

Metabolic Profile Of Leukemia Cells Influences Treatment Efficacy Of L-Asparaginase

Katerina Hlozkova

Univerzita Karlova 2 lekarska fakulta

Alena Pecinova

Fyziologicky Ustav Akademie Ved Ceske Republiky

David Pajuelo Reguera

Univerzita Karlova 2 lekarska fakulta

Marketa Simcikova

Univerzita Karlova 2 lekarska fakulta

Lenka Hovorkova

Univerzita Karlova 2 lekarska fakulta

Katerina Rejllova

Univerzita Karlova 2 lekarska fakulta

Natividad Alquezar Artieda

Univerzita Karlova 2 lekarska fakulta

Marketa Zaliova

Univerzita Karlova 2 lekarska fakulta

Tomas Mracek

Fyziologicky Ustav Akademie Ved Ceske Republiky

Jan Stary

Univerzita Karlova 2 lekarska fakulta

Jan Trka

Univerzita Karlova 2 lekarska fakulta

Julia Starkova (✉ julia.starkova@lfmotol.cuni.cz)

Univerzita Karlova 2 lekarska fakulta

Research article

Keywords: cancer metabolism, L-asparaginase, leukemia, resistance, glycolysis, mitochondrial respiration, fatty acid oxidation, mitochondrial membrane potential

Posted Date: November 20th, 2019

DOI: <https://doi.org/10.21203/rs.2.17519/v1>

License:  This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Version of Record: A version of this preprint was published at BMC Cancer on June 5th, 2020. See the published version at <https://doi.org/10.1186/s12885-020-07020-y>.

Abstract

Background Effectiveness of L-asparaginase administration in acute lymphoblastic leukemia treatment is mirrored in overall outcome of patients. Generally, leukemia patients differ in their sensitivity to L-asparaginase; however, the mechanism underlying their inter-individual differences is still not fully understood. We have previously shown that L-asparaginase rewires the biosynthetic and bioenergetic pathways of leukemia cells to activate both anti-leukemic and pro-survival processes. Herein, we investigated the relationship between the metabolic profile of leukemia cells and their sensitivity to currently used cytostatic drugs.

Methods Altogether, 19 leukemia cell lines and primary leukemia cells from 11 patients were used. Glycolytic function and mitochondrial respiration were measured using Seahorse bioanalyzer. Sensitivity to cytostatics was measured using MTS assay and/or absolute count and flow cytometry. Mitochondrial membrane potential was determined as TMRE fluorescence.

Results We characterized the basal metabolic state of the cells derived from different leukemia subtypes using cell lines and primary samples and assessed their sensitivity to cytostatic drugs. We found that leukemia cells cluster into distinct groups according to their metabolic profile, which is mainly driven by their hematopoietic lineage of origin from which they derived. However, majority of lymphoid leukemia cell lines and patients with lower sensitivity to L-asparaginase clustered regardless their hematopoietic phenotype together with myeloid leukemias. Furthermore, we observed a correlation of specific metabolic parameters with sensitivity to L-asparaginase. Greater ATP-linked respiration and lower basal mitochondrial membrane potential in cells significantly correlated with higher sensitivity to L-asparaginase. No such correlation was found in other tested cytostatic drugs.

Conclusions These data support the prominent role of the cell metabolism in the treatment effect of L-asparaginase. Based on these findings metabolic profile could identify leukemia patients with lower sensitivity to L-asparaginase with no specific genetic characterization.

Introduction

Leukemia is the most common malignancy and the second most frequent general cause of childhood death. Leukemia is classified as acute lymphoid of B- or T-lineage (B-ALL, T-ALL), being the most prevalent type in children, and as acute myeloid. Although tremendous improvement has been made in the treatment of leukemia in the last few years, there is still a large proportion of patients who do not benefit from the therapy. The overall survival of patients treated with current chemotherapy regimens is above 80% in children with acute lymphoblastic leukemia (ALL) and is approximately 70% in patients with acute myeloid leukemia (AML) (1,2).

The standardized treatment protocols that have been used for decades consist of the same repertoire of cytostatic drugs, which differ in the dosage, time of administration and combination with other drugs. New genomic techniques have enabled the identification of new genetic alterations, which could be

targeted by new compounds; however, genetic-based approaches have not fully revealed the direct cause of inter-individual differences in sensitivity to cytostatic drugs (3–6). Therefore, understanding the mechanism of action and prediction of the impaired effect of commonly administered drugs is crucial for improving patient outcomes.

We turned our attention to cellular metabolism as a limiting process in cell proliferation and survival. Recent data have indicated that some cytostatic drugs trigger metabolic reprogramming, which impairs their effects and, in the long run, could cause resistance. One of the examples was described by our group in which leukemia cells treated with L-asparaginase (ASNase) reprogrammed their metabolism and increased fatty acid oxidation (FAO) and autophagy to compensate for asparagine and glutamine depletion (7). Pharmacological inhibition of FAO increased the sensitivity to ASNase in leukemia cells, which supported its pro-survival effect and its potential role in the mechanism of resistance. A similar mechanism was seen in chronic lymphoblastic leukemia, where administration of dexamethasone led to increased FAO (8). We assume that since treatment can dramatically influence the metabolic setup, reciprocally, the metabolic predisposition of cancer cells could also interfere with their response to treatment.

Our aim was to investigate how the basal metabolic profile of leukemia cells interferes with the effectiveness of current treatment compounds. We focused on cytostatic drugs used in the treatment of childhood leukemia with an emphasis on ASNase. ASNase is a crucial drug used in the treatment of acute lymphoblastic leukemia; it was recently also incorporated in the front-line treatment of adult leukemia (9,10). Unfortunately, sensitivity to ASNase differs among ALL patients as was shown by Ramackers et al. (11). A specific phenotypic subgroup of ALL deriving from a T lymphoid lineage was also shown to be more resistant to ASNase treatment (12,13). In the current study, we have determined the metabolic phenotype of different types of leukemia in order to better understand their differences, metabolic demands, and their connection with ASNase selective sensitivity.

Materials And Methods

Cell culture

Human B-cell precursor leukemia cell lines (TOM-1, HB11;19, RS4;11, UOC-B6, REH, SUP-B15, NALM6), T-cell leukemia cell lines (HPB-ALL, CCRF-CEM, JURKAT, MOLT-4), AML cell lines (MV4;11, KASUMI-1, NB-4, THP-1, MOLM-13) and cell lines derived from the blast crisis of chronic myeloid leukemia (CML), which manifest as AML (K-562, LAMA-84) and as ALL (BV-173), were used (Table S1). The HB11;19 cell line was kindly provided by Dr. Anthony Ford from the Institute of Cancer Research (London, UK), and the UOC-B6 cell line was provided by Dr Ondrej Krejci (Massachusetts General Hospital, Boston) (14). The rest of the cell lines were purchased from German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The cell lines were negative for mycoplasma contamination and cultivated in RPMI-1640 medium with GlutaMAX™ supplemented with 10% fetal calf serum, penicillin (100U/mL) and

streptomycin (100µg/mL) under controlled conditions (37°C, 5% CO₂). The cultured cells were split every two to three days and maintained in exponential growth phase.

Patient samples

Bone marrow or peripheral blood samples from untreated children initially diagnosed with B-cell precursor ALL, T-ALL or AML were collected from the Czech Pediatric Hematology Centers. The inclusion criteria were percentage of blasts higher than 80% and high cellularity. Within 24 hours after aspiration, without freezing, the mononuclear cells were isolated by density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare, UK). All the samples were obtained with the informed consent of the children's parents or guardians as well as the approval of the Ethical Committee of the University Hospital Motol, Prague, Czech Republic, study no. 201528848A.

