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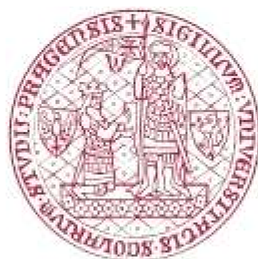

Natividad Alquezar Artieda, M.Sc

Cancer metabolism and its role in the sensitivity of leukemic cells to L-asparaginase

Nádorový metabolismus a jeho role v citlivosti leukemických buněk k L-asparagináze

Dissertation Thesis

Supervisor: RNDr. Júlia Starková, Ph.D.

Prague, 2023

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Cancer metabolism and its role in the sensitivity of leukemic cells to L-asparaginase

ABSTRACT

No ultimate treatment strategy exists for relapsed or non-responsive (15-20%) children with acute lymphoblastic leukemia (ALL). In this study, we aimed to elucidate the impact of metabolic rewiring in leukemic cells on poor therapy response and the emergence of resistance. This dissertation focuses on l-asparaginase (ASNase), a crucial chemotherapeutic agent and its effect on leukemia, using models of leukemic cell lines and primary cells of ALL patients. Cell metabolism was assessed by measuring metabolic pathways and nutrient influx using a Seahorse analyzer and stable isotope tracing. Main findings of the study demonstrated that the ASNase-therapy response was mitigated by the activity of the mechanistic target of rapamycin (mTOR)-regulated biosynthetic pathways. This phenomenon was induced by the bone marrow environment, which enabled the activation of the resistant mechanism in leukemic cells. We next found a correlation between the following metabolic features and lower sensitivity to ASNase: low ATP-linked respiration, high mitochondrial membrane potential and high glycolytic flux before therapy. The latter was shown to have prognostic implications. Moreover, high glycolytic flux was detected in T-ALL to be responsible for ASNase resistance and modulated by the phosphoinositide 3-kinase (PI3K)/Akt pathway. Further, we demonstrated metabolic rewiring plays a role in leukemogenic process driven by deregulated JAK/STAT signaling. In conclusion, we identified several metabolic features implicated in resistance, presenting novel therapeutic targets. Moreover, we have proposed a re-evaluation of patient stratification using cellular metabolism.

Keywords

Asparagine, PI3K/Akt pathway, cellular metabolism, childhood acute lymphoblastic leukemia, glutamine, l-asparaginase, mesenchymal stromal cells, metabolic profile, mTOR signaling pathway.

LIST OF ABBREVIATIONS

2-DG	2-deoxy-D-glucose
AA	Amino acid
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
AMPK	AMP-activated protein kinases
Asn	Asparagine
ASNase	L-asparaginase
ASNS	Asparagine synthetase
ATP	Adenosine triphosphate
B-ALL	B-lymphoblastic leukemia
B/M MPAL	B and myeloid mixed phenotype ALL
BCP	B-cell precursor
BM	Bone marrow
BSA	Bovine serum albumin
C-kit BMC	C-Kit positive bone marrow cells
CAD	Carbamoyl-phosphate synthetase
CLP	Common lymphoid progenitors
CML	Chronic myeloid leukemia
CMP	Common myeloid progenitors
CyTOF	Mass cytometer
d	Day
DNA	Deoxyribonucleic acid
E. chrysanthemi	Erwinia chrysanthemi
E. coli	Escherichia coli
ECAR	Extracellular acidification rate
ETC	Electron transport chain
FAO	Fatty acid oxidation
FCCP	Carbonylcyanide p-trifluoromethoxyphenylhydrazone
Gln	Glutamine
GLUT 1	Glucose transporter 1
GMP	Granulocyte-macrophage progenitor
GS-MS	Gas chromatography-mass spectrometry
GSK-3 beta	Glycogen synthase kinase-3 beta

HAP1	Huntington-associated protein 1 gene
HCA	Hierarchical clustering analysis
HCT	Hematopoietic-cell transplantation
HEPES	4-(2-hydrozyethyl)-1-piperazineethanesulfonic acid
Hex-1	Hexokinase 1
HIF-1	Hypoxia-inducible factor 1
HPLC	High-performance liquid chromatograpy
HSC	Hematopoetic stem cells
IS	Internal standard
ITS	Insulin, Transferrin, Selenium
JAK	Janus kinase
LDHA	Lactate dehydrogenase
MEP	Megakaryocyte-erythroid progenitor
min	Minute
MMP	Mitochondrial membrane potential
MPAL	mixed phenotype ALL
MPP	Multipotent progenitors
MRD	Minimal residual disease
MSCs	Mesenchymal stromal cells
mTOR	Mechanistic target of rapamycin
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NaCl	Sodium chloride
NF-kB	Nuclear factor kappa B
OCR	Oxygen consumption rate
OPRM1	Opioid receptor µ1
OXPHOS	Oxidative phosphorylation
p-	Phosphorylated
PEG	Polyethylene glycol
PES	Polyethersulfone
PI3K	Phosphoinositide 3-kinase
pre-B	Precursor B
PTEN	Phosphatase and Tensin homologue deleted
RNA	Ribonucleic acid

RPMI	Roswell Park Memorial Institute Medium
RTK	Receptor tyrosine kinase
SREBP	Sterol responsive element binding protein
STAT	Signal transducer and activator of transcription
T-ALL	T acute lyphoblastic leukemia
T/M MPAL	T and myeloid mixed phonotype ALL
TCA	Tricarboxylic
TMRE	Tetramethylrhodamineethyl ester perchlorate
ToF	Time to flight
USA	United States

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1. INTRODUCTION

1.1 Acute lymphoblastic leukemia in children

Acute lymphoblastic leukemia (ALL) is the most frequently diagnosed childhood cancer worldwide (Smith et al., 2010; Ward et al., 2014). It is defined as a malignant neoplasm of lymphocytes that suppresses the normal function of hematopoiesis. The suppression occurs through the clonal accumulation of immature lymphoid cells in the bone marrow. These cells are arrested at the lymphoid stage (common lymphoid progenitors, CLP) or later stage of development, and do not continue their normal maturation pathway to natural killer cells, lymphoid T cells and lymphoid B cells (Fig. 1). Without mature cells of these lineages, the innate and adaptive branches of the immune system are impaired, losing the defense of the organism against harmful substances and pathogens (Olson et al., 2020). Moreover, it affects other cell lineages. The counts of erythrocytes and megakaryocytes are reduced, which compromises oxygen transport throughout the body and the development of platelets, respectively (Gurbuxani et al., 2021).

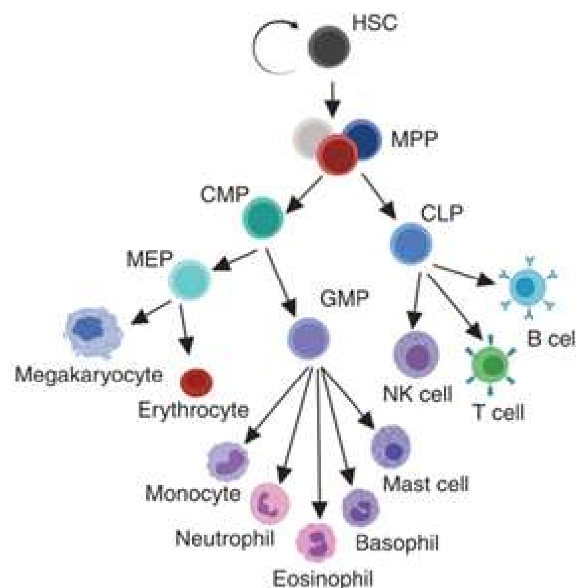

Figure 1. The model of normal hematopoietic differentiation. Of hematopoeitic stem cells (HSC) arise multipotent progenitors (MPP), which produce two different lineage-restricted progenitors, common myeloid progenitor (CMP) and common lymphoid progenitor (CLP). The first gives rise to megakaryocyte-erythroid progenitor (MEP) and granulocyte-macrophage progenitor (GMP). Ultimately, MEP, GMP and CLP produce all mature hematopoietic cells. Each bifurcating lineage choice is regulated either in a stochastic manner or in response to cytokines. Unchanched figure from (Olson et al., 2020).

The responsible events of ALL development are still not completely described. However, experts in the field agree that exist at least two phases of genetic mutation acquisitions to overt leukemia (Fig. 2). The initiating genetic lesions in the cell of origin, often chromosomal translocations, induce the generation of a small clonal population of pre-leukemic cells. The cell of origin could be HSC or arising progenitors from them. The clonal population gains self-renewal capacity, differentiation blockage, epigenetic reprogramming and/or deregulated signaling (Kuiper et al., 2007; Mullighan et al., 2007, 2009). Nevertheless, developing leukemia requires another hit of genetic mutation acquisition (Zelent et al., 2004). The secondary mutations are more diverse than the initiating translocations and are often generated by abnormal repetitive periods of inflammation. Excessive expansion of HSC during inflammation increases replicative stress, increasing their genetic instability (Olson et al., 2020; Søegaard et al., 2018). Ultimately, somatic mutations are accumulated and cause the clinical manifestation of leukemia. Secondary mutations cause unlimited proliferation and several deregulated pathways, including metabolism reprogramming (Hunger & Mullighan, 2015b).

The incidence of ALL is elevated compared to other pediatric cancers (Kodytkova et al., 2018; Stiller et al., 2006). It accounts for 32% of pediatric cancers in Europe for children under the age of 15 years. More specifically, it accounts for 21,6% of Czech pediatric cancer diagnoses under the age of 14 years.

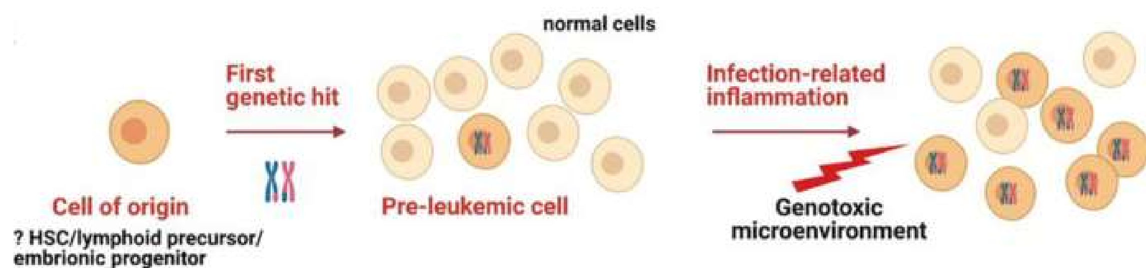

Figure 2. Genetic aberrations acquisition course into two phases to overt leukemia. HSC and arise progenitors from them gain an initiating lesion, often a chromosomal translocation, that generates a small population of pre-leukemic cells. These cells can accumulate crucial somatic mutations upon several abnormal inflammations that drive their malignant transformation to leukemia. Figure from (Dander et al., 2021) and cropped by the author of this dissertation.

ALLs are subdivided hierarchically and account for more than 30 genetic subgroups (Jeha et al., 2021). In the first level, ALLs are separated by their immunophenotype (cell-surface and cytoplasmatic markers), namely B and T cells. About 85% are of the B-cell lineage, also called precursor B-ALL (BCP-ALL) and the rest 15% are T-ALL. This first

level reflects in which lymphoid lineage, the cells became malignant. This specific differentiation is required because it has prognostic implications. Patients with T-ALL have worse treatment outcomes than B-ALL. In the second classification level, each cell lineage group is subdivided into recurrent initiating genetic lesions (Fig. 2)(Hunger & Mullighan, 2015a; Teachey & Pui, 2019).

The genetic subtypes of both ALL lineages also have prognostic value and therapeutic implications (J. F. Li et al., 2018). BCP-ALL commonly arises from aneuploidy (alterations in chromosome number) and chromosomal translocations. When the number of chromosomes increases above fifty, it is called hyperdiploidy and is associated with a favorable outcome. Its incidence is approximately 25.8% of the total cases with childhood ALL. The loss of chromosomes, known as hypodiploidy, accounts for 1% of pediatric ALL. Chromosome numbers between 31-39 indicate a bad prognosis and 24-30 chromosomes are associated with intermediate prognosis. Intrachromosomal amplifications are also observed in ALL, as in the case of the *iAMP21* subtype, which has a partial amplification of the chromosome 21. It represents 0.8% of the ALL and has a good prognosis. The best identified chromosomal translocations with a good prognosis are *ETV6-RUNX1* (21.4%) and *TCF3-PBX1* (2.8%). For the poor prognosis, there are *KMT2A* (MLL) rearrangements (4.7%) and *BCR-ABL1* (2.2%). The new subtypes based on the newly identified chromosomal translocations are *BCR-ABL1*-like (2.5%), *ETV6-RUNX1*-like (1.5%), *DUX4*-rearranged ALL (3.3%), *MEF2D* (0.5%), *ZNF384*-rearranged (1.2%) and other B-ALL (14.8%), which have diverse prognoses. Other B-ALL includes *PAX5*-altered and *NUTM1* rearrangements, among others (Fig. 3) (Gu et al., 2019; Inaba & Pui, 2021).

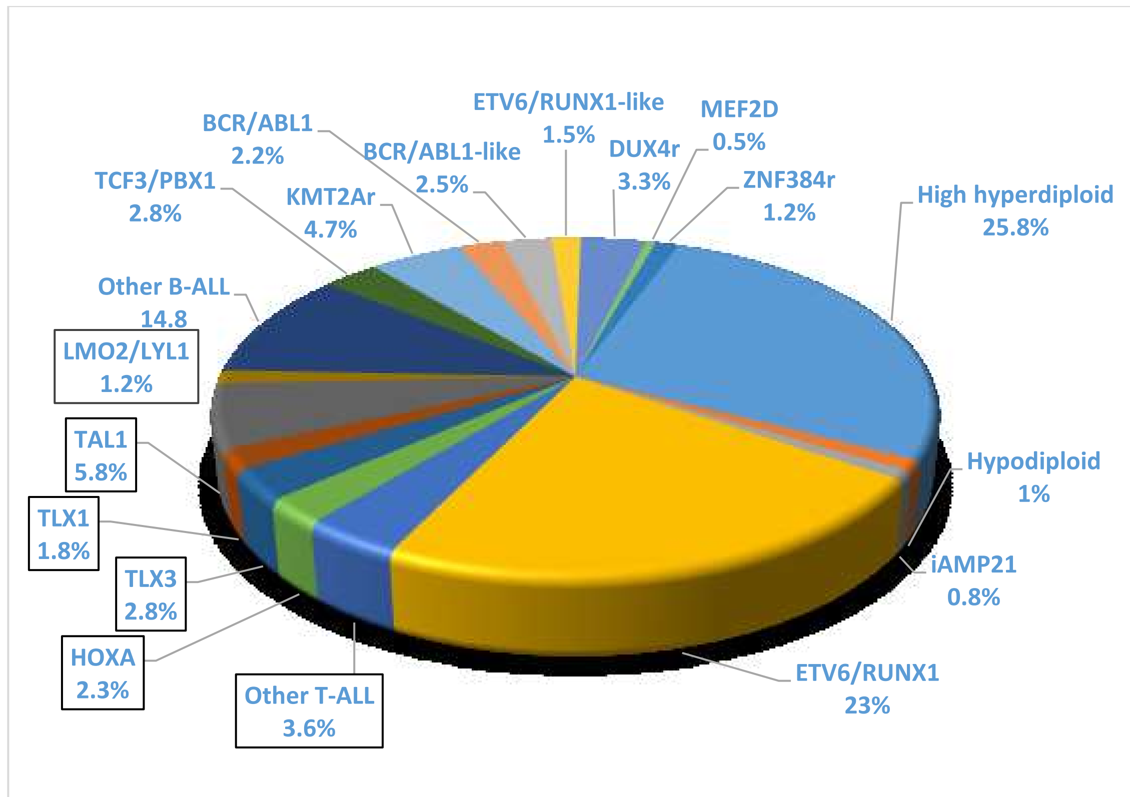

Figure 3. Genetic subtypes of pediatric ALL. Percentages are the approximate incidence of the most common genetic subgroups. Labels with surrounded by square shapes are T-ALL and the rest B-ALL. Figure from (Inaba & Pui, 2021)

Of note, not all the subtypes mentioned above have a recurrent initiating translocation, as is the case of *BCR-ABL1*-like ALL. The latter has a similar transcriptional profile to *BCR-ABL1*+ ALL but lacks the *BCR-ABL1* fusion gene. Instead, it has diverse genetic translocations, copy number alterations and sequence mutations that deregulate some signaling pathways (M. L. Den Boer et al., 2009; Mullighan et al., 2009; Roberts et al., 2012; Van Der Veer et al., 2013). The Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathways is one of the examples that is aberrantly activated in *BCR-ABL*-like ALL (J. M. Boer et al., 2017; Inaba & Pui, 2021). *JAK2* fusions are one of the mutations that cause that effect in JAK/STAT signaling. JAK2 is a tyrosine kinase, and the chimeric protein formed by *JAK2* fusions retains its catalytic domain, which is responsible for the deregulated pathway (Schinnerl et al., 2015). In normal cells, cytokines and growth factors activate the JAK/STAT signaling pathway, which interacts with their corresponding receptors, leading to receptor dimerization (Fig. 4). The latter recruits the JAK to be activated. Subsequently, p-JAK phosphorylates the tyrosine on the receptors and forms a docking site for the corresponding STATs. The affected STATs are phosphorylated on their tyrosine residues and dissociated from the receptor in the form of homodimer or heterodimer.

