Exploring L-Asparaginase treatment response in an Acute Lymphoblastic Leukemia cell model: bioinformatics analysis of single-cell transcriptomics data and experimental validation

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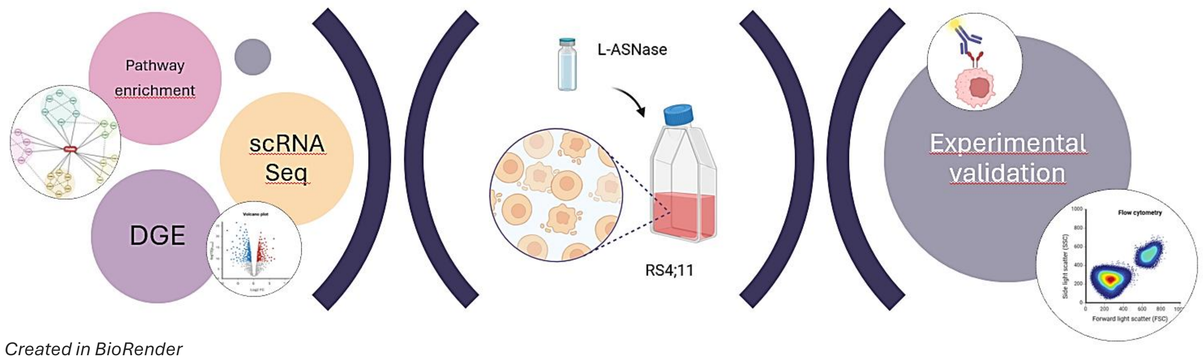
BACKGROUND

Acute Lymphoblastic Leukemia (ALL) cells commonly display a metabolic vulnerability due to low expression of asparagine synthetase (ASNS), making them unable to synthesize asparagine *de novo*. As a result, these cells rely on extracellular asparagine and glutamine to sustain protein synthesis and proliferation. This dependency is currently targeted therapeutically using **L-asparaginase** (L-ASNase), an enzyme which depletes circulating asparagine (and, to a lesser extent, glutamine), leading to impaired protein synthesis, cell-cycle disruption, metabolic stress and apoptosis in susceptible leukemia cells [1].

While these effects are well documented, a deeper characterization of these pathways is essential to fully exploit the drug's clinical potential while minimizing collateral effects, as well as to understand resistance mechanisms, which remain a major limitation.

AIM

To understand the **heterogeneity of responses** to L-ASNase treatment, we investigated the transcriptional response in **RS4;11** (ALL cell line model) and experimentally validated key pathways.



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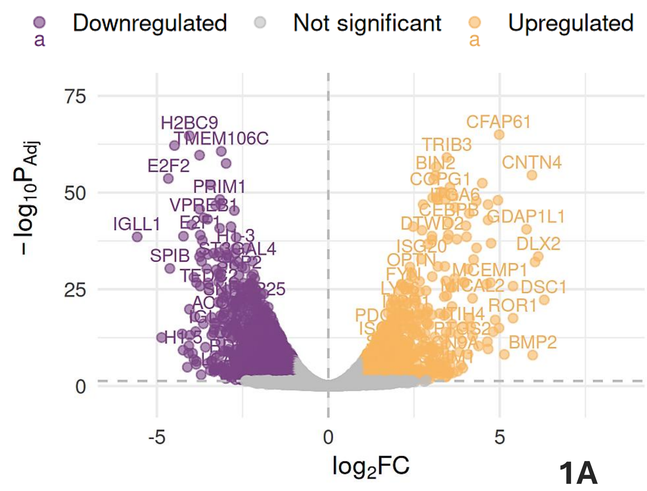
RS4;11 cells (ATCC CRL-1873) were cultured in suspension and treated with L-ASNase (**0, 0.0005, 0.001, 0.01 U/ml**, N=3) for **72h**

SINGLE CELL RNA SEQUENCING (scRNA seq)