The isolated blasts were maintained in RPMI-1640 medium with GlutaMAX™ supplemented with 10% fetal calf serum, penicillin (100U/mL) and streptomycin (100µg/mL). For the MTS assay, insulin-transferrin-sodium selenite supplement was added to the culture media (Sigma-Aldrich, St Louis, MO, USA).

Mitochondrial FAO measurement

The cells were incubated for 4 hours in culture medium containing 100µM palmitic acid, 1mM carnitine and 1.7µCi [9,10(n)-³H]palmitic acid (GE Healthcare, UK) in the presence or absence of etomoxir (100µM, Sigma-Aldrich, MO, USA). Medium was collected to analyze the amount of released ³H₂O that was formed during the cellular oxidation of [³H]-palmitate (15,16). The procedure was performed as described previously (7). The measurement was performed in three independent experiments.

TMRE staining

The cells were incubated for 30 minutes in culture medium with or without 1µM tetramethylrhodamine ethyl ester (TMRE; ThermoFisher Scientific Inc., MA, USA) and were then washed with PBS and analyzed on a flow cytometer according to the manufacturer's instructions. The level of TMRE staining was expressed as the mean of the TMRE signal in the live cells.

Cell survival and proliferation

To evaluate the cytotoxicity of ASNase, vincristine (VCR), and daunorubicin (DNR), MTS (dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium) assays were performed using a CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega Corporation, Wisconsin, USA) according to the

manufacturer's instructions. To evaluate the combined cytotoxicity of the two drugs, the number of live cells was determined by flow cytometry using DAPI (ThermoFisher Scientific Inc., MA, USA) and AccuCount Blank Particles (Spherotech Inc., IL, USA).

Seahorse extracellular flux analysis

Glycolytic and mitochondrial respiration parameters of leukemia cell lines were measured on a Seahorse analyzers XFe24 and XFp (Agilent Technologies, Inc., CA, USA) using a Glycolysis stress test and a Cell mito stress test. For the Glycolysis stress test, cells were seeded in XF Base medium, pH 7.4, and for the Cell mito stress test, cells were seeded in XF Assay medium, pH 7.4, supplemented with 10mM glucose, 1mM HEPES, pH 7.4, 1mM pyruvate and 0.1% BSA. The cells were plated at a density of 300,000 cells/well in XFe24 or 40,000 cells/well in XFp tissue culture plates coated with CellTak (Corning GmbH, Wiesbaden, GER) according to the Agilent Seahorse protocol for seeding suspension cells.

The glycolytic and mitochondrial respiration parameters of the primary leukemia cells were measured on the Seahorse analyzer XFp using the same tests and media as in the case of the cell lines. The primary cells were plated at a density of 500,000 cells/well in XFp tissue culture plates. The procedure was performed as described previously (17).

Final concentrations of the injected drugs were 10mM glucose, 1 μ M (for cell lines) or 2 μ M (for primary cells) Oligomycin A and 100mM 2-deoxy glucose (2-DG) in the Glycolysis stress test and 2 μ M Oligomycin A, 1–4.5 μ M FCCP (depending on the cell line) and 1 μ M Rotenone combined with 1 μ g/ml Antimycin A in the Cell mito stress test.

Genomic DNA isolation and mtDNA quantification

Genomic DNA was isolated from leukemia cell lines using the QIAamp DNA Mini Kit (Qiagen GmbH, Germany) according to the manufacturer's instructions. To quantify the mtDNA content, two genes as mitochondrial targets (*16S rRNA* and *D-loop* genes) and the *GAPDH* gene as a nuclear target were used. Quantification was performed using real-time PCR as described elsewhere (18).

Electrophoresis and western blotting

Protein lysates were prepared as previously described (19). The proteins (30 μ g per well) were resolved by NuPAGE Novex 4–12% Bis-Tris Gels (ThermoFisher Scientific Inc., MA, USA) and transferred to a nitrocellulose membrane (Bio-Rad, CA, USA). The membrane was probed overnight with the primary antibodies listed in Table S2. The bound antibodies were detected with the appropriate secondary antibodies conjugated to horseradish peroxidase (Bio-Rad, CA, USA) and visualized using an enhanced chemiluminescence reagent and documented by Uvitec (Cambridge, UK).

Statistical analysis

Hierarchical clusters were generated in R using the Pheatmap package (distance measure: “Euclidean”, clustering method: “ward.D2”). Linearization method was used to calculate adjusted (bonferoni) p-values for Oligomycin A effect to ASNase, VCR and DNR sensitivity of leukemia cells (Figure 3). Spearman rank correlations were calculated in R using the method “spearman”. p-values in Figure S1K were calculated using an unpaired, two-tailed, Mann-Whitney test in GraphPad Prism 6. Canonical Correlation analysis was done in R.

Results

Characterization of the basal metabolic state of leukemia cell lines

We used 19 human leukemia cell lines of different origin (8 B-ALL (one of them ALL from the blast crisis of CML), 4 T-ALL and 7 of myeloid origin (five of them AML; two of them blast crisis from CML manifesting as AML); in all of them, we determined the glycolytic function, mitochondrial respiration and FAO (Figure S1). Glycolytic and mitochondrial functions were measured on a Seahorse analyzer XFe24 using Glycolysis stress test and Cell mito stress test, respectively. This allowed us to measure and calculate different parameters within the glycolytic (basal acidification, glycolysis, glycolytic capacity, glycolytic reserve) and mitochondrial (basal respiration, ATP-linked respiration, maximal respiration, spare capacity) functions. FAO activity was measured as oxidation of [³H]palmitate.

A hierarchical clustering analysis (HCA) revealed that glycolysis and mitochondrial respiration, but not FAO, clustered leukemia cells separately according to the hematopoietic lineage of origin from which they were derived (Figure 1A, B). Myeloid leukemia and T-ALL cell lines had overall higher values of glycolytic and cell respiration parameters and clustered together, while the B-ALL cell lines with general lower values gathered into the second cluster. Nevertheless, there were some exceptions. The first exception was HPBALL, a TALL cell line, which clustered with the B-ALL cell lines. The second exception was the NALM-6 and BV-173 B-ALL cell lines, which clustered together with the myeloid leukemia and T-ALL cell lines. In the case of NALM-6, this result was only achieved according to glycolytic function. Next, the OCR (oxygen consumption rate)/ECAR (extracellular acidification rate) ratio was calculated from the OCR and ECAR values, which were determined during the measurement of glycolytic function after glucose addition (Figure S1). We plotted OCR against ECAR, which divided leukemia cell lines according to their energy status. The aerobic status represents the cells that preferentially oxidize glucose in mitochondria while cells utilizing glucose for fermentation to lactate have a glycolytic status. When both OCR and ECAR values are low, cells are quiescent, whereas if both values are high, the cells are energetic, meaning they are very actively using both glycolysis and oxidative phosphorylation (OXPHOS, Figure 1C).

Next, we tested which cellular processes could participate in a given metabolic state. The relative activity of major signaling pathways influencing basal metabolism were determined by western blot in the 15 leukemia cell lines of different origins (6 B-ALL, 4 T-ALL, 5 AML; Figure S1K). We quantified the western blots by densitometry (normalized to β -Actin level) and, importantly, we found higher levels of constitutively phosphorylated AKT (p-AKT) in all the T-ALL cell lines compared to the B-ALL cell lines ($p = 0.0095$). Furthermore, the levels of phosphorylated GSK-3 β (glycogen synthase kinase 3 beta; p-GSK-3 β) at Ser9 in the B-ALL cell lines were significantly lower compared to the levels in the AML cell lines ($p = 0.0455$). Activation of GSK-3 β by dephosphorylation inhibits glycogen synthesis. We also detected elevated phospho-S6 (protein S6; p-S6), a downstream target of the PI3K/AKT/mTOR pathway, in the AML cell lines compared to the B-ALL cell lines ($p = 0.0303$). Moreover, we found significant difference in the levels of c-MYC (an activator of glycolysis) between B-ALL and T-ALL cell lines ($p = 0.0190$). We did not see any significant difference in the levels of p-AMPK (an AMP-activated protein kinase that has a role in cellular energy homeostasis) among leukemia types.