STAT dimers enter the nucleus and regulate transcription or interact with other pathways as phosphatidylinositol 3-kinase (PIK3)/Akt/mTOR, e.g. (X. Hu et al., 2021).

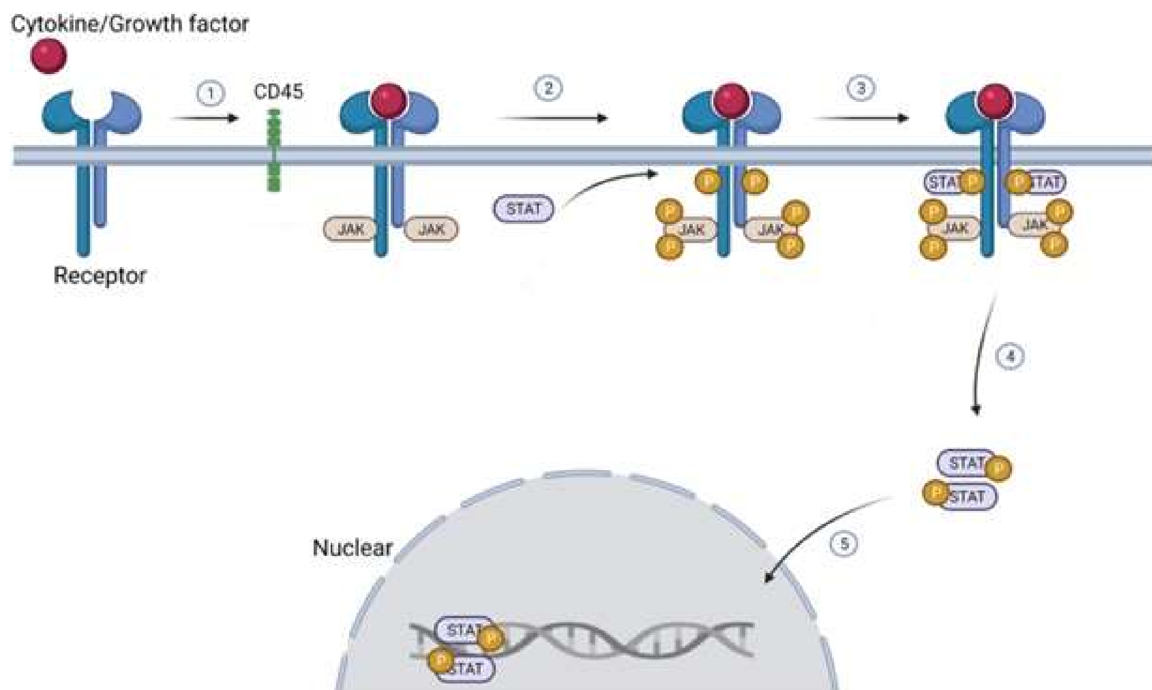

Figure 4. Activation of JAK/STAT signaling pathway by cytokine or growth factors. Dark arrows represent the activating process. Figure from (X. Hu et al., 2021)and edited by the author of this dissertation

T-ALL classification is not based on the recurrent initiating genetic aberrations since no clear association between them and patient outcomes was observed. Nonetheless, recurrent mutations can be found in specific transcription factors and oncogenes (Inaba & Pui, 2021). The most common genetic alterations are: *TAL1* (5.8%), *LMO2/LYL1* (1.2%), *TLX1* (1.8%), *TLX3* (2.8%) and *HOXA* (2.3%) from the total cases of ALL (Fig. 3). These mutations are combined with other aberrant alterations as activating mutations in Notch signaling pathways in 70% of T-ALL or JAK/STAT in 25% of the cases (Y. Liu et al., 2017). Notch is a transmembrane receptor involved in T-cells precursor development in normal hematopoiesis (Ellisen et al., 1991). In T-ALL, cases with mutated Notch have a truncated form whose activation was associated with cell proliferation, metabolism regulation, and protein synthesis (T. Palomero et al., 2006; Teresa Palomero & Ferrando, 2009).

There exists an additional subtype of leukemia, which does not fall into either B-ALL or T-ALL group and it is called mixed phenotype ALL (MPAL). MPAL is characterized by expression of cell surface proteins from multiple lineages, specifically B and myeloid (B/M MPAL) or T and myeloid (T/M MPAL). Immunophenotypically, they can be single (biphenotypic) or multiple (bilineal) subpopulations. This subtype manifests a

great plasticity based on multiple lineage characteristics, translating into poor prognosis (Alexander et al., 2018; Takahashi et al., 2018; Xiao et al., 2018).

1.2 Treatment of pediatric acute lymphoblastic leukemia

ALL treatment has been optimized over the years by employing carefully-curated prognostic factors, which led to an overall improved survival. It is considered one of the most significant achievements of modern oncology medicine. From the 1960s until today, ALL survival increased from approximately 10% to more than 90% in developed countries (Hunger & Mullighan, 2015a). Such improvement was caused by the introduction of risk stratification of patients, based on the prognostic factors. Different compositions and drug dosages of the multi-agent chemotherapy regimen are prescribed to individual risk groups. As previously described, the recurrent initiating genetic aberration and immunophenotype are prognostic factors, together with secondary mutations, age, white blood cell count at the time of diagnosis, and evaluation of treatment response. The latter is assessed by measurement of the minimal residual disease (MRD) and by studying the pharmacodynamics and pharmacogenomics of the patient (Inaba & Pui, 2021). MRD, the most informative factor, is a threshold parameter determined by the percentage of leukemic cells in bone marrow (BM) compared to healthy cells (Berry et al., 2017; Ribera et al., 2014). It is measured after the first of the four phases of the chemotherapy regimen. Patients with an MRD higher than 0.01% are facing a higher chance of treatment failure; therefore, they require a higher intensity of the chemotherapy regimen (Borowitz et al., 2008; Pui et al., 2015).

Chemotherapy regimens are divided into four essential phases: remission induction, consolidation, reinduction (delayed intensification), and continuation (Inaba & Pui, 2021). Each phase has standard basic components. First, induction therapy lasts 4 to 6 weeks, and leads to remission in almost all patients. This stage is characterized by the use of the following chemotherapeutic agents as a backbone of the treatment: glucocorticoid (prednisone or dexamethasone), vincristine, and asparaginase preparation. It can be optionally combined with anthracyclines. Second, remission must be consolidated with treatments with intensive combination chemotherapy for 6 to 8 weeks. Third, the reinduction phase lasts for 8 weeks and includes repeated courses of methotrexate administered through short intravenous infusions or high doses over 24 hours. Finally, in the continuation phase, patients receive low-intensity therapy for 18 to 30 months consisting of a daily dose of mercaptopurine or thioguanine, and a weekly oral dose of methotrexate. Some regimens also

include periodic 5-to-7-day administration of glucocorticoids and vincristine (Hunger & Mullighan, 2015a).

Since 2000, the backbone therapy, briefly described above, has included molecularly targeted treatment. Conventional chemotherapy targets the proliferation and mobility of leukemic cells. The drugs of the therapy impair nucleotide and protein synthesis and destroy the integrity of cells in the dividing stage, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). The incorporation of drugs that target molecularly abnormal events in leukemic cells led to an increase in the overall survival from approximately 80% to more than 90% (Inaba & Pui, 2021). Some of the examples of such new chemotherapeutic agents incorporated, for instance, inhibit apoptosis blockage, proliferation upregulation, and overuse of the proteasome of leukemic cells (Diaz-Flores et al., 2019; Horton et al., 2019; Khaw et al., 2016; Shen et al., 2020; Tanasi et al., 2019).

Patients who do not respond to the chemotherapy regimen can relapse during any phase of the therapy and eventually become candidates for hematopoietic-cell transplantation. The faster the patient relapse, the worse the prognosis (Raetz & Bhatla, 2012). If relapse occurs during the primary treatment (induction, consolidation, reinduction and continuation therapies), the leukemic cells of these patients usually harbor mutations that decrease the sensitivity to chemotherapeutic agents (Meyer et al., 2013; Mullighan et al., 2011). Only 50 to 70 % of such patients attain second remission, and only approximately 20 to 30% are cured. If the relapse occurs after the primary treatment, most patients will achieve a second remission, and the chance of a cure is about 50%. Allogenic hematopoietic-cell transplantation (HCT) is performed in more than 50% of the relapsed patients, and the decision is made based on the MRD negativity during the second remission (Peters et al., 2015). In conclusion, relapses occur in 15-20% of pediatric ALL cases, and cure rates are significantly decreased in patients after relapse (K. Nguyen et al., 2008).

The experts in the field agree that new approaches targeting pro-survival molecular mechanisms of leukemic cells and immunotherapy will improve overall survival further to 100% (Inaba & Pui, 2021). The new approaches are highly appealing since the intensities of conventional chemotherapy are set at the tolerance limit. Even though, further increase in the chemotherapy regimen intensity may potentially improve the overall outcome, it comes at a price of simultaneously increasing the adverse effects. Nowadays, it exists many ongoing investigations to optimize the use of new molecular targeted drugs in combination with or as a substitute for conventional chemotherapy. Therefore, a better understanding of

leukemogenesis and its several molecular responses to chemotherapeutic drugs could be helpful (Starkova et al., 2018).

1.3 Metabolism

Metabolic rewiring is a part of the malignant transformation of hematopoietic progenitors and leads the cells to obtain maximal profit from the nutrients while avoiding redox damage (Pavlova et al., 2022). In malignant cells, unlimited proliferation requires unlimited nutrient oxidation. This phenomenon is incompatible with the metabolic phenotype of differentiated cells. The metabolic phenotype of the latter is limited to a certain influx of nutrients because its primary goal is to generate energy. In differentiated cells, the based-carbon nutrients are degraded. Their products are then metabolized by the electron transport chain (ETC) in the mitochondria to form adenosine triphosphate (ATP), the energy carrier. In case of a massive influx of nutrients, the oxidative capacity of ETC could be overwhelmed, causing reductive damage. To avoid that, cancer and proliferating cells modify the metabolic pathways to use additional carbons for biomass synthesis. Thus, cancer cells undergo a metabolic rewiring during the malignant transformation to cope with their unlimited cell proliferation capacity.

1.3.1 Glucose metabolism and glycolysis

The metabolism of glucose, the primary substrate promoting cell proliferation, is strictly regulated in healthy cells. Pro-proliferative activating signals come from extrinsic factors. In general, the mentioned factors are generated by tissue needs to maintain homeostasis, regardless of individual cell needs. However, the situation changes with cancer cells since their mutations allow them to escape from the tissue controls. In healthy cells, glucose uptake is activated by environmental growth factors and positional cues (Thompson, 2011), (Fig. 5). These stimulate the activation of downstream signaling events like the receptor tyrosine kinase (RTK)-PI3K-Akt1 cascade. Its primary function is to promote the expression of glucose transporter 1 (GLUT 1) on the cell surface (Barthel et al., 1999; Meyer et al., 2013; Wieman et al., 2007). Once the glucose is imported, Akt1 increases the activity of the hexokinase 1 (first enzyme of glycolysis), and glucose enters the metabolic pathway (Rathmell et al., 2003). Extrinsic factors are not the only molecules controlling cell proliferation in healthy cells. In order to strictly regulate cell growth, negative regulators act as a secondary barrier to avoid massive expansion. For instance, phosphatase and tensin homolog deleted (PTEN) dephosphorylate PI3K, inhibiting the signaling cascade (Fig. 5)

(Chu & Tarnawski, 2004). Mutations of this negative regulator and PI3K are often involved in the driving events in tumorigenesis. They are responsible for PI3K/Akt1 signaling activation independently of external factors along with other alterations, which allows the unlimited proliferation of cancer cells (Lawrence et al., 2014; Pavlova et al., 2022).

Glycolysis is the pathway through which glucose is oxidized. It comprises numerous catabolic chain reactions that convert glucose into pyruvate, producing two ATP molecules and an electron carrier. Notably, glycolysis yields approximately fifteen times less ATP per glucose molecule than ETC. In resting cells, pyruvate follows one of two different fates depending on oxygen availability. In normoxia, it is introduced into the tricarboxylic (TCA) cycle while converted into lactate in hypoxia (Pavlova et al., 2022)That is not a strict rule since proliferative and cancer cells were reported to convert pyruvate to lactate in normoxia as well (Brand et al., 1986; Chambers et al., 2010; Noch & Khalili, 2012). Dr Otto Warburg was the first to describe this phenomenon in cancer cells. He observed that cancer cells consume abnormal amounts of glucose and rely on glycolysis to produce ATP instead of ETC in normoxic conditions (Warburg, 1956; Warburg et al., 1927). This event was named the Warburg effect. It describes the conversion of glucose into lactate even when oxygen is available.

The Warburg effect is redox neutral (Fig 5). It means that an electron carrier, generated by the oxidation of glucose to pyruvate, is used to synthesize lactate. By reusing the electron carriers, it becomes independent of ETC. Without the limitations of ETC, glucose is constantly imported and oxidized, increasing ATP production efficiency and accumulating glycolytic intermediates (Pavlova et al., 2022; Sullivan et al., 2018). Such a metabolic state is beneficial for cancer cells, and they enhance it by genetic alterations, for instance, amplification mutation in the oncogene c-myc. C-myc increases the expression of many enzymes that promote the conversion of pyruvate to lactate, like lactate dehydrogenase (LDHA) (Pavlova & Thompson, 2016). Another way cancer enhances glycolysis flux is by loss of function mutation in transcription factors that suppress access to glucose, which is the case of PAX5 in B-ALL (Chan et al., 2018).

The accumulated glycolytic intermediates are then used for biomass production. The large excess of glycolytic intermediates in transformed cells prompts the branching of glycolysis to generate nucleotides, di- and triglycerides, phospholipids and proteins (Fan et al., 2014; Patra & Hay, 2014).

