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Energy metabolism is co-determined by genetic variants in chronic lymphocytic leukemia and influences drug sensitivity

Junyan Lu,[1*] Martin Böttcher,[2*] Tatjana Walther,[3] Dimitrios Mougiakakos,[2] Thorsten Zenz[3,4] and Wolfgang Huber[1]

[1]European Molecular Biology Laboratory (EMBL), Heidelberg, Germany; [2]Department of Internal Medicine 5, Hematology and Oncology, University of Erlangen-Nuremberg, Erlangen, Germany; [3]Molecular Therapy in Hematology and Oncology, National Center for Tumor Diseases and German Cancer Research Centre, Heidelberg, Germany and [4]Department of Medical Oncology and Hematology, University Hospital Zürich and University of Zürich, Zürich, Switzerland

**JL and MB contributed equally to this work.*

ABSTRACT

Chronic lymphocytic leukemia cells have an altered energy metabolism compared to normal B cells. While there is a growing understanding of the molecular heterogeneity of the disease, the extent of metabolic heterogeneity and its relation to molecular heterogeneity has not been systematically studied. Here, we assessed 11 bioenergetic features, primarily reflecting cell oxidative phosphorylation and glycolytic activity, in leukemic cells from 140 chronic lymphocytic leukemia patients using metabolic flux analysis. We examined these bioenergetic features for relationships with molecular profiles (including genetic aberrations, transcriptome and methylome profiles) of the tumors, their *ex vivo* responses to a panel of 63 compounds, and with clinical data. We observed that leukemic cells with mutated immunoglobulin variable heavy-chain show significantly lower glycolytic activity than cells with unmutated immunoglobulin variable heavy-chain. Accordingly, several key glycolytic genes (*PFKP, PGAM1* and *PGK1*) were found to be down-regulated in samples harboring mutated immunoglobulin variable heavy-chain. In addition, 8q24 copy number gains, 8p12 deletions, 13q14 deletions and *ATM* mutations were identified as determinants of cellular respiration. The metabolic state of leukemic cells was associated with drug sensitivity; in particular, higher glycolytic activity was linked to increased resistance towards several drugs including rotenone, navitoclax, and orlistat. In addition, we found glycolytic capacity and glycolytic reserve to be predictors of overall survival ($P<0.05$) independently of established genetic predictors. Taken together, our study shows that heterogeneity in the energy metabolism of chronic lymphocytic leukemia cells is influenced by genetic variants and this could be therapeutically exploited in the selection of therapeutic strategies.

Correspondence:

WOLFGANG HUBER
wolfgang.huber@embl.de

THORSTEN ZENZ
thorsten.zenz@usz.ch

DIMITRIOS MOUGIAKAKOS
dimitrios.mougiakakos@uk-erlangen.de

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Introduction

Resistance to apoptosis rather than aberrant proliferation is regarded as the reason for chronic lymphocytic leukemia (CLL) cell accumulation. However, active proliferation also contributes to CLL pathogenesis, as sizable clonal birth rates were observed in this disease.[1,2] This suggests a substantial bioenergetic demand for proliferating subsets of CLL cells in order to support cell growth and division. Deregulated energy metabolism is considered to be one of the hallmarks of cancer.[3] While molecular mechanisms promoting survival and proliferation of CLL cells have been extensively studied, fewer studies have addressed energy metabolism in CLL. Garcia-Manteiga *et al.* suggested oxidative phosphorylation as the primary

Table 1. Results of multivariate Cox regression model for overall survival (n=119, events =18) by including either glycolytic reserve or glycolytic capacity as a predictor.

Multivariate Cox model including glycolytic reserve Factor	*P*	Hazard Ratio	Lower 95% CI	Upper 95% CI
Glycolytic reserve	0.033	1.10	1.00	1.20
U-CLL	0.095	3.00	0.83	11.00
Treatment	0.206	2.50	0.61	9.90
Trisomy12	0.265	2.40	0.52	11.00
Age	0.413	1.20	0.79	1.80
TP53 mutations	0.504	1.60	0.42	5.90
11q22.3 deletions	0.629	0.71	0.17	2.90
17p13 deletions	0.790	0.80	0.16	4.00
Multivariate Cox model including glycolytic capacity **Factor**	***P***	**Hazard Ratio**	**Lower 95% CI**	**Upper 95% CI**
Gycolytic capacity	0.046	1.10	1.00	1.10
U-CLL	0.101	2.90	0.81	10.00
Treatment	0.178	2.60	0.65	10.00
Trisomy12	0.312	2.20	0.48	9.70
TP53 mutation	0.469	1.70	0.42	6.50
11q22.3 deletions	0.494	0.61	0.15	2.50
Age	0.546	1.10	0.76	1.70
17p13 deletions	0.644	0.68	0.13	3.60

CI: Confidence Interval; U-CLL: chronic lymphocytic leukemia cells with unmutated IGHV genes.

source of energy.[4] This hypothesis is supported by subsequent findings that aerobic mitochondrial respiration results in high levels of oxidative stress of circulating CLL cells[5] and that targeting the respiratory machinery can be therapeutically exploited to achieve selective toxicity.[6] However, MacIntyre *et al*. reported increased concentrations of pyruvate and glutamate in serum samples from CLL patients as compared to healthy donors, which suggests active glycolysis.[7]

It has been well established that genetic heterogeneity contributes to the variable clinical outcomes of CLL. Based on the somatic mutation status in the variable regions of the immunoglobulin (Ig) heavy chain (IGHV) genes, CLL can be divided into two subgroups with distinct prognosis: CLL cells with unmutated IGHV genes (U-CLL) display higher B-cell receptor (BCR) signaling activity and are more aggressive than CLL cells with mutated IGHV genes (M-CLL). Serum samples from U-CLL patients were found to contain higher levels of lactate, fumarate, and uridine than those from M-CLL patients,[7] suggesting U-CLL cells might have higher rates of aerobic glycolysis. This finding is in line with the observation that normal B cells undergo a metabolic switch from oxidative phosphorylation towards glycolysis upon BCR stimulation.[4] However, considering the number of clinically relevant genetic alterations documented in CLL,[8,9] the relationship between genetic heterogeneity and energy metabolism remains largely unexplored. Our previous work showed that many of the recurrent mutations influence drug sensitivities of CLL.[10] As metabolic reprogramming has been shown to affect drug responsiveness of various cancers,[2,11,12] metabolism may serve as a promising target for overcoming drug resistance in CLL.

To gain a better understanding of the metabolic landscape of CLL tumor cells in relation to their genetic profile, and to determine the role of metabolism in the response to drug treatments, we assessed the bioenergetic features of primary CLL samples (n=140 patients) through extracellular flux assays investigating two major metabolic processes: 1) aerobic glycolysis; and 2) oxidative phosphorylation. We performed an integrative analysis of these data with previously recorded *ex vivo* responses of the same samples to a panel of 63 drugs, somatic genome mutations, tumor transcriptomes, DNA methylomes, and clinical data.[10] We found multiple associations between the mutational status and bioenergetic features, and found glycolysis activity of CLL cells contributed to resistance towards compounds targeting mitochondria-related biological processes that include rotenone, orlistat, venetoclax, and navitoclax. In addition, glycolytic capacity and glycolytic reserve features were shown to provide additional information to known genomic markers, such as IGHV and *TP53*, for predicting overall survival (OS).