Association of the metabolic profile of leukemia cells and the sensitivity to cytostatic drugs

Next, we investigated if there was a relationship between the metabolic phenotype of leukemic cells and treatment sensitivity. First, we measured the sensitivity of all 19 leukemia cells to ASNase and to VCR and DNR. The last two drugs are included in the treatment of both ALL and AML, which is contrary to ASNase's sole administration in ALL. First, sensitivity to ASNase did not correlate with sensitivity to Vincristine (VCR) and Daunorubicin (DNR), which demonstrated that the tested leukemia cell lines are not generally sensitive or resistant to cytostatic drugs but due to their intrinsic properties they respond independently to drugs with different mechanisms of action (Table 1A; Figure 2A). We observed that those least sensitive to ASNase are myeloid cell lines followed by T-ALL cells, with B-ALL cell lines being the most sensitive ones (Table 1B).

We next asked if there was any relationship between the overall metabolic state of the leukemia cells (including all parameters measured) and sensitivity to cytostatic drugs. For this purpose, we used Canonical Correlation analysis (CCA), which explores the relationship between two multivariate sets of variables. In our case, set 1 was all the metabolic parameters, and set 2 consisted of the lineage origins and IC50s (Figure 2B, C). Higher sensitivity to ASNase presented preferentially in lymphoid leukemias correlated the most significantly with lower basal respiration, higher ATP-linked respiration and with a preference for oxidative (OCR/ECAR) over glycolytic metabolism ($p = 0.0075$, Figure 2B). Higher sensitivity to DNR represented in AML correlated with lower basal respiration, higher ATP-linked respiration and lower FAO ($p = 0.0295$; Figure 2C). Sensitivity to VCR did not correlate with any metabolic activity.

Functional study of the correlation between ATP synthase activity and sensitivity to ASNase

To functionally confirm the results from the CCA, we chose to inhibit ATP synthase activity to test the relationship between ATP-linked respiration and sensitivity to ASNase. First, we determined the optimal concentration of Oligomycin A (a specific inhibitor of ATP synthase) that would still effectively decrease the level of ATP but at the same time did not completely inhibit the proliferation of cells. Thus, we counted live cells after 48 h and 72 h treatment with different concentrations of Oligomycin A (20nM–2μM). The results showed that 20nM Oligomycin A inhibited the proliferation of leukemia cells to the same level as higher concentrations (Figure S2A). When measuring mitochondrial respiration, cells treated with 20nM Oligomycin A had lower basal respiration than untreated cells. Moreover, this lowered respiration was not further reduced after 2μM Oligomycin A injection, which means that ATP synthase was already inhibited (Figure S2A). OCR of untreated cells dropped dramatically after 2μM Oligomycin A injection, reaching the level of 20nM Oligomycin A-treated cells. Based on these data, we selected 20nM Oligomycin A for further experiments.

Oligomycin A treatment was tested on five human leukemia cell lines of different origin (2 B-ALL, 2 TALL, 1 AML). Leukemia cells were pretreated with 20nM Oligomycin A for 1 hour and then co-treated with different concentrations of ASNase or left untreated for 48 or 72 hours. Then, live cells were counted using flow cytometry. The results showed that Oligomycin A pretreatment increased the resistance of leukemia cells to ASNase compared to cells treated with ASNase alone (adjusted (bonferroni) p value $<10^{-4}$; representative graphs of all treated cell lines are shown in Figure 3A;). This result suggests that cells with lower ATP synthase activity have lower sensitivity to ASNase. The effect was evident in all tested cell lines. However, the cytostatic effect of VCR and DNR was not disturbed by Oligomycin A pretreatment in the tested leukemia cell lines (Figure 3B). Surprisingly, pre-treatment with 10ng/ml of Antimycin A (inhibitor of ETC complex III) did not decrease the sensitivity of leukemia cells to ASNase compared to cells treated with ASNase alone (Figure S3). These results attest that cells pre-treated with Oligomycin A have decreased sensitivity to ASNase not due to a reduced cell growth since cells treated with both agents, i.e. Antimycin A (10ng/ml) and Oligomycin A (20nM), show similar growth reduction compared to non-treated cells (Figure S2).

Association of the mitochondrial membrane potential with the sensitivity to ASNase

Basal mitochondrial membrane potential (MMP) was measured as TMRE fluorescence in ten human leukemia cell lines of different origin (3 B-ALL, 4 T-ALL, 3 AML). Additionally, the content of the mitochondria was determined as the amount of mtDNA by real-time PCR. TMRE is an MMP-dependent dye and its fluorescence intensity is in proportion to both MMP and mitochondrial mass. TMRE

fluorescence alone or TMRE fluorescence normalized to mtDNA amount clustered leukemia cell lines regardless their origin (data not shown). On the other hand, in the tested leukemia cell lines, TMRE fluorescence significantly correlated ($\rho = 0.806$; $p\text{-value} = 0.00824$) with sensitivity to ASNase (Figure 4A). This means that cell lines with a higher TMRE fluorescence have in general a lower sensitivity to ASNase. Nevertheless, because TMRE fluorescence did not correlate with mtDNA quantity, it can be concluded that the observed TMRE changes are a representation of basal MMP in leukemia cells (Figure 4B).

Characterization of the metabolic profile of leukemia patients

Although measuring the metabolic state of primary leukemia cells is quite challenging, we assessed the glycolytic and cell respiration activity of cells from eleven childhood leukemia patients (4 BCPALL, 4 T-ALL, 3 AML). We also determined the sensitivity of primary leukemia cells to ASNase by MTS assay (Table 2). HCA gathered patient samples according their metabolic profile into two main clusters. The first cluster consisted exclusively of ALL cases. The second cluster included all AML patients as well as three ALLs (Figure 5A, B). These three ALL patients were less sensitive to ASNase in comparison to ALL cases from the lymphoid cluster (pattern mainly seen according the glycolytic activity). Interestingly, we observed that the profiles of the curves generated by Seahorse measurement (both the glycolytic and mitochondrial function) were related to their sensitivity to ASNase (Figure 5C, D). The BCP-ALL patient (P11) who clustered with the AML patient (P5) both displayed a similar profile of the curve and had a higher IC50 of ASNase (>0.1 UI/ml) in comparison to the BCP-ALL patient (P6) with IC50 ASNase <0.1 UI/ml, who showed a similar profile as the ALL patient (P10) from the first cluster.

Discussion

The aim of this study was to elucidate if metabolic predispositions of leukemia cells (both cell lines and primary cells) influence their response to the therapy. We wondered whether there is a basal metabolic profile that would predict which leukemia patients are sensitive or resistant to the given treatment. Since our previous work showed that ASNase affected the bioenergetics of leukemic cells, we were particularly interested in the link between sensitivity to ASNase and the basal metabolic status of the cells as assessed before the treatment. In addition to ASNase, we also tested the sensitivity to VCR and DNR, cytostatic drugs with different mechanism of action which are used in the treatment of broader spectrum of cancers. We first examined metabolic activity, including glycolysis, mitochondrial respiration and FAO in 19 cell lines. We performed HCA in order to cluster the cell lines with similar metabolic profile. Based on both glycolytic function and mitochondrial respiration, cell lines gathered to clusters very similarly. On the other hand, FAO clustered cell lines differently. The first cluster associated cell lines with lower glycolytic function and lower mitochondrial respiration and consisted exclusively of lymphoid leukemia cell lines. The second cluster, prevalently myeloid, grouped cell lines with higher glycolytic function and higher

mitochondrial respiration and contained all myeloid leukemia cell lines as well as some T-ALL cell lines. Next, aiming at the confirmation of this phenomenon in the primary samples, we assessed glycolytic function and mitochondrial respiration in leukemia cells isolated from ALL and AML patients and performed HCA. First cluster consisted exclusively of samples from ALL patients and the second one combined both AML and ALL patients. After subsequent examination of the samples for their susceptibility to cytostatics, we found that these ALL clustering into the predominantly myeloid metabolic cluster were less sensitive to ASNase treatment than their counterparts in the lymphoid cluster. Moreover, we observed similar association in cell lines where three T-ALL lines from the myeloid cluster were resistant to ASNase. Interestingly however, some AML patients were sensitive to ASNase to the same extent as sensitive ALL patients. This could be potentially clinically interesting since the incorporation of ASNase is still discussed in the treatment of AML.