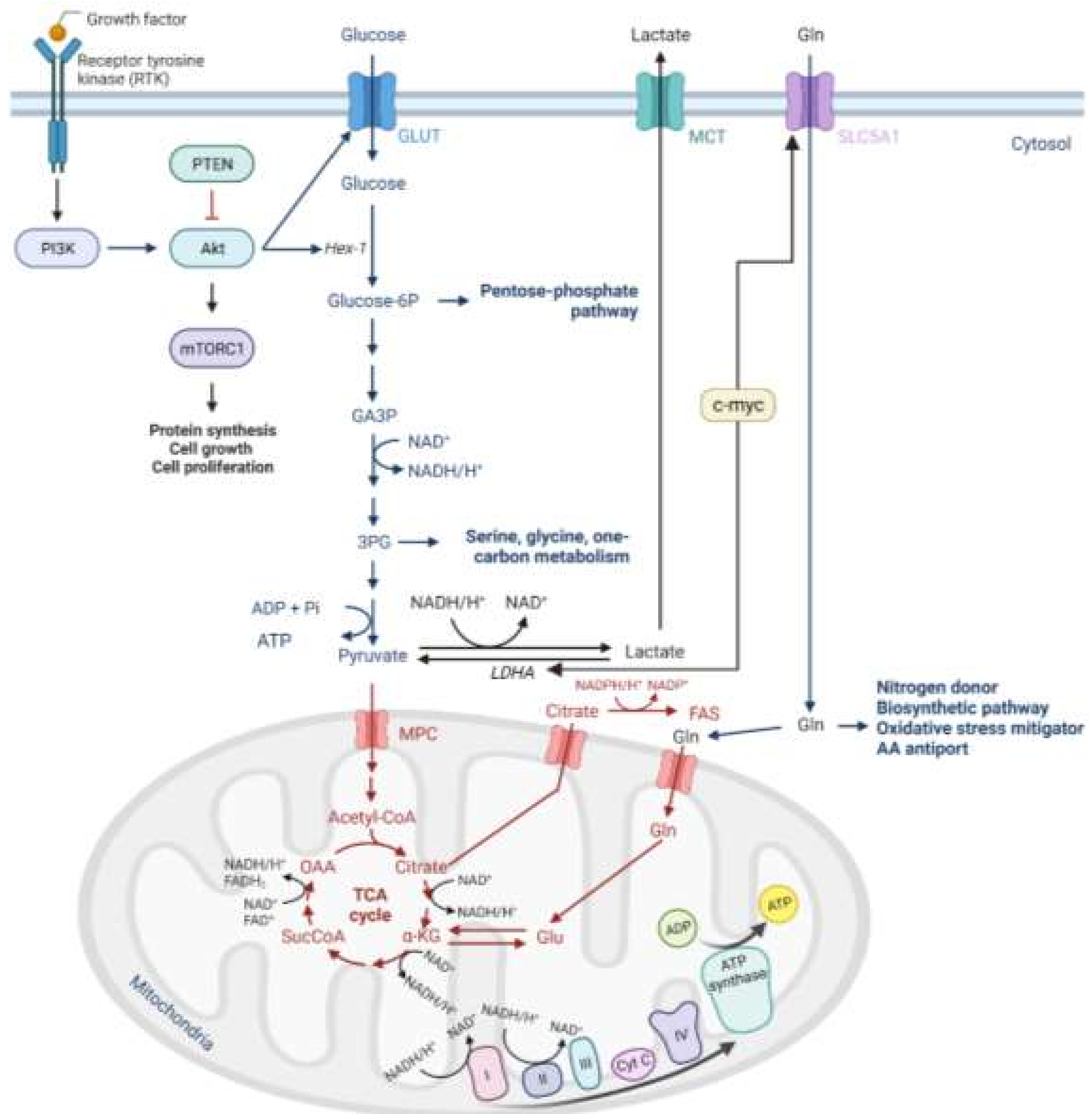

Figure 5. Metabolic circuits of proliferative cells. Figure designed by the author of this dissertation using Biorender

1.3.2 TCA cycle and glutamine metabolism

The TCA cycle is a catabolic process in which glucose-derived pyruvate is oxidized to generate the majority of electron carriers in the metabolism of resting cells (Fig. 5). Under pro-proliferative stimuli, the amount of glucose-derived pyruvate is reduced due to its utilization in branched pathways. As a result, cells become dependent on glutamine (Gln) import to fuel the TCA cycle. The main driver of Gln utilization in proliferative and transformed cells is c-myc, introduced before as the lactate synthesis regulator (Avissar et al., 2008; Reynolds et al., 2014; Wise et al., 2008). C-myc induces the expression of transmembrane Gln transporters and the enzymes for its catabolism. Gln is converted into

glutamate and enters the TCA cycle (Gao et al., 2009; Mannava et al., 2008). In addition to their role in fueling the TCA cycle, Gln and glutamate have essential roles as nitrogen donors for other biosynthetic pathways, biomass for protein synthesis, mitigators of oxidative stress, and antiport for non-essential amino acids (AAs) from the extracellular matrix (Daemen et al., 2018; Nicklin et al., 2009).

As a response to high levels of nutrients like glucose and Gln entering the TCA cycle, the accumulation of the TCA cycle's intermediates leads to the activation of biosynthetic pathways. TCA intermediates are precursors to lipids, cholesterol and AA synthesis (Hatzivassiliou et al., 2005; Porstmann et al., 2008; Shao et al., 2020).

1.3.3 Oxidative phosphorylation

Oxidative phosphorylation (OXPHOS) is the pathway through which the electron carriers are oxidized to generate ATP (Fig. 5). It takes place in the ETC and encompasses four complexes and ATP synthase, localized in the inner mitochondrial membrane. The electron carriers from glycolysis and the TCA cycle interact with ETC and release protons (H^+) and electrons (e^-). Protons accumulate in the interspace of the membrane, and electrons travel through all the complexes. As a result, the charge difference generates an electrochemical gradient that forces the protons through the ATP synthase, producing ATP (Zorova et al., 2018). Noteworthy, the accumulation of protons in the interspace space generates a mitochondrial membrane potential (MMP). When MMP levels are not sufficient, the cells enter apoptosis (Elmore, 2007; Liou & Storz, 2010).

The oxidation capacity of OXPHOS has limitations (Pavlova et al., 2022). The electron carriers oxidized in the ETC are reused to pick up electrons from the catabolism of new nutrients through those biosynthetic pathways. Thus, the cell keeps oxidizing nutrients constantly. However, OXPHOS can only oxidize a limited number of electron carriers. In case of excessive production of these carriers in proliferating cells, they accumulate in the cells, causing redox damage. Ultimately, the cells initiate the cell death program if the redox damage is not reduced (Fulda et al., 2010). Nevertheless, proliferative cells relieve the overload of ETC by using electron carriers for lactate and lipid synthesis.

In proliferating cells, both OXPHOS and glycolysis maintain ATP production. The latter is needed to power actin filament remodeling during cell division. Therefore, it is crucial for cancer cells. Any decreasing fluctuation of ATP will activate the AMP-activated protein kinases (AMPK), which increase the expression of proteins involved in glycolysis and fatty

acid oxidation (FAO). Simultaneously, it switches off biosynthetic pathways that require ATP (Rashkovan & Ferrando, 2019).

1.3.4 Fatty acid oxidation

FAO is more efficient regarding ATP generation than any other pathway oxidizing carbohydrates (Schafer et al., 2009). However, it is reserved for metabolic stress situations. FAO is a set of cyclical reactions of oxidation during which the fatty acids drop two carbons and two electrons in each cycle. The two carbons are released as acetyl-CoA. A fatty acid molecule gets degraded through this pathway until only four carbons are left in the chain. Then, two acetyl-CoA molecules are released in the last round of the oxidation process. Two electron carriers pick up electrons in each cycle. Since fatty acids are the main components of the cell membrane, their synthesis is essential in cell division for the formation of two daughter cells. Specifically, TCA cycle intermediates are precursors to lipid synthesis. Therefore, fatty acids are not wasted as an energy source under non-stress conditions (Carracedo et al., 2013). Noteworthy, in nutrient abundance conditions, cancer cells store the surplus of carbon in lipid structures as a reservoir of energy for periods of metabolic stress (Tan et al., 2022; Zhang et al., 2019). FAO is activated when metabolic stress of the cell arises. It was observed in the resistance of leukemic cells to various chemotherapeutic agents (German et al., 2016; Han et al., 2019; I Hermanova et al., 2016; Prew et al., 2022).

The main functions of FAO are the maintenance of ATP and the generation of electron carriers. Acetyl-CoA molecules can enter the TCA cycle and are used as a precursor to fatty acid synthesis or continue the usual TCA degradation to produce electron carriers. The latter can be used in the biosynthetic pathways for generating biomass or metabolized in the ETC, which leads to ATP production (Carracedo et al., 2013; Prew et al., 2022).

1.3.5 Heterogeneity of metabolic phenotype in cancer cells

Cancer cells might share similar metabolic processes as proliferating cells. However, oncogenic mutations and nutrient availability are factors that determine the metabolic phenotype of cancer, which can be very heterogeneous among cancer types (Kim & DeBerardinis, 2019). For instance, cancers that genetically enhance glucose metabolism can have high or low OXPHOS activity, resulting in different phenotypes. Cancer cells with a glycolytic phenotype (Warburg effect) provide sufficient ATP for cell division from glycolysis, which decreases OXPHOS activity (Birsoy et al., 2015; Lucas Sullivan et al., 2015). When high glycolysis is combined with high OXPHOS flux, the phenotype is known

as energetic (Fiorillo et al., 2019). Cancers without high glycolytic flux, such as leukemic stem cells and a subset of diffuse large B-cell lymphoma, appear to depend predominantly on OXPHOS. Instead of using glucose as a nutrient, they use alternative fuels such as fatty acids, and their phenotype is aerobic (Jones et al., 2018; Ye et al., 2016).

Furthermore, metabolic phenotypes can be changed due to nutrient availability. An aberrant capacity of nutrient uptake is typical in all cancers, giving them the plasticity to rewire the metabolic circuits based on the nutrients in the environment (Hui et al., 2017; Mashimo et al., 2014; Palm & Thompson, 2017; Pavlova et al., 2018; Pusapati et al., 2016), Hui et al, 2017; Mashimo et al., 2014). Metabolic phenotypes in cancer are both heterogeneous and plastic, and their investigation is vital to design therapeutic strategies based on metabolism to treat cancer (Kim & DeBerardinis, 2019).

1.4. L-asparaginase

L- asparaginase (ASNase) is an enzyme used as a chemotherapeutic agent in ALL therapy since 1974. Its anti-leukemic effect was randomly discovered through experiments with animal sera. In 1953, John Kidd published that lymphoma treatment complemented with guinea pigs serum induced a robust regression of engrafted mouse lymphoma (KIDD, 1953). He used several animal sera, but only guinea pig serum caused such an effect. In 1963, JD Broome identified the enzyme ASNase in the serum as responsible for its anti-leukemic effect (BROOME, 1963). Subsequently, the finding was confirmed by Mashburn & Wriston (1963), who additionally provided evidence about cancer growth inhibition induced by *Escherichia Coli* (*E. coli*) ASNase (Mashburn & Wriston, 1964). Besides *E. coli* and guinea pig, there are other sources of ASNase. Plants can produce it, as well as animals (not humans) and microorganisms (Verma et al., 2007). Nevertheless, ASNases from bacterial sources were uniquely included in therapy due to their adequate cost-effectiveness, accelerating their clinical use (Joachim Boos & Pinheiro, 2004).

From the first clinical trials, ASNase demonstrates to be an essential component of ALL treatments. As monotherapy, it inhibited 20 to 60% of cancer growth in ALL patients (Haskell, 1969; Jaffe et al., 1971; Tallal et al., 1970). In the current drug combination, it is applied during the induction and consolidation phases of the treatment (Hunger & Mullighan, 2015a). Notably, the high dose of ASNase in the consolidation period demonstrated excellent results with relatively low morbidity (Pession et al., 2005; Silverman et al., 2001). Therefore, ASNase is an irreplaceable drug for ALL treatments.

1.4.1 Enzymatic activity of l-asparaginase

The enzymatic activity of ASNase is a deamination process in which l-asparagine (Asn) and Gln are irreversibly converted into l-aspartic acid and l-glutamic acid, respectively (Fig. 6). Initially, Asn was the only described target of ASNase (Haley et al., 1961). However, further investigation showed that it also has a weak affinity for Gln. Indeed, it is the case of *E. coli* ASNase. In contrast, ASNases isolated from other organisms do not necessarily target both AAs, or neither has the same range of affinity as the one derived from *E. coli* (Verma et al., 2007). In general, several different kinetics were observed among the ASNases with different origins (Ghasemian et al., 2019). The cause of different kinetics among ASNases is their biochemical structures. ASNases showed diverse structures, which are translated into various affinities for their targets. Consequently, not all ASNases demonstrated toxic effects on leukemic cells and cancer inhibitory activity (Ghasemian et al., 2019). Even the isoenzymes of ASNase isolated from *E. coli* are different. The enzyme isolated from the cytosol has a greater affinity for Gln than Asn, while the enzyme isolated from periplasmic spaces has a more substantial effect on Asn (Izadpanah et al., 2018). Therefore, only one additional ASNase was tested and included in ALL therapy, *Erwinia chrysanthemi (E. chrysanthemi*) ASNase. Both ASNases are currently approved for clinical use and have remained as component of ALL treatment since 1964 (Ghasemian et al., 2019; Wade et al., 1968).

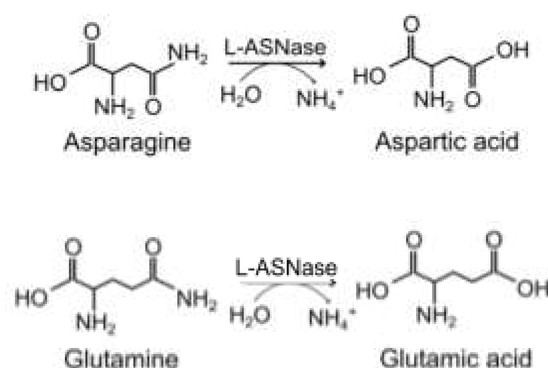

Figure 6. Reaction scheme of the enzymatic activity of ASNase. Figure prepared by the author of this dissertation thesis

Approved ASNases differ in their kinetics as well. Therefore, they cannot be interchangeable in terms of dosage recommendation (Joachim Boos & Pinheiro, 2004). The dosage scheme per each stratified group of ALL was optimized in dose-finding studies using *E. coli* ASNase (Ertel et al., 1979; M. Nesbit et al., 1979; M. E. Nesbit et al., 1981).

Subsequently, the dosage scheme was tested in another study using *E. coli*- and *E. chrysanthemi* ASNase. In this study, these therapeutic agents from different bacterial sources differed in concern of the *in vivo* half-life and duration of Asn depletion in treating newly diagnosed pediatric ALL. The half-life of a drug is defined as the time required for a reduction of 50% in the concentration of a drug in plasma. Individually, *E. coli* ASNase was 1.24±0.17 d of the half-life, while *E. chrysanthemi* ASNase was a 0.65± 0.13 d (B. L. Asselin et al., 1993; Barbara L. Asselin, 1999; Pinheiro et al., 1999). Since the primary goal of the ASNase treatment is complete Asn depletion, the dosage recommendation for the clinical application of *E. chrysathemi* ASNase was modified (Joachim Boos & Pinheiro, 2004). This change was introduced after testing different dosages of *E. chrysanthemi* ASNase in another clinical trial (J. Boos et al., 1996). As detailed, both bacterial ASNases required a dose-finding study, which means it is not beneficial to incorporate new native ASNases from other origins into ALL therapy.