Methods

Extracellular flux assays

Extracellular flux analyses (illustrated in *Online Supplementary Figure S1*) were performed on 152 CLL samples and nine B-cell samples from healthy donors on a Seahorse XFe96 system as previously described.[13] The resulting data files (*.asyr) were converted to comma-separated value (CSV) files using the Wave Desktop software package (Agilent/Seahorse Bioscience) and imported into R for quality assessment and further analysis. The data for 140 of the 152 CLL samples passed quality control and were used for subsequent analyses. A detailed description of the workflow and criteria for quality control are provided in the *Online Supplementary Methods*.

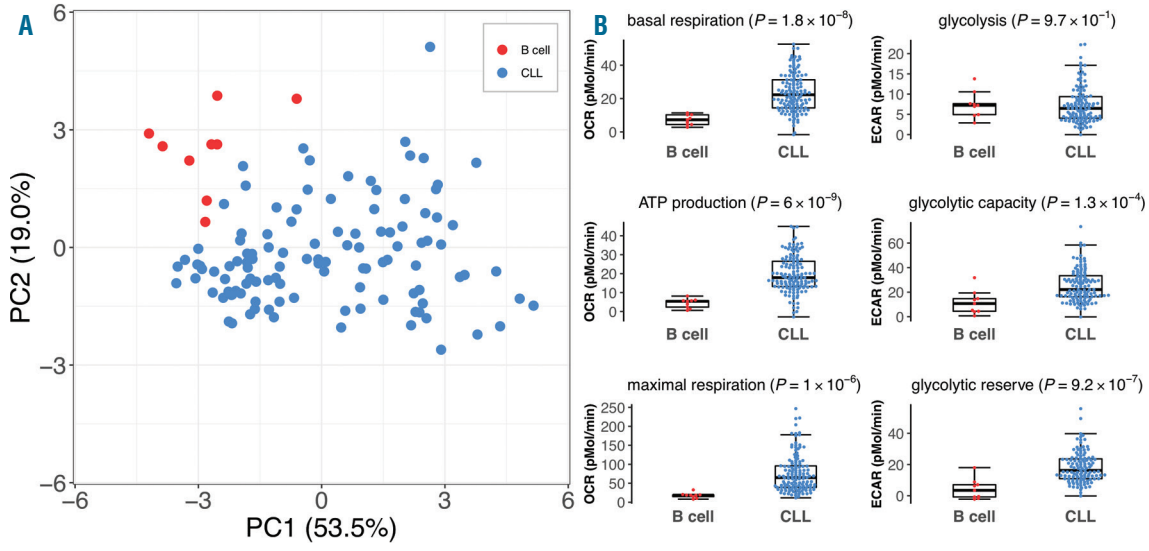

Figure 1. Difference in energy metabolism between chronic lymphocytic leukemia (CLL) cells and normal B cells. (A) Scatterplot of the top two principal components of the 11 tested bioenergetic features. Each dot represents a CLL patient sample (blue) or a healthy-donor derived B cell (red). (B) Beeswarm plots showing differences of six of the bioenergetic features between B-cell samples (n=9) and CLL samples (n=140).

Integrative data analysis

Analyses were performed using R 3.4 and included univariate association tests, multivariate regression with and without lasso penalization, Cox regression, generalized linear models, principal component analysis, and gene set enrichment analysis. For association tests between bioenergetic features and genetic variants (i.e. copy number variants and gene mutations), only those with five or more variant cases were included. Summary statistics of patients' demographic and clinical features are provided in *Online Supplementary Table S1*. All *P*-values from association tests were adjusted for multiple testing by applying the Benjamini-Hochberg procedure to control false discovery rate (FDR). Further details are provided in the *Online Supplementary Methods.*

Data availability

Our data and analysis are provided as a reader-reproducible pipeline supported by the R package seahorseCLL (*https://github.com/lujunyan1118/seahorseCLL*). An online platform based on R Shiny (*http://mozi.embl.de/public/seahorseCLL*) is also provided for reference and to visualize our dataset.

Study approval

The study was approved by the Ethics Committee Heidelberg (University of Heidelberg, Germany; S-206/2011; S-356/2013). Patients who donated tumor material provided written informed consent prior to study.

Results

Chronic lymphocytic leukemia cells and B cells show distinct energy metabolic phenotypes

We first compared the energy metabolic profiles of the 140 CLL samples and nine B-cell samples from healthy donors. In a principal component analysis (PCA) (Figure 1A), the CLL samples were clearly separated from the B-cell samples, which indicates that CLL cells have a distinct metabolic phenotype. Nine of the 11 bioenergetic features showed altered levels between CLL cells and B cells (ANOVA test, Benjamini and Hochberg multiple testing method for FDR = 5%) (*Online Supplementary Table S2*). In accordance with a previous report,[6] mitochondrial respiration-related features, including basal respiration, maximal respiration, and ATP production were increased in CLL cells (Figure 1B).

With regard to aerobic glycolysis, no significant differences were seen in basal glycolysis activity between CLL and B cells. However, CLL cells showed elevated glycolytic capacity and glycolytic reserve (Figure 1B). As these two features measure the maximum capability of cells for glycolysis and the flexibility of cells to respond to energetic demands, this observation suggests an increased adaptability of CLL cells to use glycolysis as an energy source when needed, although they do not primarily rely on it.

Molecular determinants of energy metabolism in chronic lymphocytic leukemia

Figure 1 shows a variability among the bioenergetic profiles of the CLL samples. We hypothesized that this variability may be related to the molecular heterogeneity of CLL.[8,9] Therefore, we tested the tumor-to-tumor variations of the bioenergetic features for possible correlations with 20 molecular features, including recurrent somatic mutations and copy number variations, IGHV status and methylation clusters (Figure 2A and *Online Supplementary Figure S2*).

The most prominent association identified was IGHV status: IGHV mutated CLL (M-CLL) samples had lower glycolytic activity and glycolytic capacity than IGHV unmutated CLL (U-CLL) samples (Figure 2B). Patients with M-CLL and U-CLL have been observed to have distinct serum metabolite profiles; U-CLL patients have higher lactate level in serum, which can be considered a sign of elevated glycolysis.[7] To our knowledge, our large sample size study provides the first direct proof that U-CLL do indeed have a higher glycolytic activity than M-CLL.