In cell lines, IC₅₀ of ASNase did not correlate with IC₅₀ of VCR or DNR and also, sensitivity to these cytostatics were not associated with any specific metabolic phenotype demonstrating that their effect was less influenced by the basal metabolic status of leukemia cells.

We have previously described specific signaling pathways that regulated bioenergetic processes after ASNase treatment (7). We were therefore interested if distinct metabolic profile in leukemia cells will be associated with different prerequisite activity of signaling pathways. Indeed, we found a profound difference in p-AKT and c-MYC, the regulators of glycolysis (20,21), between T- and B-ALL cells and decreased level of p-GSK3b in B-ALL cell lines.

Since we found an association between metabolic predisposition and consecutive response to ASNase treatment, we next asked which metabolic parameter correlates with IC₅₀ of ASNase and therefore with the potential treatment efficacy. Statistical analysis based on CCA revealed that higher ATP-linked respiration, higher OCR/ECAR and lower basal respiration significantly correlated with increased sensitivity to ASNase. To functionally confirm this relationship we used a specific ATP synthase inhibitor, Oligomycin A. After Oligomycin A administration, we treated the cells with ASNase and examined the changes in cell survival and growth. We confirmed that cells with lower ATP synthase activity, i.e. lower ATP-linked respiration, are more resistant to ASNase. The sensitivity to VCR and DNR did not change which corresponded with the results of the CCA. Reduced ATP synthase activity in relation to a resistant phenotype has already been described in colorectal carcinoma cells and 5-fluorouracil (22). One assumption is that cells with less active TCA cycle followed by reduced ATP-linked respiration are less dependent on the glucose or glutamine supply. Thus, these cells could better tolerate glutamine depletion and glucose uptake impairment, both of which are cytotoxic effects of ASNase.

Since Oligomycin A treatment leads to increased MMP (23), we examined the so-called basal MMP of the cells using TMRE labeling. We have shown that higher MMP significantly correlates with higher resistance to ASNase. Since TMRE fluorescence measuring is standardized cytometric method, it could be established as an additional diagnostic marker to help characterize patients' sensitivity to ASNase.

Even though ASNase is a crucial component in the treatment of ALL, its precise administration is still being tested in some specific prognostic-risk groups of ALL (24). Moreover, its incorporation into the therapy in other types of cancer is still under investigation (25–28). This is the first study describing the correlation between the sensitivity to ASNase and the metabolic profile of leukemia cells. Our results revealed that myeloid and lymphoid cells cluster based on their glycolytic activity and mitochondrial respiration. Interestingly, ALL cells resistant to ASNase resembled “myeloid”, less sensitive metabolic profile. Further characterization of the metabolic state of the leukemic blasts at the time of diagnosis may help to identify patients with lower sensitivity to ASNase and therefore more likely to fail on the conventional therapy without any other detectable high-risk factors.

Conclusions

Our study provides novel findings on the role of cellular metabolism in the treatment response of leukemia patients. We have shown that distinct leukemia types preferentially use different bioenergetics processes. However, cell lines and patients less sensitive to ASNase resemble metabolic profile typical for myeloid leukemias which are in general resistant to ASNase treatment. We also found correlation between ATP-linked respiration and basal MMP with sensitivity to ASNase. These data support the role of cancer metabolism and open new potential ways in the diagnostic and treatment management of resistant leukemia patients.

Availability Of Data And Materials

The datasets used and/or analyzed during the current study are available from the corresponding author at reasonable request.

Abbreviations

ALL: acute lymphoblastic leukemia

AML: acute myeloid leukemia

CML: chronic myeloid leukemia

ASNase: L-asparaginase

FAO: fatty acid oxidation

VCR: vincristine

DNR: daunorubicin

OCR: oxygen consumption rate

ECAR: extracellular acidification rate

MMP: mitochondrial membrane potential

References

1. Creutzig U, Zimmermann M, Bourquin J-P, Dworzak MN, Fleischhack G, Graf N, et al. Randomized trial comparing liposomal daunorubicin with idarubicin as induction for pediatric acute myeloid leukemia: results from Study AML-BFM 2004. *Blood*. 2013 Jul;122(1):37–43.
2. Stary J, Zimmermann M, Campbell M, Castillo L, Dibar E, Donska S, et al. Intensive chemotherapy for childhood acute lymphoblastic leukemia: Results of the randomized intercontinental trial ALL IC-BFM 2002. *Journal of Clinical Oncology*. 2014;32(3):174–84.
3. Mullighan CG, Goorha S, Radtke I, Miller CB, Coustan-Smith E, Dalton JD, et al. Genome-wide analysis of genetic alterations in acute lymphoblastic leukaemia. *Nature* [Internet]. 2007 Apr 12 [cited 2016 Dec 22];446(7137):758–64. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/17344859>
4. Piovan E, Yu J, Tosello V, Herranz D, Ambesi-Impiombato A, DaSilva A, et al. Direct Reversal of Glucocorticoid Resistance by AKT Inhibition in Acute Lymphoblastic Leukemia. *Cancer Cell*. 2013;
5. Tzoneva G, Dieck CL, Oshima K, Ambesi-Impiombato A, Sánchez-Martín M, Madubata CJ, et al. Clonal evolution mechanisms in NT5C2 mutant-relapsed acute lymphoblastic leukaemia. *Nature* [Internet]. 2018 Jan 17 [cited 2018 Apr 17];553(7689):511–4. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/29342136>
6. Tzoneva G, Perez-Garcia A, Carpenter Z, Khiabani H, Tosello V, Allegretta M, et al. Activating mutations in the NT5C2 nucleotidase gene drive chemotherapy resistance in relapsed ALL. *Nature Medicine* [Internet]. 2013 Mar 3 [cited 2018 Apr 17];19(3):368–71. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/23377281>
7. Hermanova I, Arruabarrena-Aristorena A, Valis K, Nuskova H, Alberich-Jorda M, Fiser K, et al. Pharmacological inhibition of fatty-acid oxidation synergistically enhances the effect of L-asparaginase in childhood ALL cells. *Leukemia* [Internet]. 2016;30(1):209–18. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/26239197>
8. Tung S, Shi Y, Wong K, Zhu F, Gorczynski R, Laister RC, et al. PPAR α and fatty acid oxidation mediate glucocorticoid resistance in chronic lymphocytic leukemia. *Blood* [Internet]. 2013 Aug 8 [cited 2016 Dec 22];122(6):969–80. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/23814018>
9. Liang J, Shi P, Guo X, Li J, He L, Wang Y, et al. A retrospective comparison of *Escherichia coli* and polyethylene glycol-conjugated asparaginase for the treatment of adolescents and adults with newly diagnosed acute lymphoblastic leukemia. *Oncology Letters* [Internet]. 2017 Oct 26 [cited 2018 Apr 18];15(1):75–82. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/29387211>
10. Siegel SE, Stock W, Johnson RH, Advani A, Muffly L, Douer D, et al. Pediatric-Inspired Treatment Regimens for Adolescents and Young Adults With Philadelphia Chromosome–Negative Acute