Years after mentioned clinical trials, a modified version of *E. coli* ASNase was approved for its clinical application in ALL. The new ASNase has a pegylation, which causes a reduction of the immunogenic response boost by exogenous proteins (Joachim Boos & Pinheiro, 2004). Pegylation is a standard procedure in the pharmaceutical chemistry field. This modification extended the half-life of ASNase, which was four times more compared to its native form (B. L. Asselin et al., 1993). The immunogenic response is one of the speculated reasons why native ASNase is fast eliminated from the bloodstream, reducing the Asn depletion period ((Barbara L. Asselin, 1999; Joachim Boos & Pinheiro, 2004). When used as a front-line therapy with a lower dosage, pegylated ASNase showed comparable efficacy to its native form (Silverman et al., 2001). Thus, pegylated ASNase increases the therapeutic options for children with ALL (Maese & Rau, 2022; Salzer et al., 2007).

1.4.2 Advances in the mechanism of action of ASNase in acute lymphoblastic leukemia

ASNase's depletion effect on Asn and Gln selectively impairs leukemic cell proliferation. Asn is required for protein synthesis, and its absence inhibits cell division (F. F. Becker & Broome, 1967; Frederick F. Becker & Broome, 1969). As stated, Gln is a metabolic nutrient and a precursor of many biosynthetic pathways as a donor of nitrogen and carbon. The depletion of both AAs disturbs metabolism, which leads to the impairment of leukemic cell proliferation. However, the specific mechanism of action is still an open area of investigation. Progressing in the field, Hermanova and colleagues provided a novel

description of the metabolic response of precursor (pre)-B cell ALL to ASNase treatment (I Hermanova et al., 2016).

1.4.3 Selective effect of ASNase on leukemic cells

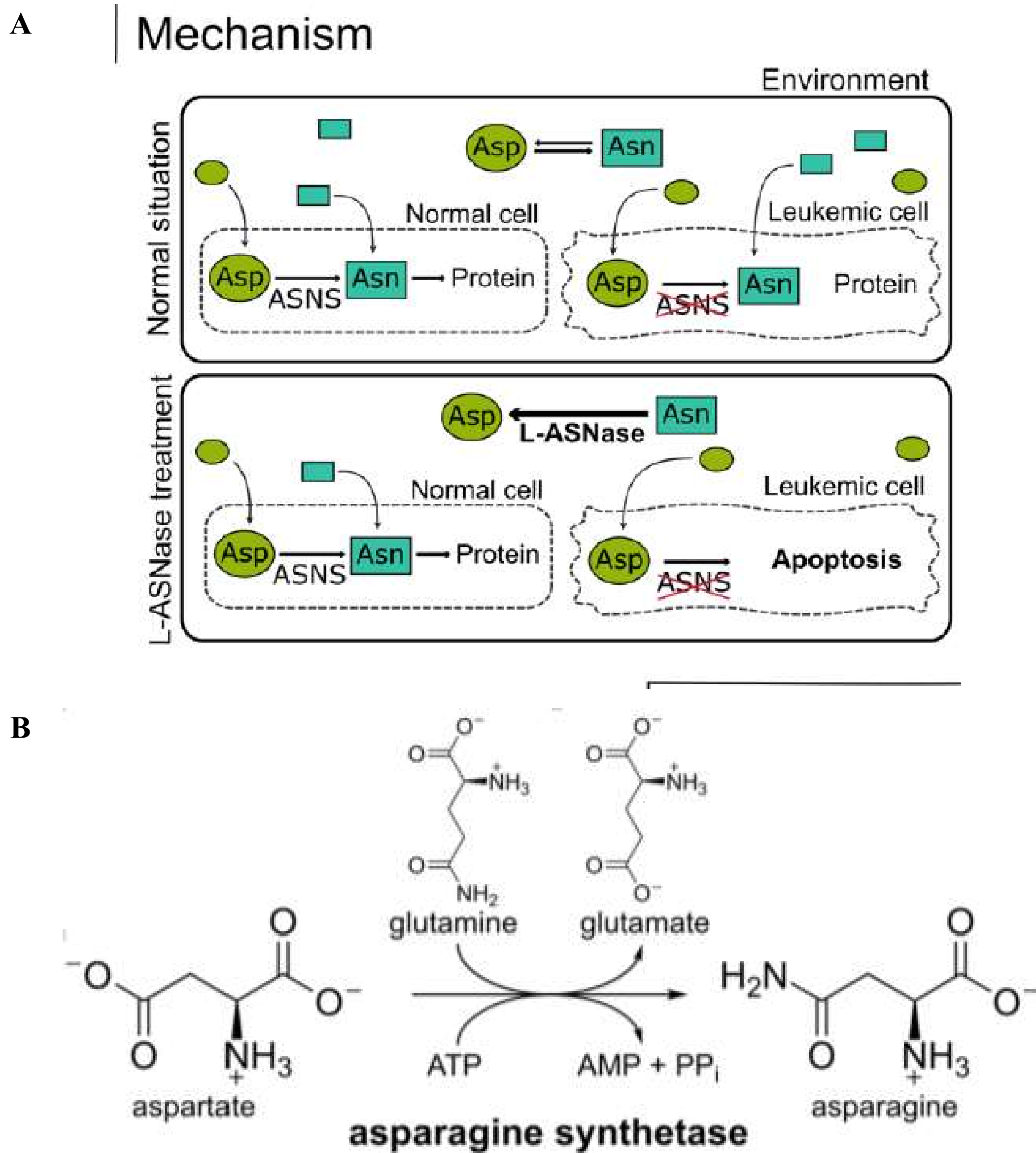

Figure 7. **A**. ASNS deficiency in leukemic cells and the way ASNase induces apoptosis. Figure from (Van Trimpont et al., 2022) and edited by the author of this dissertation. **B:** Biochemistry scheme of the synthesis of Asn from aspartate, catalyzed reaction with the enzyme ASNS (public domain from Creative Commons).

ASNase treatment exploits a deficiency in leukemic cells. Therefore, it has a minor effect on healthy cells (Van Trimpont et al., 2022). Their deficiency is the low capability of synthesizing *de novo* Asn. The gene responsible for this process is Asparagine synthetase (ASNS). While healthy cells, as mesenchymal stem cells, have sufficient gene expression

of ASNS, leukemic cells have undetectable or impaired expression (Figure 7A)(Akahane et al., 2022; Ivana Hermanova et al., 2012; Prager & Bachynsky, 1968). The ASNS enzyme uses aspartate and amino group, from degraded Gln, to synthesize Asn (, (Cooney et al., 1976)(Fig. 7B). As ASNS deficient cells, leukemic cells are dependent on extracellular Asn and its depletion triggers apoptosis activation (Boos 2004).

1.4.4 Bioenergetics metabolism of pre-B ALL after ASNase treatment

Hermanova and colleagues revealed that ASNase disturbed leukemic cell energetic metabolism. ASNase inhibits glycolysis and increases FAO and the capacity of ETC in leukemic cells. In their study, the reduced glycolytic activity was determined by measuring glucose uptake and protein levels of c-myc and glucose transporter. Due to reduced glucose uptake, leukemic cells change the preferential use of glucose for fatty acids, inducing FAO. The nutrient flow exchange leads to a slight increase in some OXPHOS parameters. FAO supplied enough electron carriers to support leukemic cell viability. When they included a FAO inhibitor (etomoxir) in ASNase treatment, the apoptosis of leukemic and primary cells (BCP-ALL patient) was increased (Hermanova et al. 2016). Results of the study showed that metabolic rewiring of leukemic cells induced by ASNase treatment could avoid apoptosis. From the pathways described, FAO upregulation has an important role in reducing the ASNase sensitivity of leukemic cells. It is not the first time FAO was reported as a rescue mechanism in cancer cells. It has been described in studies about malignant lymphoma and breast cancer (Y. J. Li et al., 2022; Sekine et al., 2022).

1.4.5 Biosynthetic pathways regulated by the mTOR signaling pathway of pre-B ALL after ASNase treatment

ASNase treatment was previously reported to inhibit the mTOR signaling pathway due to Asn and Gln depletion (Iiboshi et al., 1999). This pathway boosts cell growth and metabolism by modulating protein, lipids, and nucleotide synthesis when cells have nutrient abundance. In contrast, it regulates autophagy and lysosome biogenesis in the absence of nutrients (Saxton & Sabatini, 2017). To activate these functions, mTOR pathway needs to confirm the availability of extracellular nutrients, and it does through different signaling pathways. The key protein of these pathways is mTOR, which nucleates at least three complexes that sense nutrient availability. An example is the mTOR complex 1 (mTORC1) AA sensing pathway (Hara et al., 2002; Saxton et al., 2016; Saxton & Sabatini, 2017; Wyant et al., 2017).

In the presence of AAs, the mTOR sensing pathway activates the lysosome-resident complexes associated with Rag GTPase, which recruits and activates mTORC1 (Sancak et al., 2008; Saxton & Sabatini, 2017). The activation of this complex promotes mRNA translation (protein synthesis) by direct phosphorylation of S6K (Fig. 8)(Holz et al., 2005). The latter can activate carbamoyl-phosphate synthetase (CAD) and sterol-responsive element binding protein (SREBP). CAD induces pyrimidine synthesis, while SREBP promotes lipid and glucose synthesis (Ben-Sahra et al., 2016; Düvel et al., 2010; Robitaille et al., 2013). Furthermore, activated mTORC1 increases the expression of ATF4, which induces purine synthesis (Fig. 8) (Ben-Sahra et al., 2016; Saxton & Sabatini, 2017). In free-AA environments, lysosome-resident complexes and Rag-GTPase fail to recruit mTORC1. Therefore, it remains inactive, as well as all its downstream proteins mentioned above.

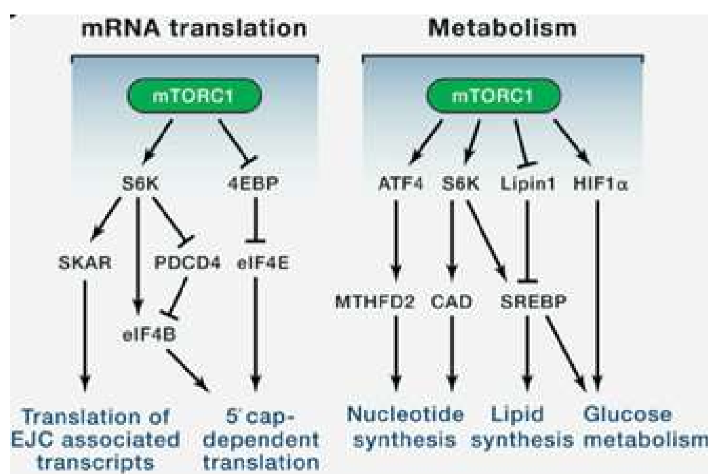

Figure 8. Downstreatm proteins of the signaling pathway of mTORC1 and their role in metabolism. Figure from Saxon & Sabatini 2017 and modified by the author of this dissertation.

While investigating the mTORC1 signaling pathway, Hermanova and colleagues demonstrated that ASNase inhibits biosynthetic pathways of leukemic cells through the RagB-mTORC1 pathway. Rags are heterodimers of RagA or RagB with RagC or RagD that have a GTPase domain which couples with the lysosome-resident complexes to active mTORC1 (Bar-Peled et al., 2012; Sancak et al., 2010). Hermanova and colleagues confirmed that ASNase inhibits pyrimidine and protein synthesis by dephosphorylating S6K and CAD in pre-B ALL cells. Using a model with activating mutation in RagB, they provide evidence that the activation of FAO required mTOR. Thus, ASNase inhibits pre-B ALL cell growth by avoiding AA-dependent mTORC1 activation. In turn, mTORC1 inactivity is sensed as metabolic stress and induces FAO upregulation. In summary, Hermanova and colleagues

identified a metabolic rewiring of leukemic cells with the potential to be responsible for treatment failure(I Hermanova et al., 2016).

1.6 Bone marrow tumor microenvironment

The tumor microenvironment (TME) was recently recognized in the latest cancer hallmark reviews to play a critical role in tumorigenesis, malignant progression and chemoresistance (Hanahan, 2022). Several studies demonstrate that some chemotherapeutic agents affect the expression of cell surface receptors that cause the migration of leukemic cells to their origin tissue (H. C. Liu et al., 2018; H. C. Liu, Gang, Kim, Abdel-Azim, et al., 2020; H. C. Liu, Gang, Kim, Ruan, et al., 2020; Wu et al., 2013). In the BM environment, non-hematopoietic cells and components of the extracellular matrix were observed to interact with the malignant cells and increase their survival both *in vitro* and *in vivo* (Iwamoto & Mihara, 2007; A. Manabe et al., 1992; Atsushi Manabe et al., 1994). The pro-leukemic BM environment is created during leukemogenesis through cell-cell and cell-matrix interactions, which modulate the conversion of this tissue to a leukemia-permissive environment (Dander et al., 2021; Ma et al., 2019; Polak et al., 2015; Vasconcellos et al., 2011). The comparison of leukemia patient biopsies with healthy controls confirmed the evidence of an existing pro-leukemic environment (Perez-Atayde et al., 1997; Pulè et al., 2002).

The leukemic BM environment has not been fully described at the moment. However, there is more information about the features of the BM in healthy conditions. Bone marrow is divided into different compartments known as niches. Particular stromal cell populations and extracellular matrix characterize each of them. The stromal cell populations in the BM are osteoblasts, osteoclasts, adipocytes, reticular cells, endothelial cells, smooth muscle cells, mesenchymal stromal cells (MSCs), and cells of the sympathetic nervous system (Dander et al., 2021; Friedenstein et al., 1987; Kassem et al., 1991; Rickard et al., 1998; Taichman et al., 1996). In healthy BM niches, stromal cells regulate HSCs by the secretion of cytokines, hormones, and growth factors, as well as the expression of receptors and adhesion molecules. The key population of stromal cells is MSCs. These cells are fibroblast-like cells with multipotent ability to differentiate into cells of the three mesodermal lineages: osteocytes, adipocytes, and chondrocytes (Dander et al., 2021).

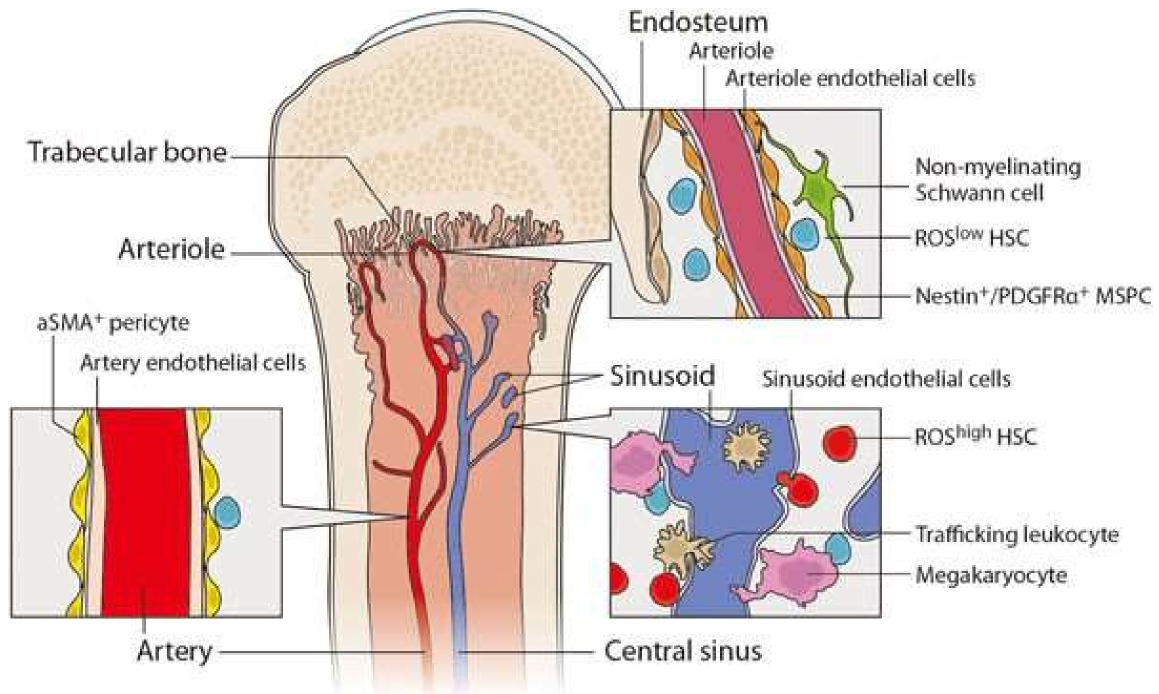

Figure 9. BM niches scheme, showing the location of these niches in the trabecular bone. Each niche has a distinct unknown stromal cell composition. Figure modified from (Zhao & Li, 2016).

