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Received: December 11, 2023.
Accepted: February 29, 2024.


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Functional and multi-omics signatures of mitapivat efficacy upon activation of pyruvate kinase in red blood cells from patients with sickle cell disease

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Running title: Sickle RBC Omics upon mitapivat treatment

Acknowledgments The clinical arm of this study is part of a Cooperative Research and Development Agreement (CRADA) with Agios Pharmaceuticals, Inc., Cambridge, MA, United States and SLT (NHLBI).

Funding Clinical research was supported by the intramural divisions of NHLBI and NIDDK at NIH. AD was supported by funds by the National Heart, Lung, and Blood Institute (R01HL146442, R01HL149714, R01HL148151, R01HL161004). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Authors’ contributions KL, SLT performed the clinical trial; clinical measurements of RBC DPG, ATP, PKR protein and activity were performed by Agios and parameters of oxygen affinity (p50) and sickling kinetics (t50) were performed by QL, EBD, TC, WAE. AG, MD, DS performed omics analyses. AD elaborated the data, generated the figures and wrote the first version of the manuscript; AD, SLT and WAE edited subsequent versions; the final version of the manuscript was reviewed and approved by all co-authors.

Disclosure of Conflict of interest The clinical arm of this study is part of a Cooperative Research and Development Agreement (CRADA) with Agios Pharmaceuticals, Inc., Cambridge, MA, United States and SLT (NHLBI). However, Agios did not sponsor the omics analyses and did not influence any of the contents of this manuscript. The authors declare that AD is a founder of Omix Technologies Inc. and Altis Biosciences LLC. AD is also a consultant for Hemanext Inc and Macopharma Inc. AJW, SP and CH: Agios – employment and shareholder. Other authors have no other conflicts to disclose.

Data sharing statement All the raw data generated in this study are available in Supplementary Table 1.
**Clinical Trial Information** Samples analyzed in this study were collected as part of an open label Phase 1/2 study (NCT04610866) on the long-term safety and tolerability of mitapivat.
Abstract

Mitapivat, a pyruvate kinase (PK) activator, shows great potential as a sickle cell disease (SCD)-modifying therapy. Safety and efficacy of mitapivat as a long-term maintenance therapy is currently being evaluated in two open-label studies. Here we apply a comprehensive multi-omics approach to investigate the impact of activating PK on red blood cells (RBCs) from 15 SCD patients. HbSS patients were enrolled in one of the open label, extended studies (NCT04610866). Leuko-depleted RBCs obtained from fresh whole blood at baseline (visit 1, V1), prior to drug initiation and longitudinal time points over the course of the study were processed for multi-omics through a stepwise extraction of metabolites, lipids and proteins. Mitapivat therapy had significant effects on the metabolome, lipidome and proteome of SCD RBCs. Mitapivat decreased 2,3-diphosphoglycerate (DPG) levels, increased adenosine triphosphate (ATP) levels, and improved hematologic and sickling parameters in patients with SCD. Agreement between omics measurements and clinical measurements confirmed the specificity of mitapivat on targeting late glycolysis, with glycolytic metabolites ranking as the top correlates to parameters of hemoglobin S (HbS) oxygen affinity (p50) and sickling kinetics (t50) during treatment. Mitapivat markedly reduced levels of proteins of mitochondrial origin within 2 weeks of initiation of drug treatment, with minimal changes in the reticulocyte counts. The first six months of treatment also witnessed transient elevation of lysophosphatidylcholines and oxylipins with depletion in free fatty acids, suggestive of an effect on membrane lipid remodeling. Multi-omics analysis of RBCs identified benefits for glycolysis, as well as activation of the Lands cycle.
**Introduction**