- Lymphoblastic Leukemia. *JAMA Oncology* [Internet]. 2018 Feb 15 [cited 2018 Apr 18]; Available from: <http://www.ncbi.nlm.nih.gov/pubmed/29450465>
11. Woerden NLR, Pieters R, Loonen AH, Hubeek I, Drunen E Van, Beverloo HB, et al. TEL/AML1 gene fusion is related to in vitro drug sensitivity for L-asparaginase in childhood acute lymphoblastic leukemia. *Blood*. 2000;96(3):1094–9.
 12. Pieters R, den Boer ML, Durian M, Janka G, Schmiegelow K, Kaspers GJ, et al. Relation between age, immunophenotype and in vitro drug resistance in 395 children with acute lymphoblastic leukemia—implications for treatment of infants. *Leukemia* [Internet]. 1998 Sep [cited 2018 Apr 18];12(9):1344–8. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/9737681>
 13. Appel IM, Kazemier KM, Boos J, Lanvers C, Huijmans J, Veerman AJP, et al. Pharmacokinetic, pharmacodynamic and intracellular effects of PEG-asparaginase in newly diagnosed childhood acute lymphoblastic leukemia: results from a single agent window study. *Leukemia* [Internet]. 2008 Sep 26 [cited 2018 Apr 18];22(9):1665–79. Available from: <http://www.nature.com/articles/leu2008165>
 14. Kim DH, Moldwin RL, Vignon C, Bohlander SK, Suto Y, Giordano L, et al. TEL-AML1 translocations with TEL and CDKN2 inactivation in acute lymphoblastic leukemia cell lines. *Blood* [Internet]. 1996 Aug 1 [cited 2018 Apr 18];88(3):785–94. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/8704231>
 15. Ito K, Carracedo A, Weiss D, Arai F, Ala U, Avigan DE, et al. A PML–PPAR- δ pathway for fatty acid oxidation regulates hematopoietic stem cell maintenance. *Nature medicine* [Internet]. 2012 Sep 19 [cited 2016 Dec 24];18(9):1350–8. Available from: <http://www.nature.com/doi/10.1038/nm.2882>
 16. Gerhart-Hines Z, Rodgers JT, Bare O, Lerin C, Kim S-H, Mostoslavsky R, et al. Metabolic control of muscle mitochondrial function and fatty acid oxidation through SIRT1/PGC-1 α . *The EMBO Journal* [Internet]. 2007 Apr 4 [cited 2018 Apr 18];26(7):1913–23. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/17347648>
 17. Hložková K, Starková J. Assessment of the Metabolic Profile of Primary Leukemia Cells. *Journal of Visualized Experiments* [Internet]. 2018 Nov 21 [cited 2019 Jun 21];(141). Available from: <https://www.jove.com/video/58426/assessment-of-the-metabolic-profile-of-primary-leukemia-cells>
 18. Pejznochová M, Tesarová M, Honzík T, Hansíková H, Magner M, Zeman J. The developmental changes in mitochondrial DNA content per cell in human cord blood leukocytes during gestation. *Physiological research* [Internet]. 2008 [cited 2018 Apr 18];57(6):947–55. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/18052680>
 19. Hermanova I, Zaliova M, Trka J, Starkova J. Low expression of asparagine synthetase in lymphoid blasts precludes its role in sensitivity to L-asparaginase. *Experimental Hematology* [Internet]. 2012 Aug [cited 2018 Apr 19];40(8):657–65. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/22542578>

20. Hung YP, Teragawa C, Kosaisawe N, Gillies TE, Pargett M, Minguet M, et al. Akt regulation of glycolysis mediates bioenergetic stability in epithelial cells. *eLife* [Internet]. 2017 [cited 2019 Oct 8];6. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/29239720>
21. Miller DM, Thomas SD, Islam A, Muench D, Sedoris K. c-Myc and cancer metabolism. *Clinical cancer research: an official journal of the American Association for Cancer Research* [Internet]. 2012 Oct 15 [cited 2019 Oct 8];18(20):5546–53. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/23071356>
22. Shin Y-K, Yoo BC, Chang HJ, Jeon E, Hong S-H, Jung M-S, et al. Down-regulation of Mitochondrial F₁F₀-ATP Synthase in Human Colon Cancer Cells with Induced 5-Fluorouracil Resistance. *Cancer Research* [Internet]. 2005 Apr 15 [cited 2019 Oct 8];65(8):3162–70. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/15833846>
23. Perry SW, Norman JP, Barbieri J, Brown EB, Harris A. *Gradient: a Practical Usage Guide*. Biotechniques. 2011;50(2):98–115.
24. Rizzari C, Conter V, Starý J, Colombini A, Moericke A, Schrappe M. Optimizing asparaginase therapy for acute lymphoblastic leukemia. *Current Opinion in Oncology*. 2013 Mar;25:S1–9.
25. Lorenzi PL, Reinhold WC, Rudelius M, Gunsior M, Shankavaram U, Bussey KJ, et al. Asparagine synthetase as a causal, predictive biomarker for L-asparaginase activity in ovarian cancer cells. *Molecular cancer therapeutics* [Internet]. 2006;5(11):2613–23. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/17088436>
26. Knott SR V, Wagenblast E, Khan S, Kim SY, Soto M, Wagner M, et al. Asparagine bioavailability governs metastasis in a model of breast cancer. *Nature*. 2018 Feb;554(7692):378–81.
27. Panosyan EH, Wang Y, Xia P, Lee W-NP, Pak Y, Laks DR, et al. Asparagine Depletion Potentiates the Cytotoxic Effect of Chemotherapy against Brain Tumors. *Molecular Cancer Research* [Internet]. 2014 May 1 [cited 2019 Jun 14];12(5):694–702. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/24505127>
28. Yu M, Henning R, Walker A, Kim G, Perroy A, Alessandro R, et al. L-asparaginase inhibits invasive and angiogenic activity and induces autophagy in ovarian cancer. *Journal of Cellular and Molecular Medicine*. 2012 Oct;16(10):2369–78.

Declarations

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by the University Hospital Motol Ethics Committee. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Written informed consent was obtained from all individual participants included in the study.

CONSENT FOR PUBLICATION

Not applicable

COMPETING INTERESTS

The authors declare no conflicts of interest.

FUNDINGS

We would like to thank the Czech Pediatric Hematology Center. This work was supported by a Grant from the Ministry of Health (NV15–28848A), by the Ministry of Health of the Czech Republic, University Hospital Motol, Prague, Czech Republic 00064203 and by the Ministry of Education, Youth and Sports NPU I nr.LO1604. This work was also supported by the Grant Agency of the Czech Republic (16–12726S). Institutional support (IPHYS) was provided by RVO: 67985823 and purchase of the Seahorse instrument was funded by the ERDF project Mitenal CZ.2.16/3.1.00/21531.

AUTHORSHIP CONTRIBUTIONS

KH, DPR and MS performed Seahorse measurements and cell cultivation; KH, AP and TM optimized the measurements on Seahorse and analyzed the data; NAA performed DNA isolation, qPCR and flow cytometry; LH performed statistical analysis; KR performed FACS analysis; JaS was responsible for the patient sample data collection and gathered the clinical information. JuS designed the project and coordinated the study. JuS, KH, JT and MZ wrote the paper and analyzed the data. All the authors revised the manuscript and approved the final version.

ACKNOWLEDGEMENTS:

We would like to thank the Czech Pediatric Hematology Center.