There are three different niches in the BM: endosteal, arteriolar, and sinusoidal. The endosteal niche is the closest to the trabecular bone and contains only 14% of HSCs (Kiel et al., 2007)(figure 9). The key orchestrators of this niche are osteoblasts (bone matrix-forming cells), which negatively regulate HSC proliferation. The arteriolar niche promotes HSCs quiescence, and this state protects them from replicative stress (Kunisaki et al., 2013). Stromal cells, endothelial cells, sympathetic nervous system nerves, and non-myelinating Schwann cells were identified to be part of the arteriolar niche. The sinusoidal niche is more permeable than arteriolar niches and promote HSCs activation. This is the place where leukocytes leave or enter the BM. It is suspected that there is a continuous exchange between the arteriolar and sinusoidal niches. That means the HSC pool stays in an equilibrium between proliferation, trafficking, and quiescence (Dander et al., 2021).

In addition to stromal cells, the peculiar oxygen tension of the BM also plays a role in regulating HSCs status. BM oxygen concentration fluctuates between 1 to 7%, and it differs between each niche (Chow et al., 2001). The BM hypoxia signaling is a central element that regulates HSCs quiescence and metabolism. It is modulated by hypoxia-inducible factor 1α (HIF1α), which promotes the utilization of anaerobic glycolysis rather than OXPHOS in HSCs (Simsek et al., 2010). Maintaining the glycolytic state is critical to

HSC quiescence, which reduces redox damage to the cells and retains their self-renewal capacity (Y. H. Wang et al., 2014).

2. AIMS

The main goal of the dissertation thesis was to elucidate the impact of metabolic rewiring in leukemic cells in relation to therapy response, resistance development and prognosis in ALL. The utilization of current prognostic factors in patient stratification facilitates the selection of suitable therapy regimens for nearly all cases. However, some patients still do not benefit from available treatment, and there are not many options for a successful clinical intervention after emerge of resistance. We propose that resistance to conventional therapy could be reduced by understanding the complex effect of the chemotherapy components, mainly ASNase. New findings could be implemented in i) therapy modulation, ii) patient stratification and iii) prediction of individual response to chemotherapy and iv) implementation of metabolism targeted therapy.

This study was divided into four connected projects:

- Explore the pro-survival metabolic processes of leukemic cells upon ASNase treatment in the BM microenvironment.

- Study of the basal metabolic profile of leukemic cells in the cellular response to ASNase treatment.

- Investigate PTEN mutations or aberrant PI3K/Akt pathway in T-ALL in the ASNase sensitivity and glucose metabolism.

- Characterize the role of metabolic rewiring in malignant transformation driven by mutated JAK2 kinases identified in *BCR-ABL*-like ALL subgroup.

3. MATERIALS AND METHODS

A detailed description of methods and experiments performed in the individual studies compiled in this dissertation is attached within corresponding publications. In this section, the crucial methods that form the main content of the investigation study have been described.

3.1 BM model

The author of this dissertation developed and optimized the BM model, which was crucial for the findings of publication 1. The model was based on a 2D cell coculture approach using Corning® Transwell® permeable supports inserts (Merck Life Science, Darmstadt, Germany) for 6- or 24-well plates. MSCs (Hamada et al., 2005; Nierste et al., 2014; Siska et al., 2017), primary MSCs or HS-5 were seeded in the insert 1 or 2 days prior to the experiment using supplemented media, ensuring correct cell adhesion. Leukemic cells were seeded into the well at the starting point of the assay, followed by replacing the media in the insert (containing the monolayer of MSCs). Assay media or transient ASNase media was used to culture the cells. The working volumes of the BM model were determined according to the manufacturer´s instructions.

Transient ASNase media was prepared by treating RPMI 1640 medium (Thermo Fisher Scientific) with *Escherichia coli* ASNase (Medac, Wedel, Germany) for 5 hours, and then the drug was filtered out using a PierceTM Protein Concentrator PES membrane (polyethersulfone) with cut-off 5 kDa (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The medium was prepared fresh and supplemented before each experiment and checked for drug absence using an ASNase activity assay kit (Sigma–Aldrich, Schnellforf, Germany) or a cell proliferation assay.

3.2 Stable isotope tracing

Stable isotope tracing was required to develop the hypothesis (Publication 1) and confirm the results (Publication 4) of the studies of this dissertation thesis. The author of this investigation project did the optimization and experimental performance.

After culturing adherent or suspension cells with the labeled isotope of interest, the culture media and cells were collected, immediately followed by the metabolite extraction (Hollinshead et al., 2018). Adherent cells were washed twice with 1ml of 0.9% NaCl in PierceTM Water, high-performance liquid chromatography (HPLC) grade (Thermo Fisher,).

The second wash included 0.5% trypsin (Thermo Fisher) to detach the cells. Suspension and detached adherent cells were centrifuged to form cell pellets at 4ºC. Before the previous step, the cells were counted for data normalization. Cell pellets were carefully washed with a salt solution (0.9% NaCl in Pierce^{TM} Water, HPLC grade, Thermo Scientific), without disturbing the pellet. The washed pellet was immediately resuspended thoroughly with 500µl of metabolism quenching reagent (Honeywell^{TM} methanol, Thermo Fisher, pre-chilled -20ºC). Samples in methanol suspension were transferred to new vials, mixed 5:2 ratio with internal standard solution (IS, 2.5µg/µl of D-glutaric in HPLC grade water, pre-chilled 4ºC) and vortex. Subsequently, HPLC grade chloroform (Honeywell^{TM}, Thermo Fisher, pre-chilled 4ºC) was added in a 7:5 ratio, followed by sample vortex for 10 minutes at 4ºC. The sample was centrifuged at 13000rcf for 10 minutes at 4ºC to collect the upper supernatant (polar phase). As part of the sample preparation for gas chromatography-mass spectrometry (GS-MS) measurement, metabolites extracted samples were evaporated in SpeedVac SPD1030 – Integrated Vacuum Concentrator (Savant, Hyannis, Massachusetts, USA).

In the case of extracting metabolites from media, 200µl of the cultured media was mixed in 1:4 ratio with acetone: isopropanol (2:1, pre-chilled at -20ºC) and centrifuged (13000rcf, 5 min., 4ºC). 400 µl of the supernatant was transferred in a new vial and mixed in a ratio 4:2:3:5:5 with IS solution, water, methanol and chloroform. From this step, samples were processed as the extraction protocol for cells.

Samples were manipulated in ice for the whole metabolite extraction process and stored at -80ºC till their measurement by GS-MS. Details on polar metabolite derivatization and data acquisition can be found in Kľučková and colleagues (Kľučková et al., 2020). Data analysis and interpretation were performed by the author of this dissertation.

3.3 Metabolism analysis using Seahorse analyzer

Metabolism analysis in these studies was required to assess glycolysis and mitochondrial respiration parameters in leukemic cells (publications 2 and 3), and metabolic phenotype identification (publication 4). The author of this dissertation performed the metabolism analysis test in primary cells from ALL patients (publication 2). Moreover, the optimization and performance of the Cell Energy Phenotype Test in transfected BMC c-kit cells were another of the author’s tasks (publication 4).

Seahorse Analyzer XFp (Agilent Technologies, Inc., CA, USA) measures the extracellular cellular acidification rate (ECAR) and oxygen consumption rate (OCR) of cells in real time. ECAR represents glycolysis, and the glycolysis stress test combines the

measurement of these parameters in basal and stress conditions. OCR represents mitochondrial respiration flux, and its function is assessed similarly to glycolytic flux. Metabolic phenotype test simultaneously measures both ECAR and OCR, and ECAR/OCR ratio identifies which pathway the cells use the most.

For the Glycolysis stress test, cells were seeded in Agilent XF Roswell Park Memorial Institute Medium (RPMI) base medium, pH 7.4, and for the Cell mito stress test, cells were seeded in XF Assay medium (10 mM glucose, 1 mM 4-(2-hydrozyethyl)-1-piperazineethanesulfonic acid (HEPES), 2mM Gln, 1 mM pyruvate, 0.1% Bovine serum albumin (BSA) and pH 7.4). The cells were plated in XFp tissue culture plates coated with CellTak (Corning GmbH, Wiesbaden, Germany), according to the Agilent Seahorse protocol for seeding suspension cells. During the glycolysis stress test, cells were treated with 10 mM glucose, 2 µM of oligomycin A and 100 mM 2-deoxy glucose (2-DG), one by one and assessing ECAR after each injection. The injection drugs in cell mito stress test were 2 µM oligomycin A, 3 and 4.5 µM of carbonylcyanide p-trifluoromethoxyphenylhydrazone (FCCP, two injections) and 1 µM rotenone combined with 1 µg/ml antimycin A.

Cell density and FCCP concentration were optimized for the Cell Energy Phenotype Test prior to the first measurement according to Agilent Seahorse protocols. Cells were seeded in coated tissue culture plates using Agilent XF RPMI Assay medium (pH 7.4, 10mM glucose, 2mM Gln, 1mM pyruvate). The inhibitors established by the protocol were injected in a unique injection (2µM oligomycin and 2µM FCCP). Data analysis and interpretation were performed by the author of this dissertation, following the manufacturer’s instructions.

4. RESULTS

4.1 Explore the pro-survival metabolic processes of leukemic cells upon ASNase treatment in the BM microenvironment

i) Alquezar-Artieda N, Kuzilkova D, Roberts J, Hlozkova K, Pecinova A et al. Restored biosynthetic pathways induced by MSCs serve as rescue mechanism in leukemia cells after L-asparaginase therapy. Blood adv. 2023; 10:2228

Author's contribution: As the first author of this study, I planned, optimized, and executed all the experiments. Moreover, I performed statistical data analysis and actively participated in manuscript writing.

This work was a continuation of the study of Hermanova et al., which demonstrated that ASNase treatment disturbs the cellular metabolism of pre-B leukemic cells. The metabolic disturbance was described as a shift in the energy metabolism, and an inhibition in the biosynthetic pathways of leukemic cells. The latter corresponded explicitly to protein and pyrimidine synthesis, which are regulated by mTOR. This intracellular mechanism of action of ASNase was reported in a conventional *in vitro* model, which does not consider one of the hallmarks of cancers: the tumor microenvironment (Hanahan, 2022). Therefore, in this study, we expanded our *in vitro* model to mimic certain aspects of the BM environment. Our aim in using it was to confirm mentioned changes in the cellular metabolism of leukemic cells and reveal new mechanisms with the potential to impair ASNase efficacy.

The included features of the BM environment model were: the BM matrix, hypoxic environment and the transient presence of ASNase simulating the drug's half-life *in vivo*. Some of them are known to induce cancer cell proliferation (Ede et al., 2018; Ehsanipour et al., 2013; Ma et al., 2019; Pillozzi et al., 2011; Polak et al., 2015). Using transwell inserts, we mimicked the BM matrix by co-culturing the leukemic cell lines (REH, NALM-6, RS4:11 and SUP-B15) with MSCs. The cultures were exposed to hypoxic environment conditions and compared to normoxia. The half-life of ASNase was achieved by transient ASNase treatment. The latter was prepared by applying the ASNase treatment in RPMI medium and drug subsequent withdrawal to exclude the constant depletion of Asn and Gln in the BM model.

Indeed, the BM environment model increased pre-B leukemic cell survival after ASNase treatment compared to the conventional *in vitro* model. The increased survival was not higher when applying low levels of oxygen. As the hypoxia condition did not have any benefit, we excluded it from the BM environment model and used only the normoxic conditions. The metabolic disturbance of leukemic cells observed in the original *in vitro* model after ASNase treatment persisted in the presence of MSCs. However, inhibition of protein and pyrimidine synthesis was significantly less profound when leukemic cells were cultured with MSCs. Protein and pyrimidine synthesis pathways were evaluated by their markers: the phosphorylated forms of S6 and CAD, which are downstream proteins of mTORC1/S6K1. The results suggested that the partial restoration of those pathways might cause increased leukemic cell survival in the BM model. We confirmed that phosphorylated S6 (p-S6) was also partially restored in primary leukemic cells when co-cultured with MSCs and treated with ASNase. Primary leukemic cells were isolated from pediatric patients with BCP-ALL. Given the known role of Asn in activating mTOR, we hypothesized that MSCs might release Asn into the extracellular environment and reactivate the mTOR signaling pathway of leukemic cells after the treatment (Meng et al., 2020). Noteworthy, healthy cells, including MSCs, should be able to synthesize Asn, while leukemic cells are not capable of that. As a consequence, this deficiency makes them particularly sensitive to ASNase treatment.

To pursue the assumption, we used stable isotope tracing to evaluate the Asn flux coming from MSCs following a three-step workflow. First, we applied Asn starvation to MSCs and supplemented them with labeled Gln (15N-Gln) for 7 days (Figure 10A). That way, we forced MSCs to *de novo* Asn synthesis using 15N-Gln and produce 15N-Asn (Figure 10B). Second, MSCs containing 15N-Asn were cultured in medium simulating ASNase treatment (without Asn and a limited concentration of Gln, Figure 10A)(Chiu et al., 2021). After 48 hours, we observed that Asn was present in the culture medium of MSCs, and from it 11.67%±0.57 was labeled. Third, independently of the first and second steps, we cultured leukemic cells in the presence and absence of MSCs with labeled Asn in the culture media (Figure 10C). NALM-6 in both cultures had 94% of intracellular Asn labeled after 24 hours. The first and second steps confirmed that in Asn-free culture conditions MSCs synthetized and released Asn, while the third step demonstrated that leukemic cells take up any available Asn.

