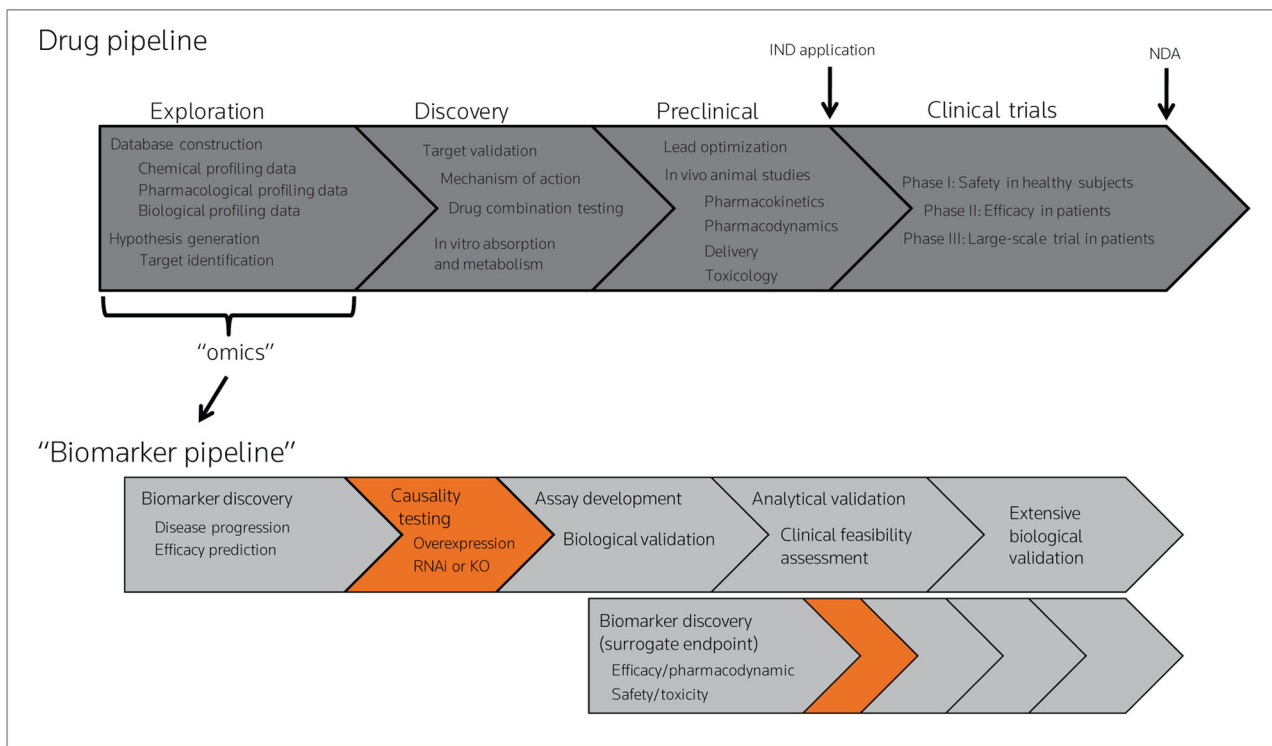
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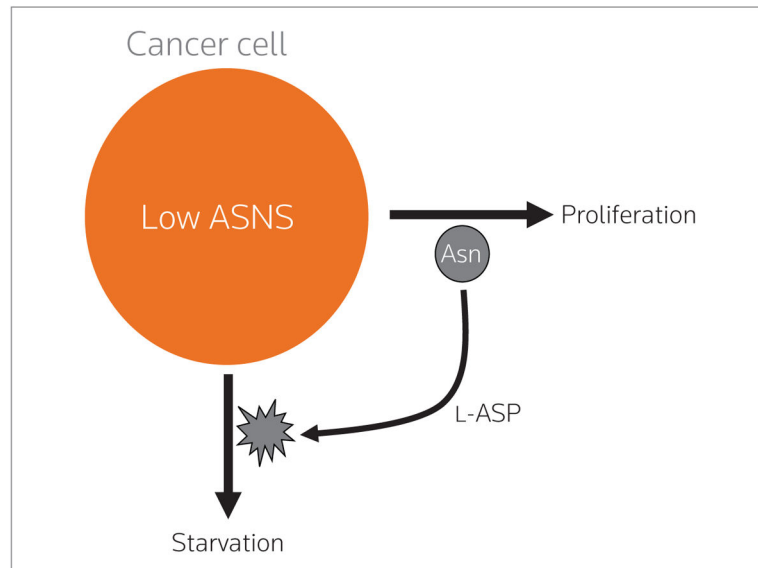
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**Figure 1.** Drug discovery and development in the postgenomic era. Top: one rendition of the modern drug pipeline, indicating typical activities conducted during Exploration, Discovery, Preclinical Studies and Clinical Trials. Bottom: “Omic” sciences, which are typically invoked during Exploration, serve the added purpose of enabling a biomarker pipeline to parallel the drug development pipeline. The upper biomarker pipeline illustrates the typical process for biomarkers of disease progression or predictors of efficacy. The bottom biomarker pipeline illustrates that the Discovery stage for surrogate endpoint biomarkers begins much later, typically in parallel with preclinical studies.



**Figure 2.** Rationale for therapy with L-asparaginase (L-ASP). Cancer cells that express low asparagine synthetase (ASNS) cannot synthesize enough asparagine (Asn) to keep up with their metabolic demands. Those cells hence become dependent upon extracellular Asn, which can be depleted by the drug L-ASP to starve the cancer.