Tables

Due to technical limitations, tables are only available as a download in the supplemental files section

Figures

Figure 1

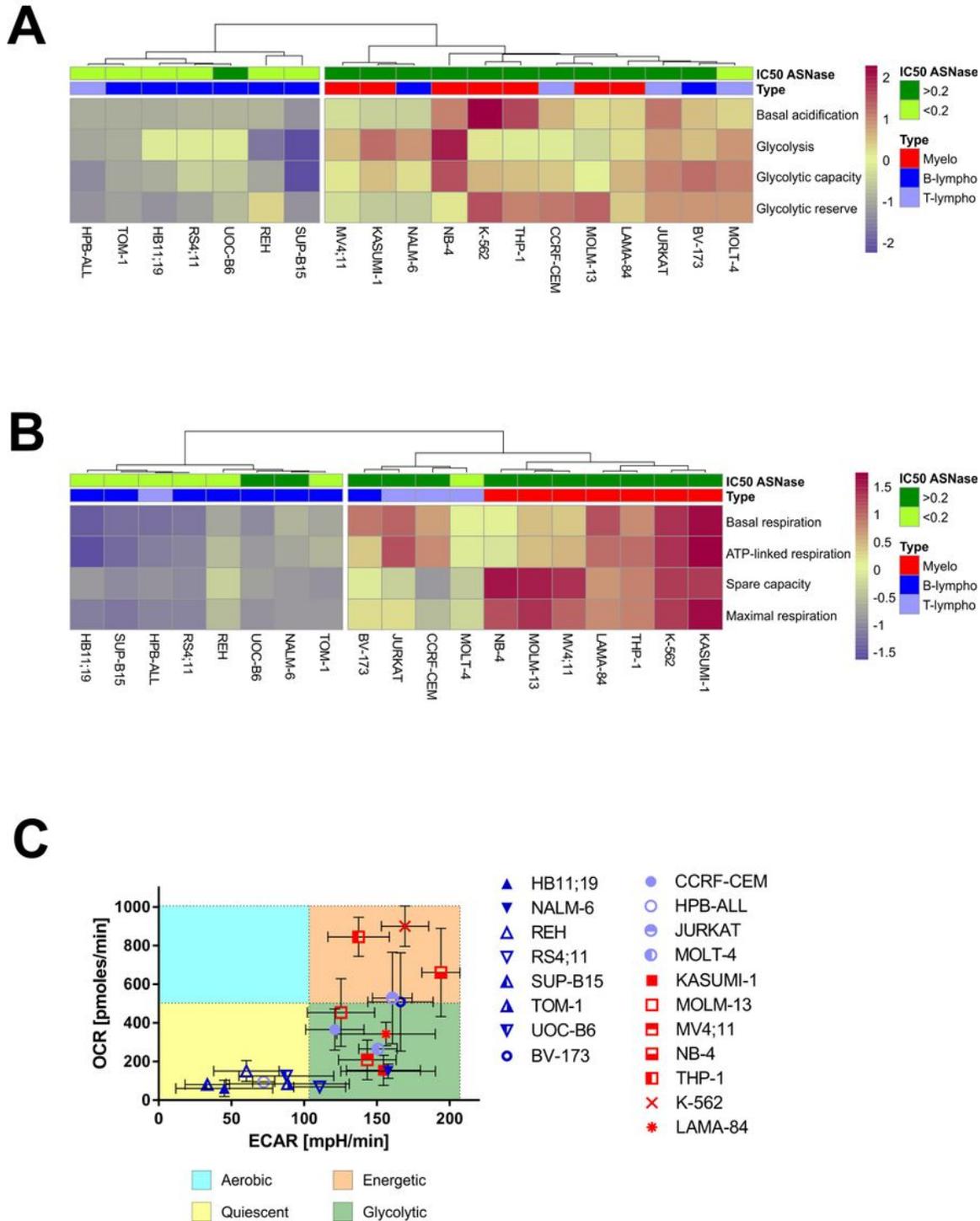


Figure 1

Basal metabolic state of leukemia cell lines (A) Hierarchical cluster analysis of leukemia cell lines based on the parameters calculated from their glycolytic function. (B) Hierarchical cluster analysis of leukemia cell lines based on the parameters calculated from their mitochondrial function. Type of leukemia and IC50 ASNase [UI/ml] are indicated for each cell line. (C) Oxygen consumption rate (OCR) level against the extracellular acidification rate (ECAR) level of the leukemia cell lines after glucose injection during the

Glycolysis Stress Test measurement. The type of leukemia is indicated in (A), (B). All the Seahorse measurements were done at least in biological triplicates and five technical replicates and are presented as a mean \pm SD.

Figure 2

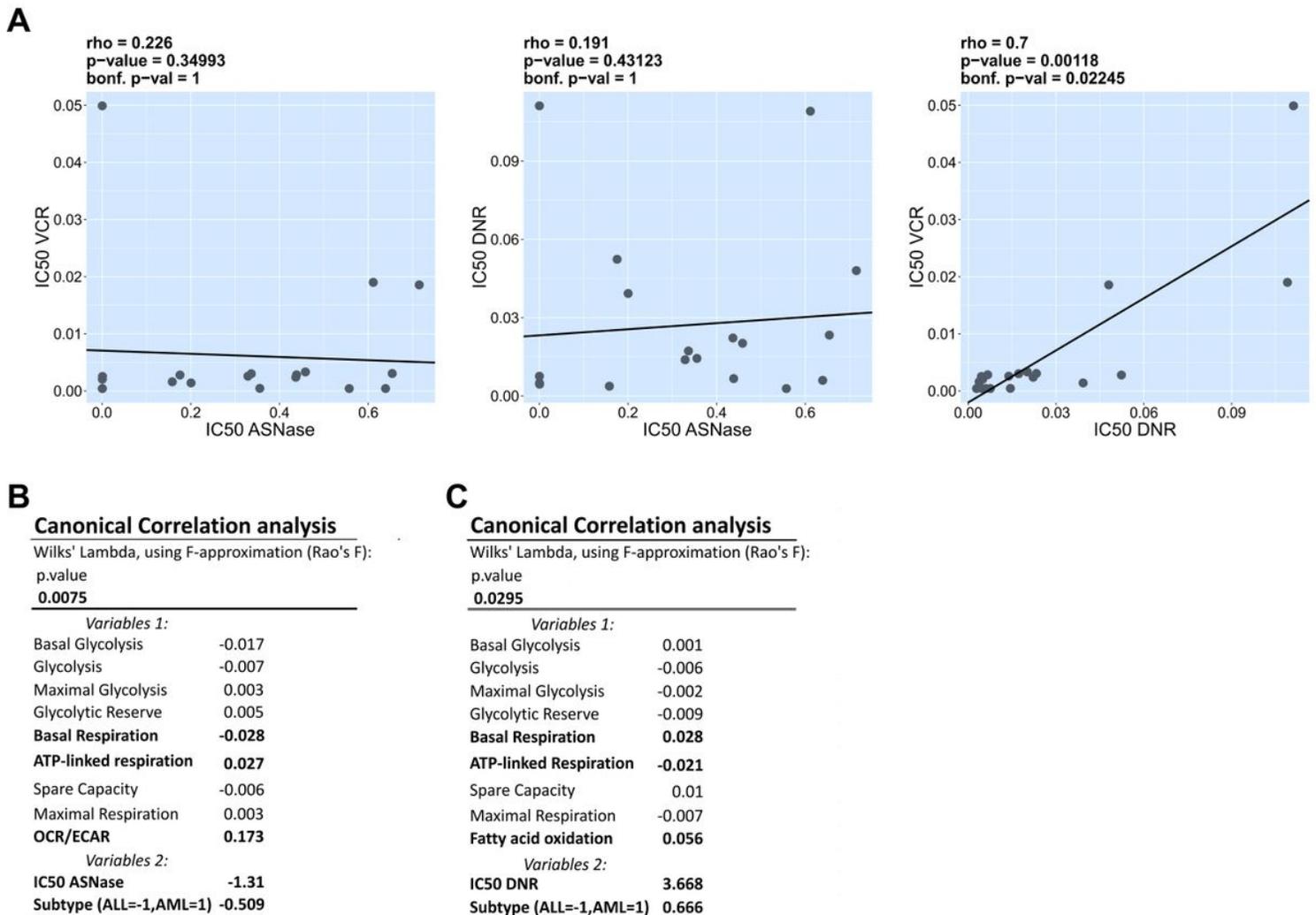
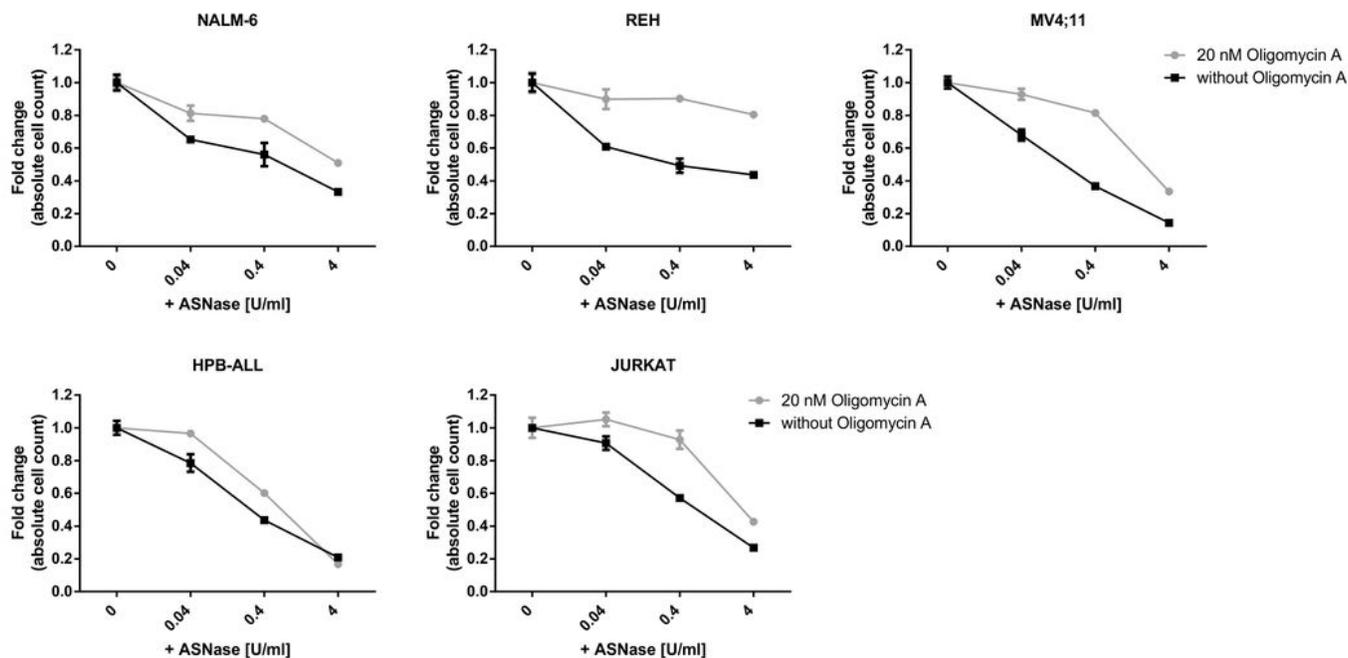