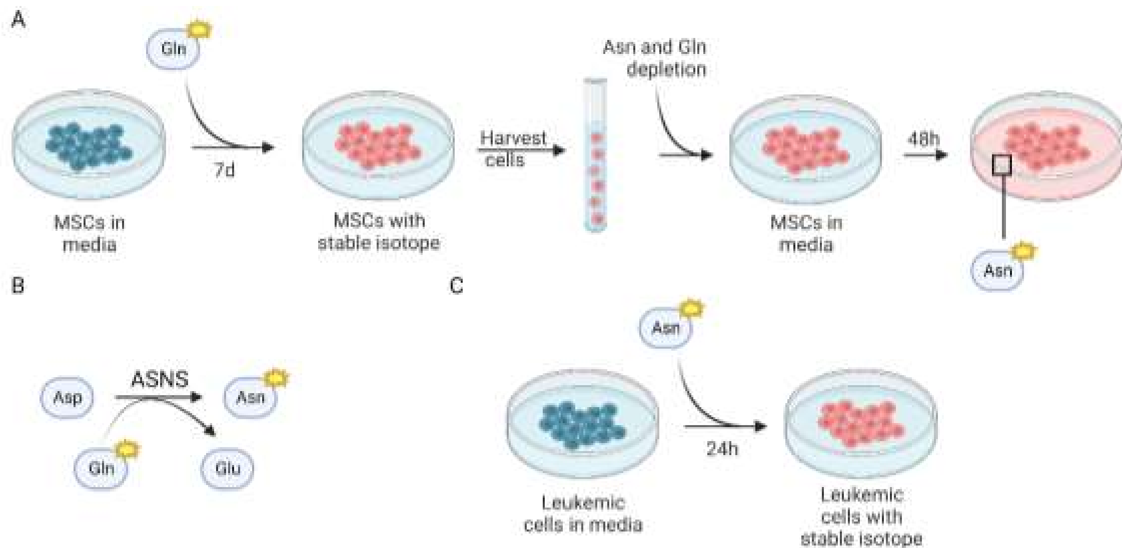

Figure 10. Stable isotope tracing workflow

To validate that MSCs restored p-S6 and p-CAD protein levels of treated leukemic cells by releasing Asn, we supplemented leukemic cells undergoing ASNase treatment with increasing concentrations of Asn. Then, we performed an 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium(MTS) assay and western blot to quantify p-S6 and p-CAD. Asn supplementation rescued leukemic cell proliferation and restored the mTOR signaling pathway. By using a co-treatment of mTOR inhibitor (rapamycin) and ASNase, the increased survival induced by MSCs was diminished. Results confirmed the key role of protein and nucleotide synthesis in the rescue mechanism using the BM microenvironment. The fact that MSCs replenished Asn in the BM environment model was also reflected in the stress levels of leukemic cells. Since AAs depletion was not complete in the co-culture, the markers of amino acid stress response (CHOP, SLC38A2 and Asn transporters) of leukemic cells were not induced as detected in the monoculture. Moreover, the indiscriminate amino acid uptake caused by ASNase in the traditional *in vitro* model was also not induced in the co-culture.

4.2 Study of the basal metabolic profile of leukemic cells in the cellular response to ASNase treatment

ii) Hlozkova K, Pecinova A, Alquezar-Artieda N, et al. Metabolic profile of leukemia cells influences treatment efficacy of l-asparaginase. BMC Cancer. 2020;20:526

Author's contribution: In this study, I have analyzed all primary samples from newly diagnosed leukemia patients. After receiving the sample from the diagnostic department, I optimized *ex vivo* culture conditions for leukemic primary cells. Moreover, primary leukemic cells were frozen and collected in pellets as a reservoir for future experiments. I measured the cells' glycolysis and mitochondrial respiration activity on the Seahorse analyzer. Moreover, I seeded the primary leukemic cells for MTS assay, by which the ASNase sensitivity was determined.

In this project, we explored the metabolic predisposition of leukemic cells of different hematopoietic lineage and its relation to therapy response.

Using a Seahorse analyzer and radioactivity assays, we initiated the study by characterization of the basal metabolic state of 19 human leukemia cell lines. From the selected cell lines, 8 were BCP-ALL (ALL derived from B-lymphoid lineage; 1 BCP-ALL originated from a CML blast crisis), 4 were T-ALL (ALL derived from T-lymphoid lineage) and 7 were of myeloid origin (5 AML; 2 CML blast crisis manifesting as AML). The first method measures glycolysis, mitochondrial function and metabolic phenotype of our cells through different types of tests. Metabolic phenotype is determined by ECAR/OCR ratio. The radioactive assay was used to determine fatty acid oxidation based on the measurement of the oxidation of [^{3}H]-palmitate. All the information about the metabolic processes of each cell line was applied to a hierarchical clustering analysis (HCA) that grouped cell lines based on their similar characteristics. The analysis based on glycolysis and mitochondrial function activity grouped the cell lines sharing the same hematopoietic lineage in two differentiated clusters. BCP-ALL cell lines were found in the first cluster with low glycolytic and cell respiration activity. The second group contained myeloid leukemia and T-ALL cell lines with high glycolytic and cell respiration values. The separation had some exceptions. HPB-ALL, a T-ALL cell line, clustered with BCP-ALL, while Nalm-6 and BV-173 (BCP-ALL cell lines) fell with the AML/T-ALL cell line group. Fatty acid oxidation activity was

different between the cell lines sharing the same hematopoietic lineage origin and did not cluster the patients as the other metabolic pathways.

Then, we evaluated the major signaling pathways of 15 leukemic cell lines (6 BCP-ALL, 4 T-ALL and 5 AML) by quantifying their marker's protein level (western blot). BCP-ALLs, which mainly formed the first cluster, showed lower levels of phosphorylated Akt (p-Akt) than T-ALL, decreased levels of phosphorylated glycogen synthase kinase-3 beta (GSK-3 beta) and S6 compared to AML, and a different expression of c-myc compared to T-ALL. GSK-3B is a negative regulator of glycogen synthesis, and S6 is a downstream protein of the PI3K/Akt/mTOR pathway. Moreover, there was no difference in p-AMPK, a modulator of cellular energy homeostasis.

In order to demonstrate whether a specific metabolic profile would interfere with ASNase treatment, we correlated the parameters of metabolic pathways of analyzed leukemic cells with their sensitivity to ASNase using Canonical correlation analysis. The latter evaluates the relationship between 2 multivariate sets of variables. First, we assessed the sensitivity to the cytostatic drug by MTS assay. Myeloid cell lines were less sensitive to ASNase, followed by T-ALLs and identified BCP-ALLs as the most sensitive ones. The results showed a correlation between higher sensitivity to ASNase and: (1) lower basal respiration, (2) higher ATP-linked respiration and (3) higher ECAR/OCR ratio. High ATP-linked respiration was functionally validated through its inhibitor, oligomycin A. We pretreated leukemic cells (2 B-ALLs, 2 T-ALLs and 1 AMLs) with oligomycin A for 1 hour before treating them with ASNase, and observed that the ASNase sensitivity was decreased using the combined treatment. Inhibition of mitochondrial respiration using antimycin A (ETC complex III inhibitor) did not affect the ASNase sensitivity of tested cell lines.

Given that oligomycin A treatment was reported to increase mitochondrial membrane potential (MMP)(Perry et al., 2011), we additionally measured it in 10 human leukemic cells (3 BCP-ALL, 4 T-ALL and 3 B-ALL). The MMP was determined using flow cytometry after staining the cells with tetramethylrhodamine ethyl ester perchlorate (TMRE) fluorescence. In our measured cell lines, the TMRE fluorescence correlated with ASNase response, meaning that leukemic cells with higher TMRE fluorescence had lower sensitivity to ASNase. TMRE is usually proportional to MMP and mitochondrial mass. The latter did not correlate with TMRE fluorescent changes in our measured cell lines. Therefore, the changes in TMRE fluorescence were given by changes in the MMP and not mitochondrial mass (measured through mtDNA). In sum, higher MMP was associated with lower sensitivity to ASNase.

To confirm our results about the metabolic profile of leukemic cells, we next measured the glycolytic and mitochondrial function, as well as sensitivity to ASNase in primary cells. Particularly, we included primary cells isolated from 26 childhood leukemia patients (15 BCP-ALL, 7 T-ALL, 4 AML, 1 T/Myelo) and 2 healthy controls (B-ALL, mononuclear cells). In contrast to the cluster analysis of cell lines, primary leukemic cells only showed slight separation by their leukemia lineage when applying glycolytic parameters. The cells were separated into two clusters. Specifically, AMLs, the T/Myelo patient and healthy controls were grouped in the same cluster, which had increased parameters of glycolysis. However, B-ALLs and T-ALLs were heterogeneously divided between the two clusters, showing both high and low glycolytic parameters. Importantly, the cluster with low glycolytic parameters showed the BCP-ALLs and T-ALLs with high sensitivity to ASNase treatment.

4.3 Investigate PTEN mutations or aberrant PI3K/Akt pathway in T-ALL in the ASNase sensitivity and glucose metabolism

iii) Hlozkova K, Hermanova I, Safrhansova L, <u>Alquezar-Artieda N,</u> et al. PTEN/PI3K/Akt pathway alters sensitivity of T-cell acute lymphoblastic leukemia to l-asparaginase. Sci. Rep. 2022;12(1):.

<u>Author's contribution</u>: As co-author of this study, I was responsible for subculturing T-ALL cell lines and analyzed changes on the ASNase sensitivity after Akt inhibition using MTS assay. In addition, I collected the primary leukemic cells from pediatric patients with T-ALL to evaluated p-Akt expression by mass cytometer (CyTOF), and the cytotoxicity of the co-treatment of ASNase with p-Akt inhibitor.

This study was elaborated on the results obtained by Hlozkova and colleagues (publication 3), summarized in Section 4.2. The results showed that T-ALL cell lines and primary cells from T-ALL patients clustered into both high and low glycolytic activity groups, accordingly to their ASNase sensitivities. Moreover, T-ALL showed a generally high expression of p-Akt. P-Akt is negatively regulated by PTEN, which is mutated in approximately 20% of all pediatric T-ALL cases (Jenkinson et al., 2016; Szarzyńska-Zawadzka et al., 2019). PTEN abnormalities are usually present in T-ALL cases with

unfavorable long-term outcomes (Bandapalli et al., 2013; Paganin et al., 2018; Tesio et al., 2017). Therefore, this study aims to elucidate the connection between PTEN deletion, ASNase sensitivity and glucose metabolism.

We first evaluated five human T-ALL cell lines with respect to their ASNase sensitivity and glycolytic function. CCRF-CEM, JURKAT and MOLT-4 were more resistant to ASNase and showed higher glycolytic function than ALL-SIL and DND-41, which were sensitive to the drug. Their protein expression assessed by western blot showed that the ASNase-resistant group had higher Akt activation due to the lack of PTEN protein expression. In contrast, sensitive T-ALL cell lines did not have Akt activation because they expressed PTEN. Moreover, the latter group expressed truncated cleaved-NOTCH1 (activated form of NOTCH1), while resistant cell lines had their wild-type protein. However, NOTCH1 expression did not reflect any pattern between the resistant and sensitive groups of cells. NOTCH1 was tested because its signaling depends on the PTEN/Akt pathway (Mendes et al., 2016). Despite these differences in Akt activation, the phosphorylation of its downstream proteins, such as p-S6 and p-GSK-3β, did not differ in connection with the ASNase sensitivity. Neither was observed in c-myc expression, the activator of glycolysis (Pavlova et al., 2022). These results made us hypothesize that hyperactivation of Akt makes T-ALL cell lines more resistant to ASNase or that truncated cleaved NOTCH1 increased the sensitivity of PTEN-positive T-ALL to the drug. A truncated form of cleaved-NOTCH1 was already reported in analyzed cells, which was caused by a premature STOP codon that prolonged the half-life of cleaved-NOTCH1 compared to wild-type (Arruga et al., 2014; O'Neil et al., 2006). Previous studies hypothesized that cells with a longer half-life of cleaved-NOTCH1 were dependent on extended active NOTCH1 signaling (Y. Wang et al., 2019).

To elucidate our hypothesis, we evaluated the effect of p-Akt and cleaved-NOTCH1 inhibitors on the ASNase sensitivity of our studied cell lines using the MTS assay. GSK690693 and Ipatasertib were the p-Akt inhibitors used, and γ-secretase inhibitor compound E targeted the cleaved NOTCH1. ASNase sensitivity was significantly increased when PTEN-null T-ALL cell lines were treated with p-Akt inhibitors. By contrast, it remained similar in PTEN-positive T-ALL cell lines, with and without p-Akt inhibitors. The inhibition of the cleaved NOTCH1 did not change the sensitivity to ASNase in studied T-ALL cell lines.

Then, we induced the expression of PTEN in JURKAT, a PTEN-null cell line, to assess the effect of its overexpression on ASNase sensitivity and glucose metabolism. The

overexpression of PTEN was performed using an established PTEN-inducible plasmid and stable expression using the pHIV-EGFP plasmid (Xu, Z., et al. 2002). Both overexpression systems showed that PTEN expression sensitized JURKAT cells to ASNase and reduced their glycolysis activity. However, inhibition of Akt was only observed in the inducible model. To confirm it in the stable PTEN expression system, we checked the downstream proteins of p-Akt: p-S6 and p-GSK-3B. Indeed, those proteins were inhibited in JURKAT cells with stable expression of PTEN, which demonstrated that p-Akt was inhibited. Taken together, we confirmed that PTEN deletion in T-ALL cell lines modulates increased glycolysis through hyperactivation of Akt, resulting in a metabolic status more resistant to ASNase treatment.

Finally, we corroborate our results in primary leukemic cells isolated from 9 pediatric patients with T-ALL before starting their treatment. Using western blot, we assessed their protein expression of PTEN and p-Akt. Some of these results were confirmed by RNA seq. Of the nine patients, 2 showed PTEN deletion and hyperactivation of Akt as in our ASNase-resistant cell line model, 2 patients with PTEN expression and p-Akt inhibition as in our ASNase-sensitive cell line model, and 2 patients with hyperactivation of Akt and also PTEN expression. These results suggested that some patients could have similar gene expression regarding PTEN and p-Akt as our cell lines, and benefit from p-Akt inhibitor treatment. Two new patients without PTEN/p-Akt information and two analyzed patients with hyperactivation of Akt were treated with ASNase in combination with a p-Akt inhibitor or monotherapy. One of the selected patients with p-Akt also had PTEN expression. After that, cells were stained using Cisplatin and measured by mass cytometry. Cisplatin staining labels non-viable cells. The combined treatment increased the number of non-viable cells compared to ASNase monotherapy. Taken together, we confirmed that T-ALL patients with activated Akt pathways could benefit from p-Akt inhibitors and improve their outcomes.

4.4 Characterize the role of metabolic rewiring in malignant transformation driven by mutated JAK2 kinases identified in *BCR-ABL*-like ALL subgroup

iiii) Lukes J, Potuckova E, Alquezar-Artieda N, et al. Chimeric JAK2 kinases trigger non-uniform changes of cellular metabolism in BCR-ALB1-like childhood ALL. (Under review in HemaSphere).

Author's contribution: I performed stable isotope tracing with labeled glucose in transformed Ba/F3 cells, with the goal to validate our observations about the effect of studied genetic translocations in metabolism. Moreover, I developed an additional overexpression models to replicate the results in the Ba/F3 cells. To achieve that, I cloned the aberrant *NPAT/JAK2* sequence into the pWCC19 vector. Moreover, I prepared a lentiviral system to overexpress *NPAT/JAK2* and *PAX5/JAK2* fusion proteins in murine c-kit BMCs (c-kit-positive bone marrow cells). After transduction, I measured their metabolic phenotype using a Seahorse analyzer.

In this study, we wanted to elucidate the role of two Janus Kinase 2 (JAK2) translocations in the metabolic reprogramming occurring during the malignant transformation. Our assumption is that some cancer-driving mutations might be essential in altering cellular metabolism, which would help in developing new therapeutic strategies based on cancer's vulnerabilities. Patients with *JAK2* fusion genes from *BCR-AB1-like* ALL subtype has a poor prognosis (Roberts et al., 2014). This subtype of leukemia has a gene expression profile similar to BCR-ABL1-positive ALL, but does not carry the BCR-ABL1 fusion gene. Most of the leukemias that fall into BCR-ABL-like ALL clusters have aberrant activation of different kinases (ABL, JAK2, etc.). The most common JAK2 mutation in this subgroup is the translocation of *PAX5/JAK2*. Recently, we have identified another translocation, *NPAT/JAK2* (Lühmann et al., 2021). Our aim was to describe NPAT/JAK2 fusion protein in detail, its oncogenic potential, and study the metabolic setting of cells carrying the *JAK2* translocations.