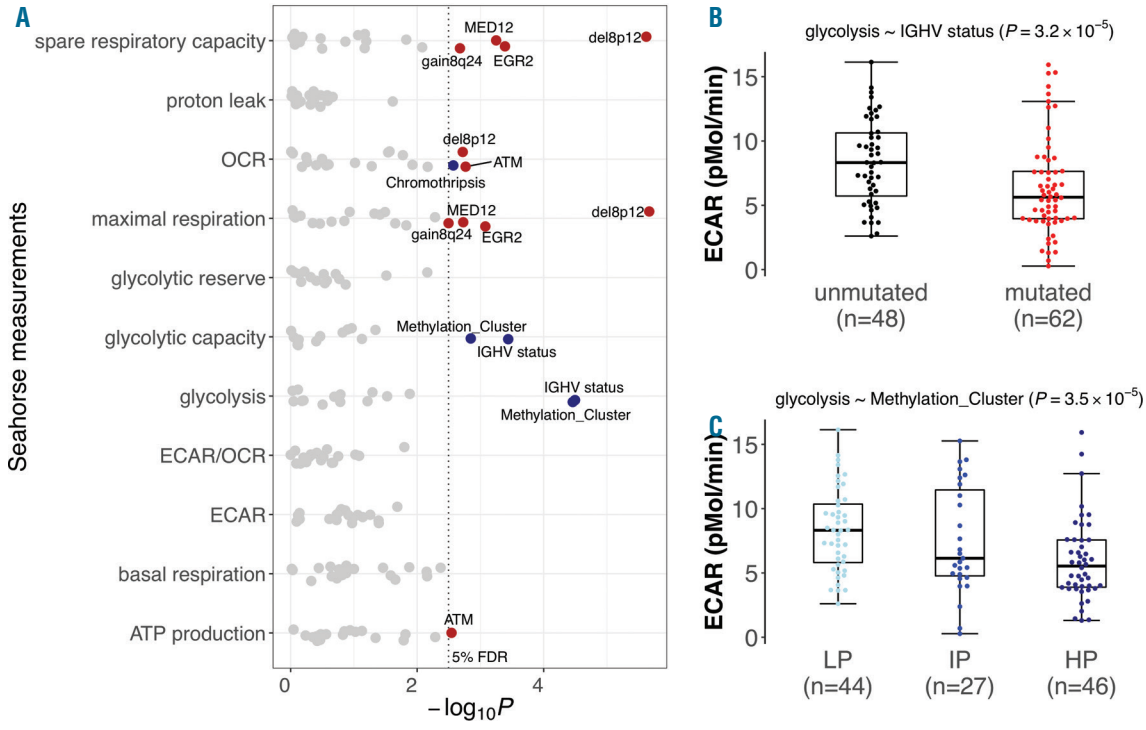

Figure 2. Associations between genetic variants and bioenergetic features. (A) The distribution of *P*-values of the associations between each genetic variant and each energy metabolic feature (ANOVA test). Gray: associations that did not pass a threshold corresponding to a 5% false discovery rate (FDR) (Benjamini and Hochberg method); red: associations with higher bioenergetic values in mutated cases; blue: associations with lower bioenergetic values in mutated cases (or high-programmed subtype). (B and C) Examples of associations, visualized in beeswarm plots. (B) Glycolysis and IGHV status. (C) Glycolysis and DNA methylation cluster.

IGHV status is strongly associated with three subtypes of CLL defined by their global levels of CpG methylation.[14] Accordingly, we found that the high-programmed CLL (HP-CLL) subtype, which has higher global methylation level, had a lower glycolysis activity than the low-programmed CLL (LP-CLL) subtype (Figure 2C).

To further dissect the role of IGHV status in metabolic reprogramming, we analyzed transcriptome data that we had measured for 120 of these patient samples (of which 111 had annotation for IGHV status). We performed gene set enrichment analysis on the genes that were differentially expressed between M-CLL and U-CLL samples using the Hallmark gene sets from Molecular Signature Database (MsigDB).[15] We found that genes down-regulated in M-CLL were enriched in the glycolysis pathway (Figure 3A). Thirty-four glycolysis-related genes were down-regulated in M-CLL (Figure 3B), including several that encode key enzymes *PFKP* (Phosphofructokinase, platelet), *PGAM1* (Phosphoglycerate Mutase 1), and *PGK1* (Phosphoglycerate kinase 1) (Figure 3C).[16-18] This analysis suggests that IGHV status directly influences the expression of genes related to glycolysis resulting in the observed difference in glycolytic parameters between M-CLL and U-CLL. As IGHV status reflects the B-cell receptor (BCR) signaling activity,[19] we referred to two published datasets for the transcriptomic signatures of BCR stimulation in CLL, either by anti-IgM antibody[20] (GEO ID: GSE49695) or unmethylated bacterial DNA (CpG) (GEO ID: GSE30105). In both conditions, genes that were up-regulated after BCR stimulation were significantly enriched in the glycolysis pathway (*Online Supplementary Figure S3*). Together these results indicate a causal link from BCR signaling to glycolysis activity in CLL, in line with previous evidence.[21,22]

We also identified several other novel associations between bioenergetic features and genetic variants (*Online Supplementary Figure S4*). Gain of 8q24, deletion of 8p12, *ATM* mutation, *EGR2* mutation and *MED12* mutation were found to be associated with higher values of respiration-related features such as ATP production and maximal respiration, while tumors with chromothripsis showed lower oxygen consumption rate (OCR) values.

Glycolytic activity contributes to drug resistance in chronic lymphocytic leukemia

Sensitivity to drugs is an informative cellular phenotype that reflects pathway dependencies of tumor cells.[10] Therefore, we asked how the 11 intrinsic bioenergetic features were related to the vulnerabilities of CLL cells towards a panel of 63 drugs applied *ex vivo*. This panel comprised clinically used drugs as well as small molecule probes of pathways important in leukemia. Using the Pearson correlation test, we identified 118 significant (FDR=10%) associations between drug sensitivities and bioenergetic features (Figure 4A and *Online Supplementary Figure S5*). Thirty-two drugs had at least one significant association with a bioenergetic feature. A significant association between a bioenergetic feature and an *ex vivo* drug response indicates that the sensitivity or resistance of CLL samples to the drug is affected by the intrinsic activity of the bioenergetic feature.

At an aggregate level, glycolysis-related features of the

CLL cells were positively correlated with the viabilities of those cells after drug treatment, while respiration-related features were negatively correlated. This suggests that higher glycolysis activity of CLL cells reduces sensitivity to drugs, while higher respiration activity contributes to increased sensitivity *ex vivo*. There were more specific patterns for drugs with different target profiles. CLL samples with higher respiration activity were more sensitive to kinase inhibitors, including the inhibitors of Bruton's tyrosine kinase (BTK), ibrutinib, and of spleen tyrosine kinase, tamatinib, both of which target the BCR pathway. In addition, two checkpoint kinase 1 (Chk1) inhibitors, AZD7762 and PF-477736, and the heat shock protein 90 (Hsp90) inhibitor AT13387 showed similar association patterns, which is in line with the report that they also target the BCR signaling cascade.[10]

Viabilities after treatment of drugs targeting mitochondria-related biological processes (rotenone, venetoclax and navitoclax) were positively correlated with the glycolysis-related features (Figure 4A and *Online Supplementary Figure S6*) for most of the drug concentrations (*Online Supplementary Figure S5*); the multivariate test results show that this finding is not merely due to confounding by IGHV status (*Online Supplementary Figure S7*). Rotenone is a mitochondrial complex I inhibitor, which disrupts the electron transport chain and thus blocks cellular respiration. Therefore, the correlation between rotenone response and glycolysis activity can be explained by the fact that higher glycolysis activity or potential (with increased metabolic flexibility) can compensate for cytotoxic effects of respiration inhibition by providing an alternative way of producing ATP. Venetoclax and navitoclax are BH3-mimetics that target the BCL2 protein and lead to mitochondrial damage and the inhibition of oxidative respiration.[23] Thus, lower reliance on oxidative respiration is a plausible explanation for the resistance to BH3-mimetics of CLL cells with high glycolysis activity. We also observed associations between glycolysis-related features and the responses to orlistat, an anti-obesity drug, which has also been identified as a pro-apoptotic agent in CLL by inhibiting lipoprotein lipase (LPL),[24] and KX2-391, an inhibitor of the proto-oncogene tyrosine-protein kinase Src (*Online Supplementary Figure S6*).