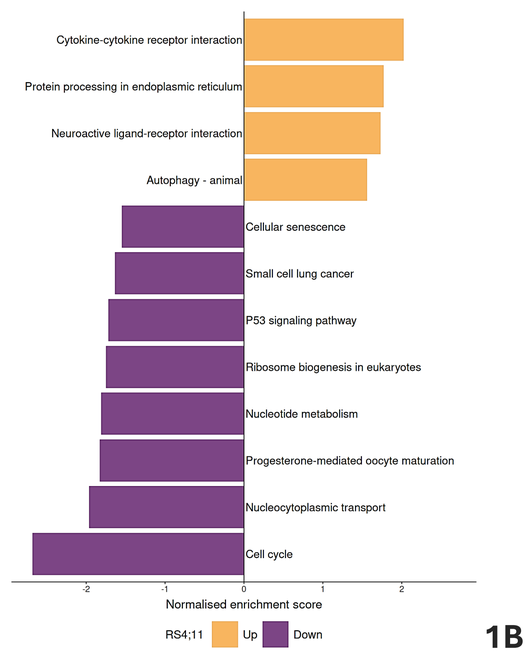
Single-cell gene Expression Flex (10X Genomics) was performed at the Italian National Facility Human Technopole, on three independent control and three treated samples. Downstream analyses were conducted in R through packages: DESeq2 (v.1.46.0), Seurat (v.5.2.1); fgsea (v.1.32.4), clusterProfiler (v.4.14.6).

Using a pseudo-bulk approach, **differential gene expression** (DGE) was assessed across all cell populations to identify genes that are significantly altered between the two conditions, with biological replicates treated independently to capture sample-specific variation. We identified 6,046 differentially expressed genes out of 11,551 with non-zero counts between control and treated samples; among them, 25% showed LFC > 0 and 27% LFC < 0 (Figure 1A).

Then, to provide a systems-level understanding of the key cellular processes affected, we performed a **pathway enrichment** analysis, referencing **Gene Ontology** (GO) and **Kyoto Encyclopedia of Genes and Genomes** (KEGG) databases for functional annotation (Figure 1B).

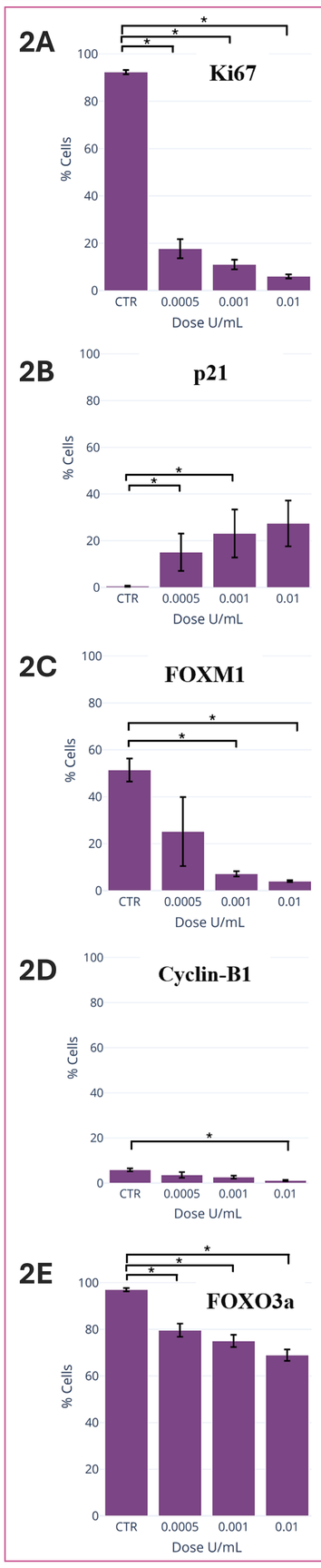


1A



1B

EXPERIMENTAL VALIDATION



Based on scRNA-seq results, pathways of interest identified through DGE and pathway enrichment analysis included "*Cellular Senescence*" (KEGG: hsa04218), "*Apoptosis*" (KEGG: hsa04210) and "*Ferroptosis*" (KEGG: hsa04216) [2]. Accordingly, markers involved in **cell-cycle control**, **proliferation**, and **cell death** were selected to validate transcriptomic changes at the protein level by **flow cytometry**.