Figure 2

Association between the metabolic profile of the leukemia cells and the sensitivity to cytostatic drugs (A) Spearman rank correlation calculations of leukemia cell lines IC50 of ASNase [UI/ml] with VCR [μ M], ASNase [UI/ml] with DNR [μ M], VCR [μ M] with DNR [μ M], followed by a Bonferroni multiple comparison test. (B, C) Canonical correlation analysis: we specified all the metabolic parameters as the first set of variables and IC50 of ASNase (or IC50 of DNR) plus leukemia cell types as the second set. Variables 1, which are mostly correlated with variables 2, are highlighted. Canonical coefficients are displayed across all sets of variables. Their values can differ due to different types of parameter. Negative or positive coefficients express negative or positive correlations with parameters from the other set (B) The strongest influence from variable set 1 were OCR/ECAR, basal respiration and ATP-linked respiration and from variable set 2 were IC50 and cell type. (C) The strongest influences from variable set 1 were FAO, basal respiration and ATP-linked respiration and from variable set 2 were IC50 and cell type.

Figure 3

A



B

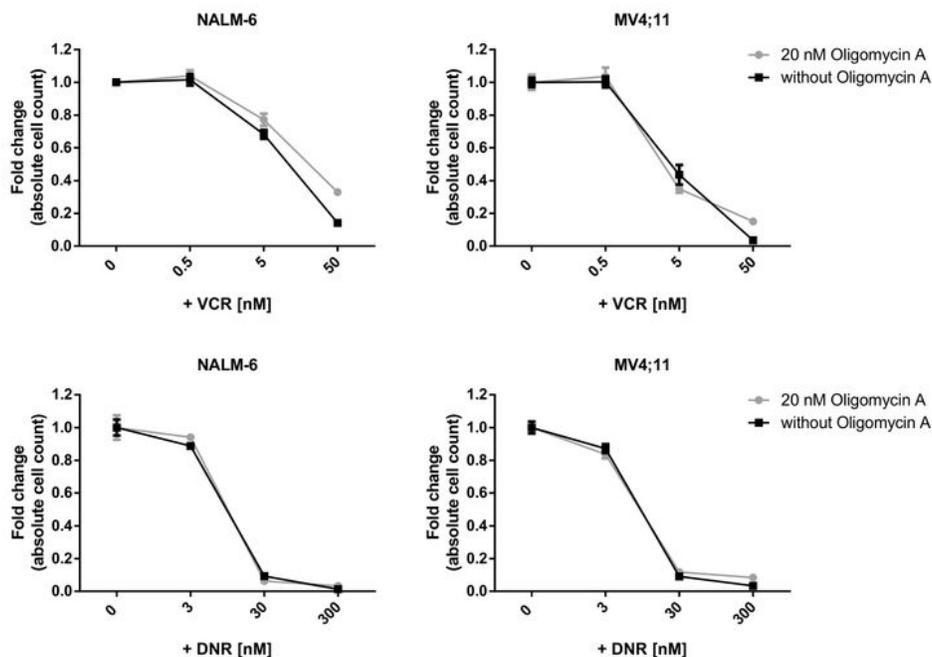


Figure 3

Functional study on the correlation between ATP synthase activity and sensitivity to ASNase (A) Effect of Oligomycin A (20 nM) on the sensitivity of leukemia cell lines (REH, NALM-6, MV4;11, JURKAT and HPB-ALL) to ASNase. (B) Effect of Oligomycin A (20 nM) on the sensitivity of leukemia cell lines (NALM-6, MV4;11) to VCR and DNR. Cells were pretreated with Oligomycin A for 1 hour or left untreated and then co-treated with ASNase (or VCR, DNR) for the indicated time (48 hours; JURKAT and HPB-ALL for 72

hours). Absolute cell counts were obtained from three independent experiments; data were normalized to untreated controls and are presented as mean \pm SD.