The *NPAT/JAK2* sequence was first transduced into HEK293T cells to confirm the encoded protein. The *NPAT/JAK2* encodes an aberrant protein with an intact JAK2 kinase domain and has a size of 74kDa. The chimeric protein was detected in both the cytoplasm and nucleus, and was phosphorylated on tyrosine Y1007/Y1008 of the wild-type JAK2 structure. When an inactivating mutation in the ATP-binding site of the *JAK2* domain was introduced, the *NPAT/JAK2* failed to be activated. Thus, the chimeric protein was aberrantly phosphorylated because of its mutated *JAK2* domain, which means that its ATP-binding site and catalytic function were preserved.

To study the impact of phosphorylated chimeric protein on the JAK/STAT signaling pathway, and its oncogenic potential, we transduced *NPAT/JAK2* and *PAX5/JAK2* in IL-3-dependent Ba/F3 murine hematopoietic cell line, used for Ba/F3 transformation assay (Kong et al., 2017). *PAX5/JAK2* translocation was already shown to have oncogenic potential and

we used it in our project as a representative of most frequent *JAK2* aberration in ALL (Jurado et al., 2022). IL-3-dependent Ba/F3 cell model identifies tumor-driving mutations. If the transduced cells show pro-survival and proliferation capacity after IL-3 withdrawal, cells carry tumor-driving mutation. Indeed, transformed cells with NPAT/JAK2 and PAX5/JAK2 fusion proteins became independent of IL-3, showing their oncogenic potential. Of note, cells with NPAT/JAK2 fusion protein showed faster IL-3-independent growth and transformation than those with PAX5/JAK2. Using western blot, we observed that both transformed cell lines showed increased phosphorylation of STAT1, STAT3 and STAT5. However, STAT3 phosphorylation was lower in PAX5/JAK2 transformed cells. Furthermore, STAT phosphorylation and proliferation were decreased after treating the cells with the JAK2 inhibitor, ruxolitinib. These results indicate that the *NPAT/JAK2* had a stronger oncogenic potential than *PAX5/JAK2*, and its chimeric protein successfully activated the JAK/STAT signaling pathway.

The metabolic phenotype of transformed cells with two *JAK2* translocations was assessed using the Seahorse analyzer, specifically the Cell Energy Phenotype Test. Compared to the phenotype of the parental cell lines, the *NPAT/JAK2*-transformed cells became more energetic, while the *PAX5/JAK2*-transformed cells were more quiescent. A shift to energetic phenotype means that cells use more glycolysis and mitochondrial respiration, and a quiescent shift is in the opposite direction, meaning less activity of both processes. Stress conditions applied in both transformed cell lines increased their glycolytic and mitochondrial function. However, the stressed metabolic phenotype of the *PAX5/JAK2*-transformed cells did not reach the non-stressed parental cell line phenotype. These results suggested that *NPAT/JAK2* and *PAX5/JAK2* transforming capacity depends on tumor-metabolic rewiring.

Then, we evaluated the mentioned dependencies by measuring cell proliferation when treated with the glycolysis inhibitor, 2-deoxy-D-glucose (2-DG). The proliferation of *NPAT/JAK2*-transformed cells was drastically inhibited by 2-DG, while PAX/JAK2-transformed cells were less affected by the inhibitor. When we used stable labeled glucose in the culture of transformed cells, we observed that *NPAT/JAK2*-transformed cells oxidized more glucose than *PAX5/JAK2*-transformed cells. Regardless of these differences, both transformed cell lines showed increased ATP production and NAD/NADH ratio compared to parental cell lines.

Even though the Ba/F3 transformation assay is a well-established method, we wanted to confirm the metabolic rewiring induced by the two JAK2 translocations in another model. Therefore, we transduced them in murine c-kit bone marrow cells (c-kit BMCs). New model confirmed that *NPAT/JAK2* transforming capacity increased glycolysis and mitochondrial function, while *PAX5/JAK2* decreased these processes.

5. DISCUSSION

Treating pediatric ALL becomes challenging once the patients relapse (15-20%). In such a case, their treatment options and prognosis drastically deteriorate. The secondary therapy for relapsed patients is a re-induction treatment consisting mostly of the same chemotherapeutic agents from the primary treatment and HCT (Hunger & Raetz, 2020). However, leukemic cells isolated from early relapsed patients carry mutations that reduce their sensitivity to front-line chemotherapeutic agents (Meyer et al., 2013; Peters et al., 2015). Moreover, not all relapse patients are good candidates for hematopoietic stem cell transplantation since it requires low or null MRD levels (Kuhlen et al., 2018).

All these facts cause a disappointing overall survival rate after secondary treatment, which accounts for 20-50% (Q. N. Nguyen et al., 2015; Oskarsson et al., 2016). Therefore, the best chance to further enhance the overall survival of ALL is the improvement of the primary therapy before chemoresistance development. Ideally, a successful primary therapy will eradicate the resistant cell population already present at diagnosis that causes the treatment failure. In this dissertation, we propose two significant approaches building on the cellular metabolism of leukemic cells that might improve the outcome of the patients. The first approach would be the combination of the current chemotherapy with therapeutic drugs targeting salvage metabolic events in leukemic cells after ASNase treatment. The second approach would be using the metabolic state of leukemic cells as a prognostic factor for possible secondary classification of non-responsive patients. Ultimately, we aim to propose precise tailoring of the treatment.

Changes in the cellular metabolism of leukemic cells are implicated in resistant development to particular chemotherapeutic drugs. Such a phenomenon is called metabolic rewiring, which is a metabolic adaptation that promotes the survival of cells. For instance, enhanced glycolysis was observed in some subtypes of T-ALL that were resistant to Daunorubicin (Stäubert et al., 2015). As well as upregulated glycolysis counteracted tyrosine kinases inhibitors (imatinib) in chronic myelogenous leukemia. Furthermore, OXPHOS became essential to acquire resistance to imatinib and cytarabine in chronic myeloid leukemia stem cells and acute myeloid leukemia, respectively. Another metabolic pathway, FAO was reported to rescue ALL of ASNase cytotoxicity (I Hermanova et al., 2016). Cytarabine, daunorubicin and ASNase are chemotherapeutic drugs in the conventional ALL treatment. Metabolism is clearly implicated in the long-term survival of leukemic cells. Evaluation of the metabolism at the moment of diagnosis and after relapse will promote the development of new strategies for non-responsive ALL patients. One example is metformin,

an inhibitor of OXPHOS, that demonstrates a large apoptotic effect on leukemic cells in ALL, chronic lymphoblastic leukemia and leukemia-initiating cell population (Bruno et al., 2015; Grimaldi et al., 2012; Leclerc et al., 2013; Rodríguez-Lirio et al., 2015)

5.1 Novel therapeutic targets raised from the understanding of ASNase driven resistant mechanism leading to relapsed leukemia

The improvement of the primary treatment cannot be achieved only from the dosage changes of the conventional chemotherapeutic agents. To date, the increased overall survival rate of ALL treatment was mainly due to dose adjustment based on the risk of relapse. The latter is determined by the prognosis factors, which stratify the patients into risk groups after the initial part of the treatment, called induction phase. The high-risk patients which have increased a probability of treatment failure receive more intensive therapy. Noteworthy, the current treatment cannot be further intensified. The cytostatic drugs' intensity and dosage in the high-risk regimen have already reached the maximum threshold of toxicity. Experts cautioned that escalating the dosage of chemotherapeutic agents could result in intolerable adverse side effects with limited benefits (Inaba & Pui, 2021). Therefore, the new strategy is to combine conventional chemotherapy with small molecules targeting the pathological mechanisms observed in leukemic cells (Diaz-Flores et al., 2019; Khaw et al., 2016; Ni Chonghaile et al., 2014; Peirs et al., 2014; Pullarkat et al., 2021; Scherr et al., 2014; Tanasi et al., 2019).

The assumption is that relapse in ALL is caused by the presence of single or multiple chemoresistant clones. Due to the chemotherapy pressure, the clone is selected and becomes dominant. Relapse clones can derive from major or minor subpopulations existing already at diagnosis, derived from initiation pre-leukemic cells or chemoresistance acquisition. Moreover, the survival of resistant clones can be favored by attenuating the dosage intensity of the chemotherapy for patients suffering from adverse side effects. One of the strategies to decrease the survival of chemoresistant clones is to therapeutically target the resistant mechanism by which cells escape from the toxicity of the chemotherapeutic drugs. Thus, combinations of front-line cytostatic drugs with inhibitors of drug-resistant mechanisms are an active area of investigation (Ramos et al., 2021).

Among the conventional chemotherapeutic drugs, improving the efficacy of ASNase treatment is more promising than other drugs. ASNase cytotoxicity has a selective effect on leukemic cells. Hence, it does not have excessive long-term side effects that will impair the quality of survivors‘life ASNase is a non-invasive agent since it depletes essential metabolic

nutrients (Asn and Gln) in an extracellular manner. Moreover, ASNase treatment is quite potent as a monotherapy. It can achieve an inhibition of approximately 60% of leukemia growth (Haskell, 1969; Jaffe et al., 1971; Tallal et al., 1970)

It has already been described that B-ALL cells can adapt their metabolic phenotype to counteract ASNase treatment (I Hermanova et al., 2016). Leukemic cells decreased glycolysis and increased FAO. Since electron carriers are compensated with FAO activation, OXPHOS function remained similar upon ASNase treatment. Using an FAO inhibitor, Hermanova et al demonstrated that leukemic cells could survive ASNase treatment by depending on FAO. In order to further validate the potential of FAO as a therapeutic target, it was necessary to measure resistant metabolic phenotype within the BM niche. Accordingly, ALL patients do relapse the most at this site, accounting for 38.5% (Tuong et al., 2020).

BM environment becomes leukemia-permissive after malignant transformation. The non-hematopoietic cells in this site were demonstrated to be implicated in chemoresistance development. In particular, MSCs are the cell type with more studies proving its association with resistant mechanism (Atsushi Manabe et al., 1994). The MSCs contact helped to induce vincristine resistance in B-ALL by the mutual activation of nuclear factor kappa B (Jacamo et al., 2014). The resistance to cytarabine, tyrosine kinase inhibitors and corticoids in ALL were modulated by the activation of signaling pathways induced by BM-MSCs. The first two types of chemoresistance were caused by abnormal Wnt signaling/B catenin, while corticoids resistance was linked with notch signaling (K. Hu et al., 2015; Kamdje et al., 2011; X. Liu et al., 2013; Mudry et al., 2000). Moreover, MSCs transferred their healthy mitochondria to ALL blasts that replaced the damaged organelles by the redox stress. The mitochondrial replacement was involved with daunomycin and cytarabine resistance (Burt et al., 2019; Polak et al., 2015; Usmani et al., 2019)

In the case of ASNase chemoresistance, MSCs were reported to increase the ASNS expression and supply Asn to ALL (Dimitriou et al., 2014; Iwamoto & Mihara, 2007). Herein, we demonstrated that mentioned Asn restored biosynthetic pathways regulated by mTORC1/S6K1 in pre-B ALL undergoing ASNase treatment (Publication 1). Asn was already reported to be one of the amino acids regulating the mTOR pathway (Meng et al., 2020). As a result of mTOR restoration, leukemic cell survival was sustained by BM model, and the intrinsic metabolic rewiring of the blasts effectively countered ASNase cytotoxicity (I Hermanova et al., 2016). Indeed, MSCs compensated for depleted Asn, which otherwise effectively induced apoptosis in leukemic cells. A similar reduction of efficient cytotoxicity

of the drugs is found in cancer with upregulated ABC transporters, which efflux the therapeutic drug from intracellular space. As a consequence, the therapeutic drug does not reach optimal concentration, and cancers becomes resistant (Kadioglu et al., 2020).

The described mechanism concerning ASNase did not require cell-to-cell contact, unlike other resistant mechanisms induced by MSCs using N-cadherins adhere binding (Nygren et al., 2009). Therefore, we measured the released Asn from MSCs in monoculture. By stable isotope tracing, we confirmed that Asn was released from MSCs to the extracellular medium and taken up by leukemic cells treated with ASNase (Publication 1). Showing the labeled Asn transfer between MSCs and the leukemic cells in a unique experiment was challenging due to isotope tracing limitations. The transferred amount of Asn is expected to be very low and immediately used by the leukemic cells for the biosynthetic pathway. In concordance, we observed that 3μM of Asn was sufficient to increase the survival of treated leukemic cells upon ASNase treatment. Thus, the transferred Asn is beyond the detection level of the method (Publication 1).

Noteworthy, the advantage of the BM model in this study was the simulation of the half-life of ASNase *in vivo*, which is approximately a day and a half. After this period, it is not ensured that Asn and Gln are still completely depleted. These conditions were mimicked in the BM model by a pre-treated media. This was prepared by treating the culture media with ASNase to exhaust its target amino acids, followed by the withdrawal of the drug using centrifugation. Without this approach, the effect of MSCs on the biosynthetic pathways could have been hidden.

The hypoxic environment was initially included in the BM model. Nevertheless, hypoxic conditions induced similar survival of pre-B ALL cells after ASNase treatment compared to normoxia. The comparable results were probably caused by the fact that hypoxia-inducible factor 1 (HIF-1) and ASNase inhibits the TCA cycle. HIF-1 inhibits the TCA cycle to regulate the metabolic shift that allows cell survival under low-oxygen conditions (Tennant & Gottlieb, 2010). After clarification of hypoxia's effect on the survival of leukemic cells after ASNase, we did not include this condition in further experiments.

Indeed, the MSCs-induced mechanism gives leukemic cells an advantage to counteract ASNase treatment. However, it partially depends on the efficiency of the intrinsic pro-survival mechanisms in leukemic cells. In addition to the FAO-dependent resistant mechanism described above (I Hermanova et al., 2016), Kang et al. demonstrated that leukemic cells could acquire ASNase resistance by losing the opioid receptor μ1 (OPRM1). This gene is required for the apoptotic induction orchestrated by ASNase treatment (Kang

et al., 2017; C. Lee et al., 2021). A similar apoptotic inhibition effect was observed in leukemic cells lacking Huntington-associated protein 1 gene (HAP1). The deletion of HAP1 prevents the Ca^{2+} release from the endoplasmic reticulum, causing the downregulation of the Calpain-1-Bid-caspase-3/12 pathway and apoptosis inhibition (J. Lee et al., 2019). Another mechanism involved with ASNase resistance was the downregulation of the Wnt pathway. The blockage of this pathway causes the degradation of proteins through GSK3-dependent protein ubiquitination and proteasome, which supply Asn to leukemic cells treated with ASNase (Hinze et al., 2019).

Moreover, the second paper presented in the dissertation demonstrated a combination of metabolic features correlated with ASNase resistance development. Those mentioned features are: high glycolytic flux, low ATP-linked respiration and high MMP (publication 2).