We previously showed that although drug response phenotypes of CLL cells were largely influenced by genetic variants, there was still substantial variance in the drug response phenotypes that were not explained by genetics. Thus, we asked whether the energy metabolism profile could add additional predictive information. For each drug, we built two multivariate linear regression models to predict its response profile: one included only the 20 genetic features shown in *Online Supplementary Figure S2* as predictors, the other included these genetic features plus 11 bioenergetic features. As a measure of predictive strength, we compared the variance explained (R2 value adjusted by numbers of predictors) between the two models. For most drugs, including bioenergetic features in the model did not increase explanatory power (Figure 4B, dots on diagonal line); moreover, responses to individual kinase inhibitors were well explained by the genetic features (blue dots in Figure 4B and *Online Supplementary Figure S8*). However, for five drugs, including venetoclax and rotenone, the variance explained increased by 10% or more upon inclusion of the bioenergetic features (red dots

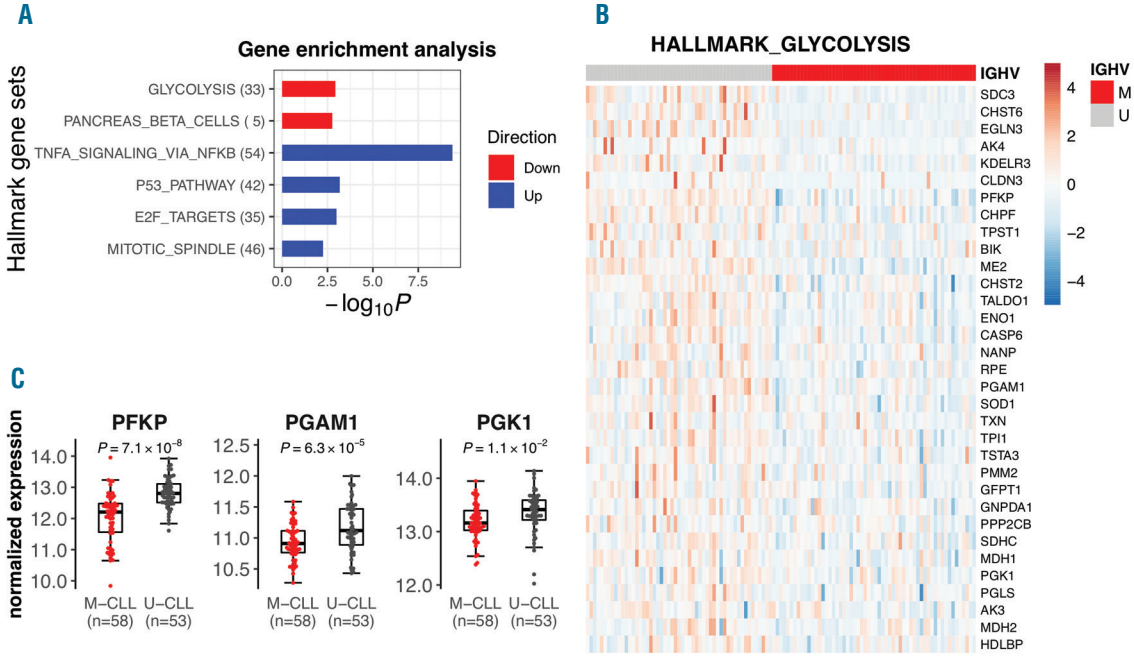

Figure 3. Genes from the glycolysis pathway are down-regulated in immunoglobulin variable heavy-chain (IGHV) gene mutated chronic lymphocytic leukemia (M-CLL) samples. (A) Hallmark gene sets that are significantly (10%; Benjamini and Hochberg method for false discovery rate) enriched among genes differentially expressed between M-CLL and unmutated CLL (U-CLL). (B) Heatmap showing z-score of the expression values of glycolysis pathway genes that are differentially expressed between M-CLL and U-CLL samples. (C) Beeswarm plots for the expression values of three key genes in the glycolysis pathway: *PFKP* (Phosphofructokinase, platelet), *PGAM1* (Phosphoglycerate Mutase 1), and *PGK1* (Phosphoglycerate kinase 1).

in Figure 4B). In addition, except for cephaeline, bioenergetic features were more significant than genetic features in the multivariate models (Figure 4C).

Association between clinical course and energy metabolism of chronic lymphocytic leukemia

The use of primary patient cells enabled us to investigate the associations between bioenergetic features with patient history or outcome in CLL. In our study cohort, 43 patients had received treatment before sample collection, in all cases with chemotherapeutic agents (*Online Supplementary Table S1*), and none of them was undergoing treatment when samples were collected. Therefore, we first asked whether these completed treatments prior to sample collection affected the energy metabolism of primary tumor samples, as studies have shown chemotherapy or targeted therapy could drive clonal evolution leading to drug resistance or oxidative stress.[25-27] We found two bioenergetic features, namely glycolytic capacity and glycolytic reserve, associated with pretreatment