CELLULAR SENESCENCE

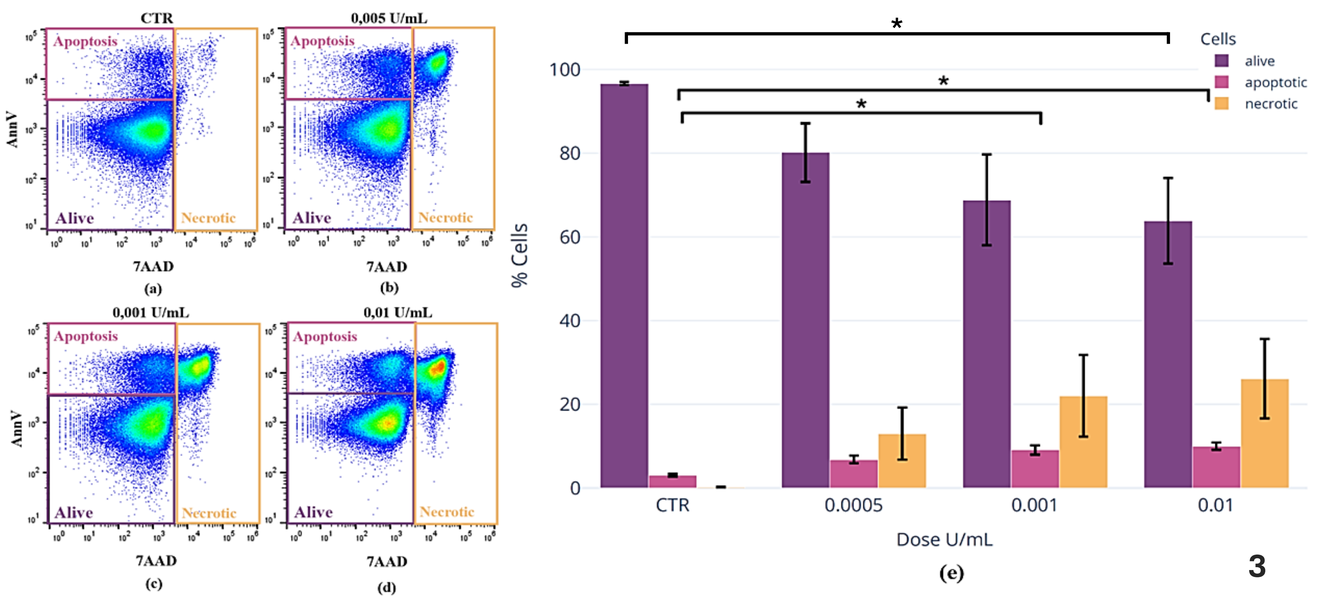
The canonical effect of ASNase treatment on the cell cycle is a **blockade in G1/S transition**, visible as an accumulation of cells in G1.

Analysis of positive populations for **p21** and **Ki-67** (Figure 2A-B) serves as primary biological confirmation of the proliferation block induced by metabolic stress, as p21 is a cyclin-dependent kinase inhibitor that enforces G1 arrest, while Ki-67 is a marker of active cell proliferation and is downregulated in non-cycling cells.
In line with these observations, additional markers of cell-cycle regulation and stress response were also affected by treatment. The pro-proliferative transcription factor **FOXM1** (Figure 2C) was progressively downregulated in a dose-dependent manner, indicating suppression of mitotic gene expression. **Cyclin B1** (Figure 2D), a key regulator of G2/M progression, was similarly reduced, reflecting an impaired ability of treated cells to enter mitosis. **FOXO3a** (Figure 2E), a stress-responsive transcription factor, showed dose-dependent decrease.

CELL DEATH

Cell viability was evaluated using a dual staining assay with **Annexin V** and **7-AAD**. This approach enabled the distinction between viable ($AnnV^-/7AAD^-$), apoptotic ($AnnV^+/7AAD^-$) and necrotic cells ($AnnV^+/7AAD^+$).

As shown in Figure 3, a slight increase in the apoptotic population is observed even at the lowest dose, reaching statistical significance only at doses 0.001 U/ml and 0.01 U/ml ($p < 0.05$).



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CONCLUSIONS

The combination of high-throughput single-cell transcriptomics with flow cytometry provides a powerful, integrative approach, merging molecular-level insights with functional validation. This strategy enhances the reliability of findings, deepens our understanding of treatment response heterogeneity in leukemia, and supports a precision medicine perspective in ALL.

Future studies will further investigate the molecular mechanisms of **ferroptosis**, **ROS production**, and **lipid peroxidation** underlying metabolic stress.

[1] Maggi M, Mittelman SD, Parmentier JH, Colombo G, Meli M, Whitmire JM, Merrell DS, Whitelegge J, Scotti C. A protease-resistant Escherichia coli asparaginase with outstanding stability and enhanced anti-leukaemic activity in vitro. Sci Rep. 2017 Nov 3;7(1):14479. doi: 10.1038/s41598-017-15075-4. PMID: 29101342; PMCID: PMC5670125
[2] Kanehisa M, Furumichi M, Sato Y, Matsuura Y, Ishiguro-Watanabe M. KEGG: biological systems database as a model of the real world. Nucleic Acids Res. 2025 Jan 6;53(D1):D672-D677. doi: 10.1093/nar/gkae909. PMID: 39417505; PMCID: PMC11701520.