Figure 4

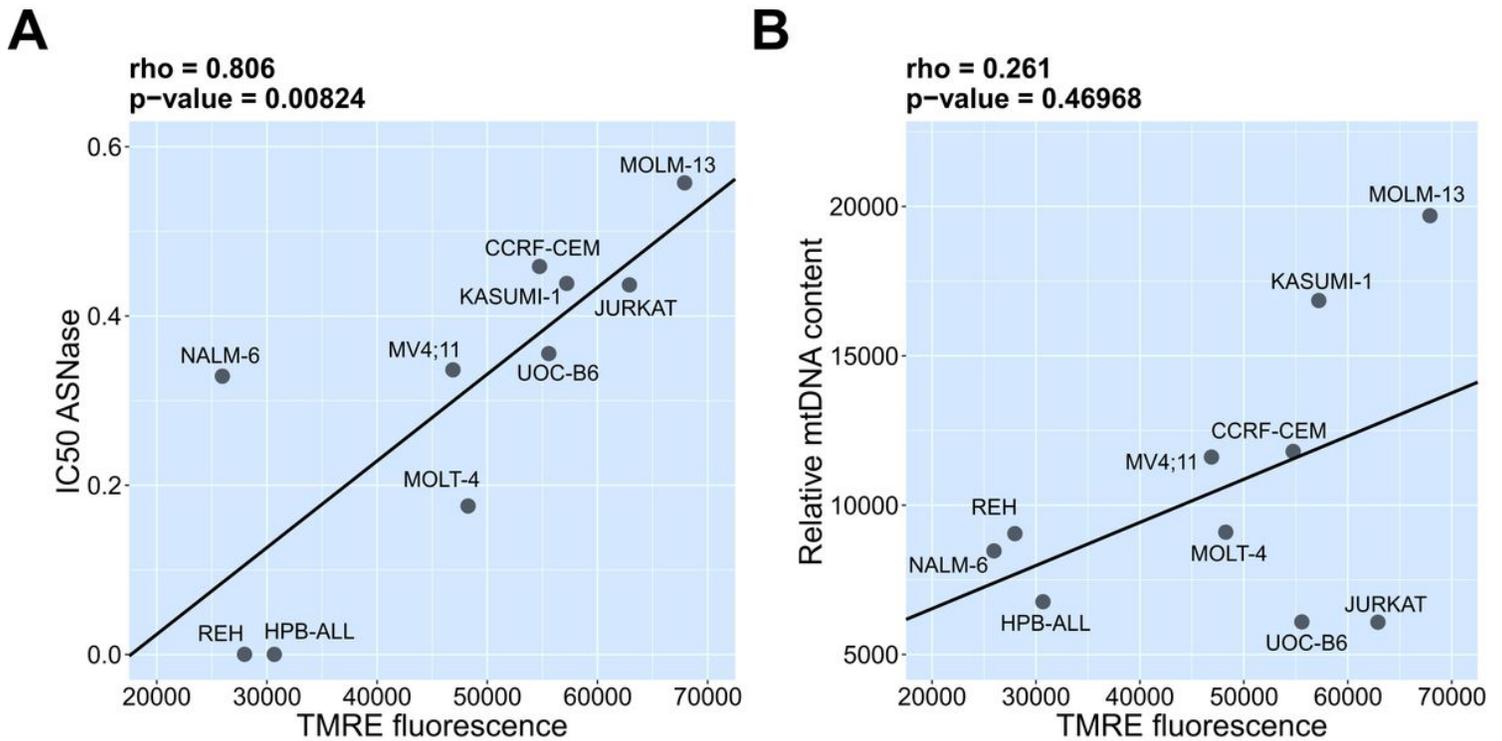
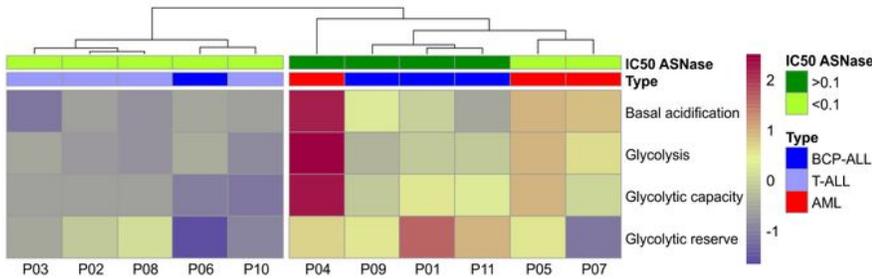


Figure 4

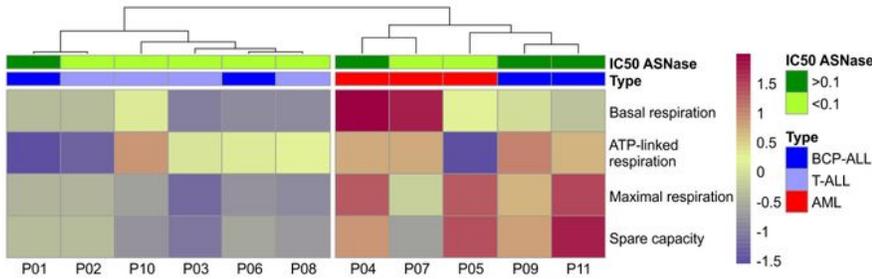
Association of mitochondrial membrane potential with sensitivity to ASNase Spearman rank correlation calculations of leukemia cell lines (NALM-6, REH, UOC-B6, CCRF-CEM, HPB ALL, JURKAT, MOLT-4, KASUMI-1, MOLM-13, MV4;11). (A) IC50 ASNase [UI/ml] with TMRE fluorescence. (B) TMRE fluorescence with relative mtDNA content.

Figure 5

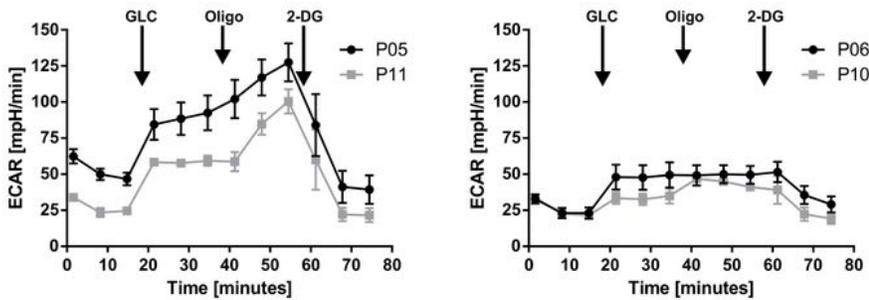
A



B



C



D

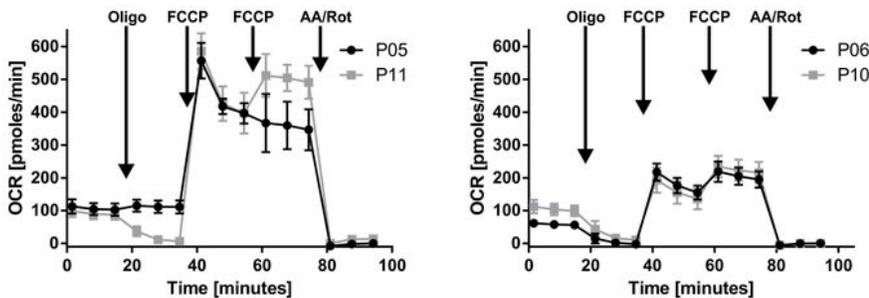


Figure 5

Characterization of the metabolic profile of leukemia patients (A) Hierarchical cluster analysis of primary leukemia cells based on parameters calculated from glycolytic function. (B) Hierarchical cluster analysis of primary leukemia cells based on parameters calculated from mitochondrial function. Type of leukemia and IC50 ASNase [UI/ml] are indicated for each patient. For more information, see Table 2. (C) Comparison of the glycolytic function courses between selected pairs of primary leukemic patients (P5–

AML, P11–BCP-ALL, P6–BCP-ALL, P10–T-ALL). (D) Comparison of the mitochondrial function courses between selected pairs of primary leukemia cells.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Sup2.jpg](#)
- [Sup1.jpg](#)
- [SupplementaryTab2.pdf](#)
- [Table2.pdf](#)
- [SupplementaryTab1.pdf](#)
- [Table1.pdf](#)
- [Sup3.jpg](#)