Firstly, we identified high glycolytic flux in the leukemic cell lines and primary cells isolated from ALL patients that were resistant to ASNase treatment. Secondly, the feature of low ATP-linked respiration (low ATP synthetase activity) was correlated with the resistant phenotype. Indeed, treatment with oligomycin A (ATP synthetase inhibitor) led the leukemic cells to become more resistant to ASNase treatment. These results confirmed the association between low ATP synthetase activity and resistance to ASNase treatment. The low function of ATP synthetase in the mitochondria was also reported in resistance to 5-fluorouracil in colorectal carcinoma cells (Shin et al., 2005). Reduced ASNase sensitivity in our results is probably connected with the low dependency of this phenotype on the TCA cycle. Therefore, they were less affected by the depletion of Gln, the substrate of the TCA cycle. As stated, Gln is depleted by ASNase treatment. We assume that leukemic cells with high glycolytic flux synthesize enough ATP through this pathway to depend on ATP synthetase function. Therefore, the mitochondria activity is minimal and requires fewer electron carriers generated in the TCA cycle (Bartman et al., 2023)(Publication 2). Thirdly, higher MMP, a key indicator of mitochondrial activity (Van Blerkom et al., 2003), correlated with higher resistance to ASNase treatment. This finding might seem controversial concerning the phenotype described above. However, there is another explanation. Not only the electron carriers from the TCA cycle can fuel the mitochondrial ETC, but FAO also generates electron carriers that enter the ETC (Carracedo et al., 2013). Therefore, ASNase-resistant leukemic cells can have a low TCA cycle and high mitochondria activity.

In the next article, we focused on the differences between ASNase-resistant and – sensitive T-ALLs cell lines. We demonstrated the involvement of Akt and its downstream

pathways in the resistance to ASNase (publication 3). Akt is part of the PI3K/Akt/mTOR signaling pathway that prompts cell growth and survival of cells (Pulido, 2015). It is negatively regulated by the tumor suppressor PTEN (Evangelisti et al., 2018; Fruman et al., 2017). However, in T-ALL pediatric cohorts, 20% of the cases contain loss-of-function mutations in PTEN. These mutations are associated with a worse prognosis and outcome in cancers, including leukemia (Bandapalli et al., 2013; Jenkinson et al., 2016; Paganin et al., 2018; Szarzyńska-Zawadzka et al., 2019; Tesio et al., 2017). In this dissertation, we demonstrated that the activation of Akt (phosphorylated form) induced glycolytic activity in leukemic cells and primary cells isolated from T-ALL patients. A Similar effect was previously reported in other cancers as well (Carnero et al., 2008). Furthermore, the evidence provided in this dissertation revealed that leukemic cells with aberrant Akt become less sensitive to ASNase, with the involvement of higher glycolysis flux.

In the case of T-ALL patients, the activation of Akt was not always related directly to the presence of PTEN mutation. This event is probably caused by some other mutations within the PI3K/Akt/mTOR pathway that phenocopies PTEN loss and deregulates cell growth. A similar situation occurs in endometrial cancers, where aberrations in the PI3K/Akt/mTOR pathway are detected in 80% of the cases (Cheung et al., 2011). Therefore, Akt hyperactivation rather than PTEN expression could be used as a marker to predict ASNase response in T-ALL. Noteworthy, phosphorylated Akt measurement was assessed by mass cytometry in our study, which could be easily applied to current diagnostic panels.

In this dissertation, the pharmacological inhibition of Akt in combination with ASNase treatment showed to be more efficient in killing the leukemic cells with aberrant Akt. Some Akt inhibitors are currently being tested for hematological malignancies in clinical trials, for instance, GSK2110183 (Guo et al., 2019).

5.2 Leukemia metabolism as a prognostic factor in non-responsive patients

In 2011, Douglas H., & Weinberg R.A recognized energy metabolism as one of the hallmarks of cancer (Hanahan, 2022). Based on that, we proposed to use the metabolic phenotype as a prognostic factor to improve the ALL treatment. Metabolic rewiring follows leukemogenesis as a response to unlimited growth signals. Mentioned changes mainly depend on nutrient availability and genotype. The latter includes the particular genetic aberrations of each patient. As part of the prognostic factors of ALL treatment, the patient's response during the primary therapy is evaluated and includes MRD levels, pharmacogenomics and pharmacodynamics (Inaba & Pui, 2021). However, it does not

predict the particular response of each patient to chemotherapy. Hence, there is a need for a novel parameter to enhance patient stratification, and our data propose that the metabolic predisposition to ASNase treatment emerges as a potential candidate. Noteworthy, new strategies to better stratify AML patients based on their metabolic predisposition were recently presented (Chen et al., 2022; F. Hu et al., 2021; Zhou et al., 2022). They are expected to improve AML outcomes.

ASNase treatment disturbs cellular metabolism. However, since it does not happen in the same way in all leukemic cells, it can influence the sensitivity to ASNase. In this dissertation, we demonstrated that the metabolism of leukemic cells influences response to ASNase treatment. The leukemic cells included in this study were cell lines and primary cells isolated from patients with B-ALL, T-ALL and AML leukemia. Specifically, according to the levels of glycolytic flux, patients clustered into two groups in which also the sensitivity to ASNase was reflected. In the case of the cell lines, the immunophenotype of the cells was reflected in the clustering. B-ALL were in the ASNase-sensitive group, while T-ALL (with one exception) and AML were in the resistant cluster (Publication 2).

The clustered analysis had some exceptions. HPB-ALL, NALM-6 and BV-173 were classified within the cell group with other immunophenotypes. Moreover, the exceptions increased in the cluster analysis of primary leukemic cells. T-ALLs and B-ALLs were heterogeneously distributed between the ASNase-resistant and sensitive groups. These results could imply that B-ALLs, which clustered with T-ALLs could benefit from more intense chemotherapy. Similarly, T-ALLs clustered with B-ALLs could benefit from a lower intensity of chemotherapy, and by that, reducing the risk of relapse derived from chemotherapy toxicity (Publication 2).

Taken together, the immunophenotype of leukemic cells provides a limited prediction concerning their response to ASNase treatment. This evidence has clear implications for the prognosis of ALL. The current patient stratification is sufficient for the majority of subtypes of leukemia. However, the non-responsive patient could benefit from adding the metabolic profile as a possible secondary strategy for patient stratification. The non-responsive patient with the worst prognosis could be detected at the beginning of the primary chemotherapy (Hunger & Mullighan, 2015a). At this time, a metabolic phenotype study could be implemented and modify the chemotherapy regimen (Publication 2).

Even when the results provided by this dissertation indicate that glycolytic flux itself could predict the response of leukemic cells to ASNase treatment, a complete evaluation of the metabolic phenotype is required. Leukemia is a heterogeneous disease formed by

subclonal populations displaying metabolic heterogeneity. Leukemia subclones share essential metabolic features to reach unlimited proliferation and are acquired by different sets of mutations (Elstrom et al., 2004; Flier et al., 1987; Shim et al., 1997). Shared metabolic features enhance the ability to generate energy, synthesize macromolecules and maintain redox homeostasis (DeBarardinis and Chandel, 2016). Their different genetic origins lead them to have different activities of the deregulated metabolic pathway. The variation in activity is combined with unshared and leukemogenesis-irrelevant aberrant pathways and creates metabolic heterogeneity within the leukemic cells population (Fiehn et al., 2016; Gao et al., 2009; Shackelford et al., 2013). Indeed, metabolic heterogeneity has implications for leukemia therapy. As we observed in the comparison of the metabolic phenotype of *JAK2* translocations, included in the next study. Malignant transformation of Ba/F3 cells (murine B-progenitors) driven by *NPAT/JAK2* translocation was accompanied by a different metabolic setting compared to PAX5/JAK2-transformed Ba/f3 cells (Publication 4). This difference caused that both *JAK2* translocations displayed different sensitivities to glycolytic inhibition. The lower dependency on glycolysis of PAX5/JAK2-transformed cells suggests a different balance between the metabolic pathways in these cells. As a result, PAX5/JAK2-transformed cells probably have other metabolic vulnerabilities compared to NPAT/JAK2-cells.

The estimation of the metabolic phenotype of leukemic cells cannot be done solely according the genetic characterization. The results showed that *JAK2* translocations with oncogenic potential carrying different fusion partners led to different metabolic rewiring. The novel fusion *NPAT/JAK2* found in *BCR-ABL1*-like ALL induced increased glycolysis and mitochondrial respiration activity. A similar effect of JAK2 activating mutation was reported in an *in vivo* model of myeloproliferative neoplasms (Rao et al., 2019). In the case of *PAX5/JAK2*, transformed cells displayed lower glycolysis and mitochondrial respiration, adopting a more quiescent phenotype than NPAT/JAK2-transformed cells. *PAX5/JAK2* translocation causes slow proliferation and weaker transforming capacity in cells than *NPAT/JAK2* mutation.

In summary, metabolic heterogeneity could be observed from cells sharing the same chimeric protein, JAK2, which drives the malignant transformation (Publication 4). Patients with these translocations could be treated similarly in the conventional patient stratification. As a result, it could increase the risk of relapse in some of these variants. Indeed, every tumor has a unique metabolomics signature, as demonstrated in a large study with patient-derived melanoma xenografts (Shi et al., 2017)

Noteworthy, multiple scenarios could cause differences in the metabolism profile between both fusions genes expressing *JAK2*. PAX5/JAK2-transformed cells have lower glycolytic function given the suppression of its STAT3 signaling (Marin-Valencia et al., 2012). Another option could be the biological impact of *PAX5* moiety. Wild-type *PAX5* activates the gene expression of genes related to the repression of glucose uptake and energy metabolism (Chan et al., 2017). Since PAX5/JAK2 aberrant protein is detected exclusively in the nucleus, it might have free access to *PAX5* target loci and partially maintain its function (Schinnerl et al., 2015). As a result, glycolysis could be partially inhibited in PAX5/JAK2-transformed cells.

The current leukemia classification, with its therapy implications, reached its plateau. It was adjusted over the years after decades of research and clinical trials with a high-efficiency rate (Inaba & Pui, 2021). Nevertheless, it needs to implement new parameters even when they can be challenging, such as cellular metabolism. We propose to use the metabolic profile of non-responding patients as a secondary patient stratification strategy. Incorporating the metabolic phenotype as a prognostic factor in primary patient stratification requires further investigation.

5.3 Study limitation and future perspectives

The studies presented in this dissertation had some limitations. First, Asn transporters inhibition could have been tested for diminishing MSCs support to leukemic cells after ASNase treatment. However, little is known about the Asn transporters and their interactions with other transporters. Second, the study of primary cell samples was biased due to the high amount of material needed to assess all the measurements. As a consequence, we only included the patients with high white blood count and sufficient material in the study. Finally, most studies aimed to elucidate novel targets to improve leukemia chemotherapy. Thus, further investigation is needed to employ them in the current therapeutic approaches, which brings new research opportunities.

For instance, the continuation of the impact of the BM microenvironment in the survival of leukemic cells by applying different BM cell types in the BM model. Indeed, adipocytes were reported to release Gln into the matrix, which potentially counteracts ASNase cytotoxicity (Ehsanipour et al., 2013). Another option is including one by one the BM cell types in order to find synergies boosting leukemic cell survival. Moreover, we could test different strategies to diminish MSCs support, like (i) using the clinical alternative of

rapamycin together with ASNase and observing toxicity in healthy cells, (ii) finding efficient inhibitors for blocking Asn uptake in leukemic cells.

6. CONCLUSIONS

By understanding the metabolism of leukemic cells upon ASNase treatment, we identified several novel metabolic features implicated in resistance. Moreover, we have proposed a re-evaluation of patient stratification using cellular metabolism as a secondary strategy for non-responsive patients. Resistance to ASNase occurs as a result of combined intra- and extracellular factors. The latter is represented by the BM microenvironment that enhances the development of resistance in leukemic cells. When simulating BM microenvironment *in vitro*, MSCs sustain the biosynthetic pathways of ASNase-treated leukemic cells, which retrieve the intrinsic resistant mechanism of leukemic cells. The efficiency of metabolic rewiring depends on the particular basal metabolic state of treated leukemic cells. Leukemic cells with high glycolytic flux, low ATP-linked respiration and high mitochondrial membrane potential (determined prior to the treatment) displayed lower sensitivity to ASNase. Additionally, ASNase resistance in T-ALL with high glycolytic flux was found in patients with mutated PTEN or PI3K/Akt pathway. Akt inhibitor combined with ASNase in patients with hyperactivated Akt kinase was indeed beneficial in their treatment outcome. Taken together, mentioned intrinsic and extrinsic metabolic features of leukemic cells can be exploited therapeutically in combination with ASNase treatment.

The stratification of non-responsive patients can be re-examined by measuring the cellular metabolism of leukemic cells. We have demonstrated that the level of glycolytic flux of leukemic cells can predict the sensitivity to ASNase treatment independently of the immunophenotype; B-ALL or T-ALL. Moreover, we found differences in metabolic dependencies between JAK2-rearranged ALL patients with different fusion partners despite their similar gene expression profiles. This difference was then translated into distinct metabolic vulnerabilities. In conclusion, leukemic cell metabolism expands the leukemia characterization, potentially improving patient stratification and treatment options.

7. SUMMARY

7.1 Explore the pro-survival metabolic processes of leukemic cells upon ASNase treatment in the BM microenvironment.

In the main project, I have elucidated the metabolic mechanism by which MSCs increase the survival of leukemic cells. Using a BM model, we have demonstrated that MSCs restore the biosynthetic pathways of leukemia cells after ASNase treatment. The restored pathway sustained the leukemic cells' survival, which retrieved the intrinsic resistant mechanisms in leukemic cells. The latter mechanism counteracts the cytotoxicity of the drug.

7.2 Study of the basal metabolic profile of leukemic cells in the cellular response to ASNase treatment.

This study investigated the connection between basal metabolic profile and the sensitivity to ASNase treatment in acute leukemia. The metabolic profile was assessed by measuring glycolysis parameters, mitochondrial respiration activity, and MMP in leukemic cell lines (19) and primary cells from both ALL and AML patients (26). Higher glycolytic flux was strongly associated with lower ASNase sensitivity. Moreover, we demonstrated a correlation between lower ASNase sensitivity with higher ATP-linked respiration and basal MMP.

7.3 Investigate PTEN mutations or aberrant PI3K/Akt pathway in T-ALL in the ASNase sensitivity and glucose metabolism.

This study focused on the link between PTEN deletion, glucose metabolism and the sensitivity of T-ALL to ASNase treatment. T-ALL with lower ASNase sensitivity displayed higher glycolysis, upregulated by the aberrant activation of the PI3K/Akt pathway. PTEN is a negative regulator of PI3K/Akt pathway, which we found was functionally lost in T-ALL cells which were resistant to ASNase. Pharmacological inhibition of Akt kinase enhanced the sensitivity to ASNase, and led to the inhibition of glycolytic flux.

7.4 Characterize the role of metabolic rewiring in malignant transformation driven by mutated JAK2 kinases identified in *BCR-ABL*-like ALL subgroup.

This study compared the metabolic rewiring during leukemogenesis induced by *JAK2* translocations with different fusion partners. We demonstrated that the translocations with altered JAK/STAT signaling displayed different metabolic phenotypes. As a result, we confirmed metabolic heterogeneity within leukemia subtypes, implying different metabolic vulnerabilities.

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9. ATTACHMENTS

- Publication 1: Alquezar-Artieda N, Kuzilkova D, Roberts J, Hlozkova K, Pecinova A et al. Restored biosynthetic pathways induced by MSCs serve as rescue mechanism in leukemia cells after L-asparaginase therapy. Blood adv. 2023; 10:2228

- Publication 2 Hlozkova K, Pecinova A, Alquezar-Artieda N, et al. Metabolic profile of leukemia cells influences treatment efficacy of l-asparaginase. BMC Cancer. 2020;20:526

- Publication 3: Hlozkova K, Hermanova I, Safrhansova L, Alquezar-Artieda N, et al. PTEN/PI3K/Akt pathway alters sensitivity of T-cell acute lymphoblastic leukemia to l-asparaginase. Sci. Rep. 2022;12(1):.

- Publication 4: Lukes J, Potuckova E, Alquezar-Artieda N, et al. Chimeric JAK2 kinases trigger non-uniform changes of cellular metabolism in BCR-ALB1-like childhood ALL. (Under review in HemaSpere)