In red blood cells (RBCs), the small molecule metabolite 2,3-diphosphoglycerate (DPG) stabilizes the deoxy (T) conformation of hemoglobin (Hb) to promote oxygen off-loading and counteract hypoxia.\(^1\) In sickle cell disease (SCD), mutation of glutamate 6 to valine in the beta subunit of hemoglobin favours the polymerization of the sickle Hb (HbS) upon deoxygenation\(^2\). Therefore, elevation of 2,3-DPG in SCD is deleterious because it promotes polymerization by stabilizing HbS fibers. High levels of DPG also promote HbS polymerization by decreasing intracellular pH\(^3,4\). DPG is an intermediate metabolite in the Rapoport-Luebering shunt off the glycolytic pathway; in the Embden-Meyerhof-Parnas glycolytic pathway, pyruvate kinase (PK) is a rate-limiting enzyme that catalyses the second adenosine triphosphate (ATP)-generating step, where phosphoenolpyruvate is converted to pyruvate\(^5\). Therapeutic enhancement of the endogenous RBC PK (PKR) activity should increase the glycolytic flux, therefore leading to increases of ATP concomitantly with decreases in DPG, both of which have anti-sickling effects. Intracellular ATP is essential for maintenance of RBC hydration and membrane integrity, which impacts the pathophysiology of SCD.\(^6\)

Mitapivat (AG-348, Agios Pharmaceuticals Inc, Cambridge, MA) is a first-in-class oral, allosteric activator of PK that was originally developed for treating patients with inherited PK deficiency (PKD) caused by mutations in the *PKLR* gene. Mitapivat is approved in the United States by the Food and Drug Administration for the treatment of hemolytic anemia in adults with PK deficiency, and in the European Union by the European Medicines Agency and in Great Britain by the Medicines and Healthcare products Regulatory Agency for the treatment of PK deficiency in adults. Its ability to enhance activity of wild type PK subsequently led to clinical trials of mitapivat in other hemolytic anemias, including thalassemia and SCD. Indeed, proof-of-concept for activating PK as a therapeutic approach was established in 2 independent studies of mitapivat, a Phase 1, open-label multiple dose ascending study\(^7\) (NCT04000165) and a Phase 2 open-label study\(^8\) ([www.trialregister.nl](http://www.trialregister.nl) NL8517). In both studies, mitapivat improved hematologic parameters, increased ATP and decreased DPG levels with decreased sickling\(^7,8\). Safety and efficacy of mitapivat as a long-term maintenance therapy for patients with SCD is currently being evaluated in both studies. In the present study, we apply a comprehensive multi-omics approach\(^9-11\) to investigate the impact of activating PK on RBCs from SCD patients on mitapivat therapy in the extended study NCT04610866. The rationale for these omics analyses
was to test for the metabolic effects of mitapivat on late glycolysis and other pathways, including ATP synthesis and redox status of the sickle RBC cytosol and membrane (lipidome). At the same time, proteomics analyses afforded the opportunity to monitor for changes in PK levels, while also monitoring the impact on the proteome and (ATP-dependent phosphoproteome) as a whole.