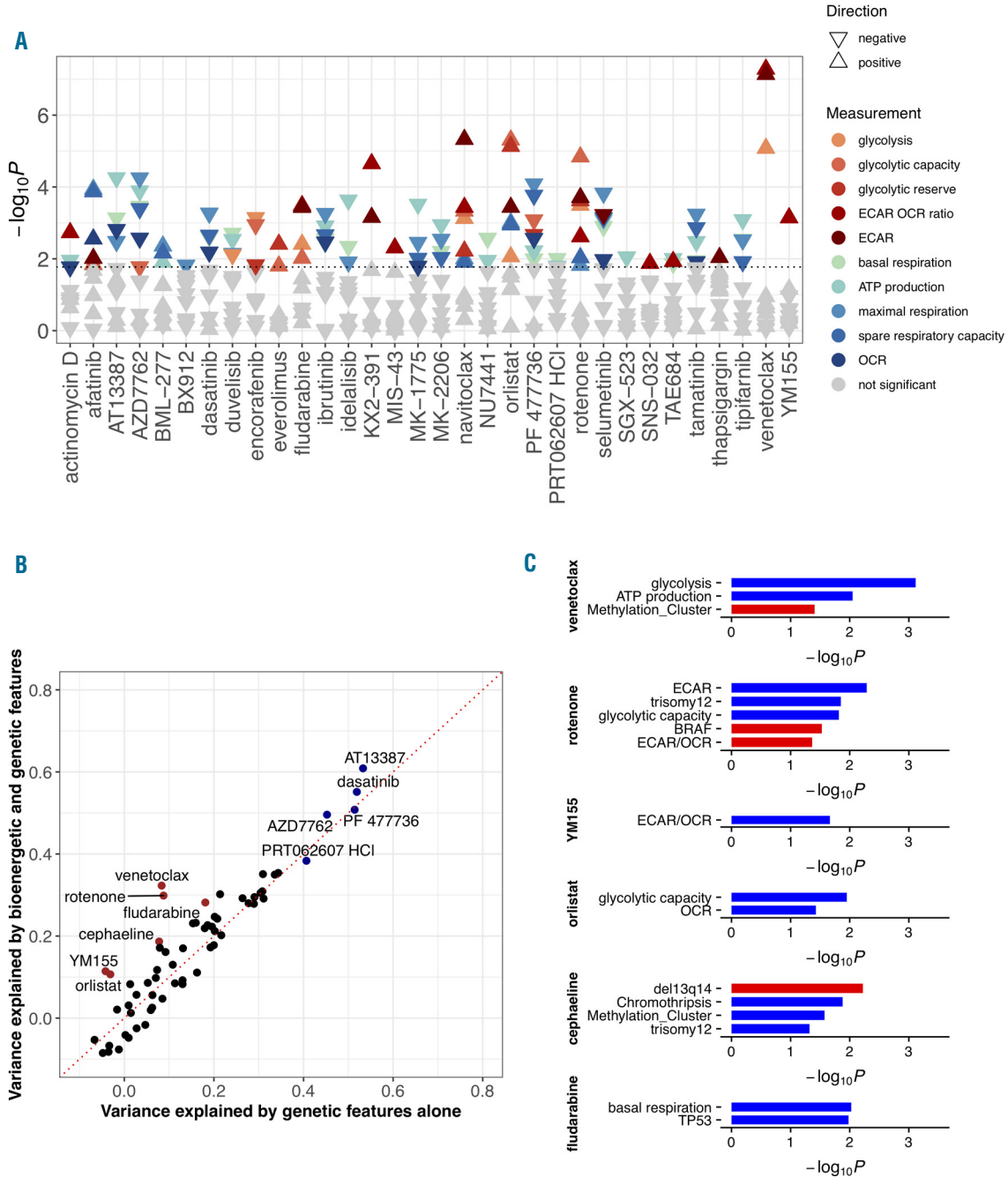

Figure 4. Correlation test results between drug response phenotypes and bioenergetic features. (A) y-axis: negative logarithm of the Pearson correlation test *P*-values. Only drugs with at least one significant association with bioenergetic features are shown (Benjamini and Hochberg method for false discovery rate (FDR)] = 10%). Viabilities across different drug concentrations were aggregated using Tukey's median polish method. Correlations with glycolysis-related features are in warm colors and correlations with respiration-related features are in cold colors. The dotted line indicates the *P*-value threshold given by the Benjamini and Hochberg method for FDR (10%). (B) Comparison of explained variance of drug responses between the multivariate model, including only genetic features, and the model including genetic and bioenergetic features. (C) Red: predictors with significant (<0.05) *P*-values in multivariate models for the drugs; red bar: a positive association with drug responses (higher drug sensitivity is associated with presence of the mutation or higher value of the bioenergetic feature); blue bar: negative association.

status at a significance threshold of $P<0.05$ (*Online Supplementary Table S3* and *Online Supplementary Figure S9*). However, pretreatment status was also highly correlated with IGHV ($P=0.0006$, χ^2 test). This reflects the fact that U-CLL patients more frequently receive treatment due to faster progression. Furthermore, glycolytic capacity and reserve are correlated with IGHV status based on our analysis (see above). Thus, to dissect confounding from more direct association, we included IGHV status as a blocking factor in a multivariate model. In this more in-depth analysis, no significant association between pretreatment status and bioenergetic features was detected ($P<0.05$). In a second analysis to assess potential roles of pretreatment status on the biology of the tumor samples, we revisited our association tests between the bioenergetic features and: i) the genetic variants; and ii) the drug responses. Including pretreatment status as a blocking factor had negligible impact on directions, strengths and *P*-values of these associations (*Online Supplementary Figure S10*). Together, these results indicate that the treatments experienced by 43 of our patients led to no detectable differences between the metabolic phenotypes of their circulating CLL cell samples and those of the other 97 patients. Therefore, we proceeded with the subsequent analysis using the combined dataset of 140 samples.

Returning to clinical outcomes, we considered two end points: time to treatment (TTT) and OS. Univariate Cox regression models indicated that glycolytic reserve, maximal respiration, and spare respiratory capacity were associated with TTT, and glycolytic capacity and glycolytic reserve were associated with OS ($P<0.05$) (*Online Supplementary Figure S11*). Samples with higher values of these features were associated with worse clinical outcomes, i.e. shorter time to treatment and OS. In multivariate Cox models including age, trisomy 12, deletion of 11q22.3, deletion of 17p13, *TP53* mutation and IGHV sta-

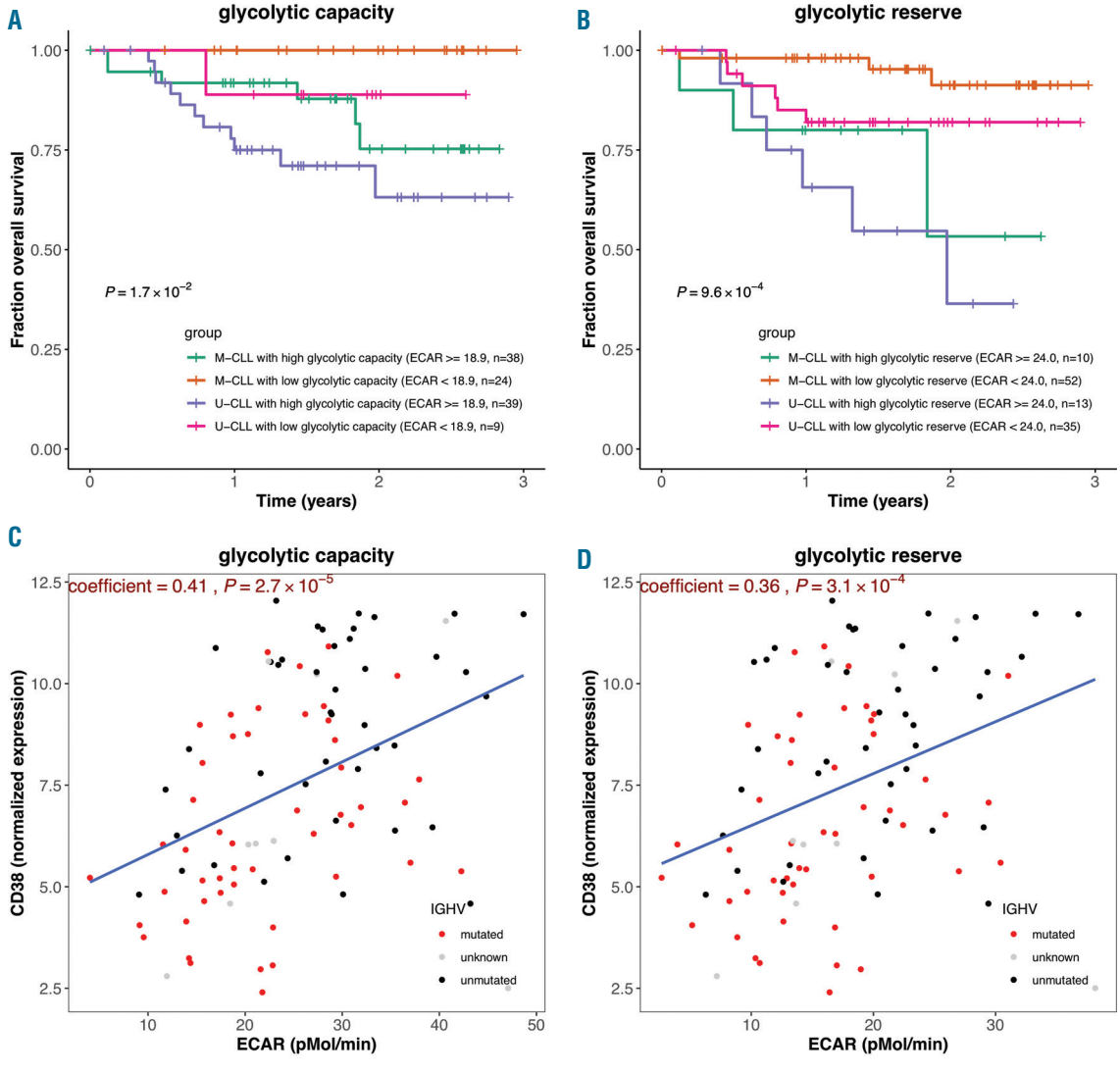

Figure 5. Associations between bioenergetic features and clinical course. (A and B) Kaplan-Meier plots for overall survival (OS) stratified by immunoglobulin variable heavy-chain (IGHV) gene status and glycolytic capacity (A) or glycolytic reserve (B). The cutoff to define high and low bioenergetic groups was estimated by maximally selected rank test. The cutoff value and number of samples in each group are shown inside the parentheses in the figure panels. (C and D) Scatter plots for associations of *CD38* expression with glycolytic capacity and glycolytic reserve.

tus as co-variates, bioenergetic features were not picked up as predictive for TTT (*Online Supplementary Tables S4-S6*). However, glycolytic capacity and glycolytic reserve were the most significant predictors for OS also in the multivariate Cox models (Table 1), indicating that these two glycolysis-related features provide additional OS-related information to established variables such as IGHV status, one of the most reliable prognostic markers in CLL. M-CLL patients with low glycolytic capacity or reserve showed best prognosis, U-CLL patients with high glycolytic capacity or reserve showed worst prognosis, while the other two groups lie in between (Figure 5A and B).

We also investigated associations of each bioenergetic feature to clinically relevant phenotypes including *CD38* expression, *CD49d* (*IGTA4*) expression, and lymphocyte doubling time (LDT), which are considered as indicators for CLL progression.[28-31] Again, we considered IGHV status as a potential confounder (*Online Supplementary Tables S7 and S8*). There were significant correlations between *CD38* gene expression with glycolytic capacity and glycolytic reserve (5% FDR) (Figure 5C and D). As well as the known fact that *CD38* expression is highly associated with IGHV status,[32] we found that it was positively correlated to glycolytic capacity or glycolytic reserve in both M-CLL and U-CLL disease subgroups (*Online Supplementary Figure S12*). This result suggests an IGHV status-independent link between *CD38* activity and adaptability of CLL cells to glycolysis as an energy source.

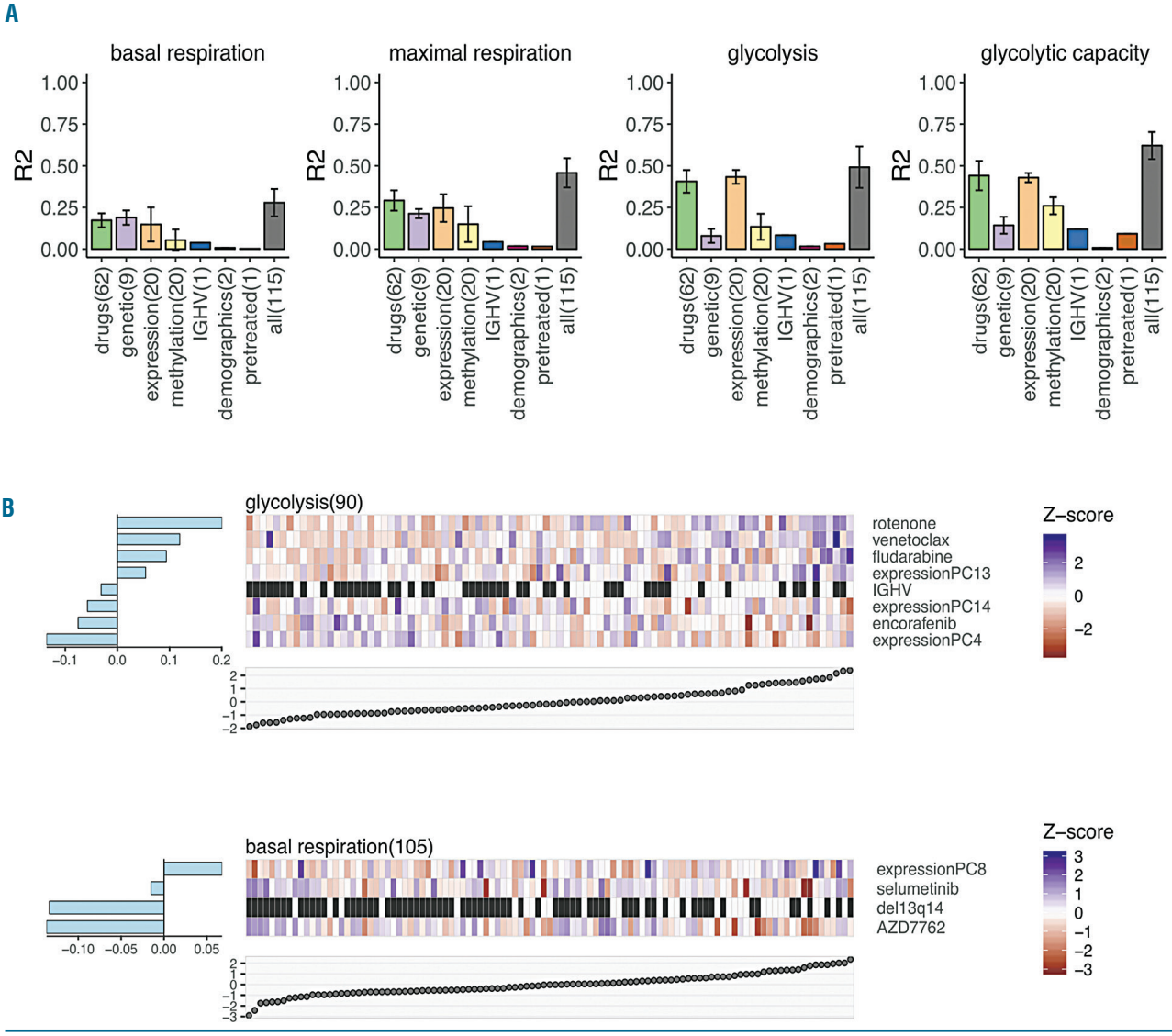

Figure 6. Multivariate regression models for energy metabolism features. (A) Explanatory power (cross-validated R^2) of datasets of different data types for the prediction of the energy metabolic features. Error bars represent standard deviations of R2 over 100 repeated cross-validations. Numbers in parentheses after dataset names indicate the number of variables in the dataset. (B) Visualization of fitted adaptive L1 (lasso) regularization multivariate models using drug responses, gene mutations, immunoglobulin variable heavy-chain (IGHV) gene status, pretreatment status, and the top 20 principal components of the gene expression (RNASeq) data. Numbers in parentheses indicate the number of samples used for the regression. (Bottom) Scatterplot of Z-scores of the energy metabolic features (i.e. centered by mean and scaled by standard deviation). (Middle) Predictor values. The continuous variables [drug responses and gene expression principal components (PC)] are shown centered and scaled using the red-white-blue color representation, the binary variables (genetic variants, IGHV status) in black and white (black: mutation present). (Left) Horizontal bars show average model coefficients over 100 repeated cross-validations. Only the features that were selected at least 80 times out of 100 repeats are shown.

The complex network of chronic lymphocytic leukemia energy metabolic predictors

While the analyses presented so far provide insights on pairwise associations between bioenergetic features and other tumor properties, we next aimed to create a systems-level map of the network of gene mutations, DNA methylation, gene expression, *ex vivo* drug responses, and bioenergetic features. We used multivariate linear regression with lasso regularization to predict each bioenergetic feature by other available biological features and measured prediction performance using cross-validated R2 (Figure 6).

We first assessed to what extent each omics data type alone, or the combination of all the datasets, explained each bioenergetic features. The gene expression data and the drug response data performed best in predicting bioenergetic features (Figure 6A). Combining all datasets slightly increased the predictive power for each metabolic feature, indicating that each set contains non-redundant information. Notably, the glycolysis-related features were better explained by the multi-omics data than the respiration-related features (Figure 6A and *Online Supplementary Figure S13*).

We visualized predictor profiles for individual bioenergetic features, focusing on the *ex vivo* drug responses, gene expressions, and genetic variants (Figure 6B and *Online Supplementary Figure S13*). In accordance with the above univariate analysis, the multivariate model identified IGHV status and response to mitochondria-targeting drugs like venetoclax and rotenone as important predictors for glycolysis-related features. In addition, *SF3B1* mutation was identified as one of the top predictors for glycolytic capacity and reserve, as its presence is associated with higher values. *SF3B1* is an mRNA splicing factor that is frequently mutated in CLL and associated with more aggressive disease and worse survival, but its oncogenic mechanism is still elusive.[33] Another genomic aberration, deletion of 13q14, was selected as one of the top predictors for basal respiration and ATP production.

Several principal components (PC) from the gene expression datasets were also identified by the multivariate modeling. PC8 was the top predictor with positive coefficient for all respiration-related features. As the genes with high positive loadings on PC8 are enriched in E2F targets, this suggests that higher expression of E2F targets associates with higher respiratory activity in CLL cells. On the other hand, PC10 was the top predictor, with negative coefficient, for maximal respiration and spare respiratory capacity (*Online Supplementary Figure S14*). Based on enrichment analysis, genes with high negative loadings on PC10 are enriched in the mTOR pathway and therefore this also suggests higher mTOR pathway activity associates with high respiration capability. These findings are in line with previous reports that E2F transcription factors and mTOR pathway are key players in regulating mitochondrial activity.[34,35]

PC 2, 4, 6 and 11 were identified as predictors for several glycolysis-related features (Figure 6B and *Online Supplementary Figure S13*). Gene set enrichment analysis highlighted TNFα-NFκB signaling as the most enriched pathway for genes with high loadings on PC2, 4 and 6 (*Online Supplementary Figure S14*). This finding is consistent with previous reports that NFκB signaling pathway controls energy homeostasis in inflammatory and cancer cells.[36] As we also found NFκB activation signatures in the two published transcriptomic profiling datasets of BCR stimulation (*Online Supplementary Figure S3*), which is in line with previous reports that BCR stimulation activate NFκB, we suggest that NFκB activation may play a role in increased glycolysis after BCR activation.[37,38]

Discussion

In this study, we identified molecular features that underlie the heterogeneity of energy metabolism in CLL and linked bioenergetic features with *ex vivo* drug responses and clinical course. We found that, although CLL cells and B cells have a similar basal glycolytic activity, CLL cells had a significantly higher glycolytic capacity and glycolytic reserve, which are both indicators for the cell's potential to switch to glycolysis as an energy source when necessary. Interestingly, we also found glycolytic capacity and reserve, but not basal glycolysis, to be novel predictors for OS in our cohort; CLL patients with higher glycolytic capacity and reserve showed worse prognosis. In addition, higher glycolytic capacity and reserve were also found to be correlated with high expression of the CD38 gene, a cell surface marker of B-cell activation and a negative prognostic marker in CLL. These observations can be viewed in the context of a recent report of the increased reliance of CLL cells on aerobic glycolysis to produce energy after a glycolytic switch induced by their contact with stromal cells.[39] Although we assayed circulating CLL cells for our study, the glycolytic capacity and reserve in the flux assay may actually measure the ability of CLL cells to adapt to glycolysis in a stimulated state, similar to the stimulation by stromal cells. Our findings thus imply that circulating CLL cells may have previously undergone such metabolic reprogramming and carry the metabolic repertoire that allows them to quickly switch to glycolysis when a suitable stimulation occurs, e.g. upon stromal contact. Our findings also suggest that the magnitude and efficiency of this switch can further impact the prognosis of CLL patients.

We showed that U-CLL has significantly higher glycolytic rates, which validates the previous hypothesis that U-CLL may have higher reliance on aerobic glycolysis due to higher BCR signaling pathway activity.[4,7] In addition, we illustrated that the glycolysis pathway is more active in U-CLL than M-CLL, accompanied by an upregulation of key enzymes regulating cellular glycolysis. This indicates that M-CLL and U-CLL have intrinsically different energy metabolisms and that the BCR signaling pathway may have a direct impact on the metabolic reprogramming. We had previously attempted to monitor circulating CLL cells *in vivo* by using fluorodeoxyglucose positron emission tomography (FDG-PET), which pinpoints anatomical locations with high rate of glycolysis.[40] This attempt failed due to insufficient sensitivity, and our results suggest that considering the difference between the M-CLL and U-CLL subtypes could increase the sensitivity of this diagnostic approach.

We found that the CLL patient samples with gain of 8q24 showed increased respiratory activity. The likely reason for this is the oncogenic activity of the extra copy of the *MYC* proto-oncogene. Previous studies have shown that *MYC* substantially contributes to mitochondrial biogenesis, and the overexpression of *MYC* leads to increased respiratory capability in several cell line models, which is

in line with our observation.[41]

In our study, we also highlighted the possibility of exploiting heterogeneity of energy metabolism to improve individualized patient care. We show that higher glycolytic flexibility can contribute to the resistance of CLL samples to treatment with drugs that affect mitochondria, such as rotenone, venetoclax, and navitoclax. We postulate that the cytotoxic effects of these drugs may partially result from restricting the energy supply by blocking cellular respiration and thus, cells with higher glycolytic potential can counteract their effect due to higher metabolic flexibility.

The current study has certain limitations. Firstly, while most of the proliferative activity of CLL cells appears in lymph node and bone marrow, in this study we only used circulating CLL cells due to the easier availability of patient material, which was instrumental in providing an adequate study size. In addition, although we observed many biologically meaningful associations, these are generally weak, as indicated by the relatively small effect sizes or correlation coefficients. While it is possible that biological variables not measured by us contribute to the heterogeneity in energy metabolism, a likely explanation could be biological noise (since we are using patient samples instead of cell lines) and technical noise of the Seahorse extracellular flux measurements, and the other assays used. Indeed, our study is, to our knowledge, the first that uses such a dynamic assay to systematically interrogate energy metabolism on such a large scale.

Taken together, our in-depth characterization of energy metabolism and integrative analyses provide valuable insights on mechanisms underlying the metabolic regulation of CLL cells, and reveal the possibilities of guiding clinical diagnosis and individualized patient care based on metabolic profiles. Our large-scale energy metabolism dataset complements the current traditional omics datasets, such as RNA sequencing, DNA sequencing, and methylation profiling, and contribute to a better understanding of CLL biology.

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References

1. Messmer BT, Messmer D, Allen SL, et al. In vivo measurements document the dynamic cellular kinetics of chronic lymphocytic leukemia B cells. J Clin Invest. 2005; 115(3):755-764.
2. van Gent R, Kater AP, Otto SA, et al. In vivo Dynamics of Stable Chronic Lymphocytic Leukemia Inversely Correlate with Somatic Hypermutation Levels and Suggest No Major Leukemic Turnover in Bone Marrow. Cancer Res. 2008;68(24):10137-10144.
3. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011; 144(5):646-674.
4. Garcia-Manteiga JM, Mari S, Godejohann M, et al. Metabolomics of B to plasma cell differentiation. J Proteome Res. 2011; 10(9):4165-4176.
5. Moran EC, Kamiguti AS, Cawley JC, Pettitt AR. Cytoprotective antioxidant activity of serum albumin and autocrine catalase in chronic lymphocytic leukaemia. Br J Haematol. 2002;116(2):316-328.
6. Jitschin R, Hofmann AD, Bruns H, et al. Mitochondrial metabolism contributes to oxidative stress and reveals therapeutic targets in chronic lymphocytic leukemia. Blood. 2014;123(17):2663v2672.
7. MacIntyre DA, Jiménez B, Lewintre EJ, et al. Serum metabolome analysis by 1H-NMR reveals differences between chronic lymphocytic leukaemia molecular subgroups. Leukemia. 2010;24(4):788-797.
8. Zenz T, Mertens D, Küppers R, Döhner H, Stilgenbauer S. From pathogenesis to treatment of chronic lymphocytic leukaemia. Nat Rev Cancer. 2010;10(1):37-50.
9. Fabbri G, Dalla-Favera R. The molecular pathogenesis of chronic lymphocytic leukaemia. Nat Rev Cancer. 2016;1 6(3):145-162.
10. Dietrich S, Oleś M, Lu J, et al. Drug-perturbation-based stratification of blood cancer. J Clin Invest. 2018;128(1):427-445.
11. Wu S-H, Bi J-F, Cloughesy T, Cavenee WK, Mischel PS. Emerging function of mTORC2 as a core regulator in glioblastoma: metabolic reprogramming and drug resistance. Cancer Biol Med. 2014; 11(4):255-263.
12. Long Y, Tsai W-B, Wangpaichitr M, et al. Arginine Deiminase Resistance in Melanoma Cells Is Associated with Metabolic Reprogramming, Glucose Dependence, and Glutamine Addiction. Mol Cancer Ther. 2013;12(11):2581-2590.
13. Böttcher M, Renner K, Berger R, et al. D-2-hydroxyglutarate interferes with HIF-1α stability skewing T-cell metabolism towards oxidative phosphorylation and impairing Th17 polarization. Oncoimmunology. 2018;e1445454.
14. Oakes CC, Seifert M, Assenov Y, et al. DNA methylation dynamics during B cell maturation underlie a continuum of disease phenotypes in chronic lymphocytic leukemia. Nat Genet. 2016;48(3):253-264.
15. Liberzon A, Birger C, Thorvaldsdóttir H, Ghandi M, Mesirov JP, Tamayo P. The Molecular Signatures Database (MSigDB) hallmark gene set collection. Cell Syst. 2015;1(6):417-425.
16. Semenza GL, Roth PH, Fang HM, Wang GL. Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. J Biol Chem. 1994; 269(38):23757-23763.
17. Hitosugi T, Zhou L, Elf S, et al. Phosphoglycerate Mutase 1 Coordinates Glycolysis and Biosynthesis to Promote Tumor Growth. Cancer Cell. 2012; 22(5):585-600.
18. Dunaway GA, Kasten TP, Sebo T, Trapp R. Analysis of the phosphofructokinase subunits and isoenzymes in human tissues. Biochem J. 1988;251(3):677-683.
19. Stevenson FK, Krysov S, Davies AJ, Steele AJ, Packham G. B-cell receptor signaling in chronic lymphocytic leukemia. Blood. 2011;118(16):4313-4320.
20. Tavolaro S, Colombo T, Chiaretti S, et al. Increased chronic lymphocytic leukemia proliferation upon IgM stimulation is sustained by the upregulation of miR-132 and miR-212. Genes Chromosom Cancer. 2015; 54(4):222-234.
21. Doughty CA, Bleiman BF, Wagner DJ, et al. Antigen receptor-mediated changes in glucose metabolism in B lymphocytes: Role of phosphatidylinositol 3-kinase signaling in the glycolytic control of growth. Blood. 2006;107(11):4458-4465.
22. Qorraj M, Bruns H, Böttcher M, et al. The PD-1/PD-L1 axis contributes to immune metabolic dysfunctions of monocytes in chronic lymphocytic leukemia. Leukemia. 2017;31(2):470-478.
23. Lagadinou ED, Sach A, Callahan K, et al. BCL-2 inhibition targets oxidative phosphorylation and selectively eradicates quiescent human leukemia stem cells. Cell Stem Cell. 2013;12(3):329-341.
24. Pallasch CP, Schwamb J, Königs S, et al. Targeting lipid metabolism by the lipoprotein lipase inhibitor orlistat results in apoptosis of B-cell chronic lymphocytic leukemia cells. Leukemia. 2008;22(3):585-592.
25. Ahn IE, Underbayev C, Albitar A, et al. Clonal evolution leading to ibrutinib resistance in chronic lymphocytic leukemia. Blood. 2017;129(11):1469-1479.
26. Udensi UK, Tchounwou PB. Dual effect of oxidative stress on leukemia cancer induction and treatment. J Exp Clin Cancer Res. 2014;33:106.

27. Malcikova J, Stano-Kozubik K, Tichy B, et al. Detailed analysis of therapy-driven clonal evolution of TP53 mutations in chronic lymphocytic leukemia. Leukemia. 2015; 29(4):877-885.
28. Bulian P, Shanafelt TD, Fegan C, et al. CD49d is the strongest flow cytometry-based predictor of overall survival in chronic lymphocytic leukemia. J Clin Oncol. 2014;32(9):897-904.
29. Ibrahim S, Keating M, Do KA, et al. CD38 expression as an important prognostic factor in B-cell chronic lymphocytic leukemia. Blood. 2001;98(1):181-186.
30. Molica S, Alberti A. Prognostic value of the lymphocyte doubling time in chronic lymphocytic leukemia. Cancer. 1987; 60(11):2712-2716.
31. Viñolas N, Reverter JC, Urbano-Ispizua A, Montserrat E, Rozman C. Lymphocyte doubling time in chronic lymphocytic leukemia: an update of its prognostic significance. Blood Cells. 1987;12(2):457-470.
32. Cruse JM, Lewis RE, Webb RN, Sanders CM, Suggs JL. Zap-70 and CD38 as predictors of IgVH mutation in CLL. Exp Mol Pathol. 2007;83(3):459-461.
33. Cazzola M, Rossi M, Malcovati L, Associazione Italiana per la Ricerca sul Cancro Gruppo Italiano Malattie Mieloproliferative. Biologic and clinical significance of somatic mutations of SF3B1 in myeloid and lymphoid neoplasms. Blood. 2013;121(2):260-269.
34. Benevolenskaya EV, Frolov MV. Emerging links between E2F control and mitochondrial function. Cancer Res. 2015;75(4):619-623.
35. Morita M, Gravel S-P, Chénard V, et al. mTORC1 controls mitochondrial activity and biogenesis through 4E-BP-dependent translational regulation. Cell Metab. 2013; 18(5):698-711.
36. Tornatore L, Thotakura AK, Bennett J, Moretti M, Franzoso G. The nuclear factor kappa B signaling pathway: integrating metabolism with inflammation. Trends Cell Biol. 2012;22(11):557-566.
37. Saba NS, Liu D, Herman SEM, et al. Pathogenic role of B-cell receptor signaling and canonical NF-κB activation in mantle cell lymphoma. Blood. 2016;128(1):82–92.
38. Weil R, Israël A. T-cell-receptor- and B-cell-receptor-mediated activation of NF-κB in lymphocytes. Curr Opin Immunol. 2004; 16(3):374-381.
39. Jitschin R, Braun M, Qorraj M, et al. Stromal cell-mediated glycolytic switch in CLL cells involves Notch-c-Myc signaling. Blood. 2015;125(22):3432-3436.
40. Karam M, Novak L, Cyriac J, Ali A, Nazeer T, Nugent F. Role of fluorine-18 fluorodeoxyglucose positron emission tomography scan in the evaluation and follow-up of patients with low-grade lymphomas. Cancer. 2006;107(1):175–183.
41. Morrish F, Hockenbery D. MYC and mitochondrial biogenesis. Cold Spring Harb Perspect Med. 2014;4(5):a014225–a014225.