**Methods**

**Study design and Preparation of Blood Samples**

This study evaluated one of the exploratory endpoints in an open label Phase 1/2 study (NCT04610866), i.e., the long-term safety and tolerability of mitapivat. This study was approved by the National Heart, Lung, and Blood Institute Institutional Review Board and was performed in accordance with the Declaration of Helsinki. Blood samples for ex-vivo studies were obtained from 15 patients with HbSS enrolled in the study. Patients were all adult (age >18 years) with confirmed SCD (HbSS) and baseline hemoglobin (Hb) 7.1 - 10.5 g/dL, with no recent blood transfusions, erythropoietin therapy, or changes in SCD-specific therapies including hydroxyurea (HU) and L-glutamine. All patients started mitapivat at 50 mg twice daily (BID), escalating after 4 weeks (wks) to 100 mg BID; dose adjustments were performed for safety and tolerability, per Principal Investigator (PI) discretion. At the time of data cutoff (March 23, 2023), all 15 patients completed the core period of 24 wks (visit 6, V6), 14 patients completed 48 wks (V8), 10 patients completed 72 wks (V10) and 6 pts completed 92 wks (V12). RBCs obtained from fresh whole blood in EDTA at baseline (V1, prior to drug initiation) and longitudinal time points were collected over the course of the study. After centrifuging 6 ml whole blood at 800g for 10 minutes at room temperature, the plasma was removed and the RBC pellets were resuspended by adding 3 ml PBS. To obtain leuko-depleted (LD) RBCs, the resuspended RBCs were subjected to leukodepletion process using NEO High-Efficiency Leukocyte Reduction Filter for RBCs (Haemonetics, PA USA). Samples were flash-frozen in a ethanol and dry ice, and kept frozen at -80°C until analysis. PKR activity was measured as described. In total, 150 (6x12, 4x10, 4x8 and 1x6) timepoint samples were analyzed (Fig 1A). Sickling kinetics were measured by counting the fraction of sickled red cells as a function of time in a 384 well-plate using a machine learning method while slowly deoxygenating cells with nitrogen to 5% oxygen in the oxygen pressure- and temperature-controlled humidified chamber of a Biotek “Lionheart FX” automated microscope system (Agilent Technologies). The t50 is
the time at which 50% of the cells are sickled. Oxygen dissociation curves were measured with a Hemox Analyzer (TCS Scientific Corp). Briefly PK-R activity was measured by a coupled enzyme system with lactate dehydrogenase (LDH) in which the pyruvate produced by PK-R was reduced to lactate with the concomitant oxidation of NADH to NAD. Reaction progress was followed by a change in the oxidation state of the cofactor spectrophotometrically at 340 nm. PK-R protein levels were determined by Mesoscale Assay (MesoScale Discovery) goat anti-PKLR antibody (Aviva) and mouse anti- PKLR antibody (Abcam). SULFO-TAG goat anti-mouse (Mesoscale Discovery) was used as detection antibody.

**Omics analyses:** Omics methods and statistical analyses are extensively reported in Supplementary Methods - Extended Metabolomics, lipidomics and proteomics analyses were performed as previously described.\(^{17-19}\) Statistical analysis was conducted using MetaboAnalyst v 5.0 and RStudio (v.4.2.3). Biorender (https://www.biorender.com/) was used to generate summary vignettes.

**Results**

*Mitapivat had significant effects on the SCD RBC metabolome, lipidome and proteome*

We performed two separate analyses of the data collected on the longitudinal samples: (i) we first analyzed all 150 samples available from all visit timepoints 1-12 (V1 to V12) samples - a breakdown of biological replicates (n) available per each time point in Figure 1.A; and (ii) 90 samples for all 15 patients up to V6 (Figure 1.A). All raw omics data and elaborations are provided in Supplementary Table 1, including complete blood counts (CBC) from the patients at the timepoints analysed in the study. Unsupervised analyses of multi-omics data were performed via repeated measure ANOVA and linear models of combined metabolomics and lipidomics data (Figure 1.B), and proteomics data (Figure 1.C), respectively. These analyses identified molecules associated with mitapivat treatment, either when testing for unadjusted variables, or upon adjustments for confounders like patient-specific responses. Mitapivat levels were detected via mass spectrometry in the LDRBCs, suggesting successful drug delivery (Figure 1.D). Of note, ATP and L-carnitine levels were identified as the top two metabolites with the strongest positive and negative responses, respectively, across all patients throughout the whole duration of the study (Figure 1.D). First, we performed supervised analysis of combined multi-omics data via linear discriminant analysis (LDA). In Figure 1.E results were
plotted based on the top two major components (LDA1 and 2 – x and y, respectively), while discriminating the samples across visits along the z axis. Plotting of the same results using LDA3 as a sample clustering factor for the z axis revealed patient specific responses to the treatment, with a confounded, yet still observable clustering of the samples by visit number (Supplementary Figure 1.A). This patient specific heterogeneity can be at least in part explained by the heterogeneity of mitapivat levels as per design of the clinical protocol, as detected by mass spectrometry (Supplementary Figure 1.B). Overall, the temporal trends of omics responses to mitapivat treatment across visits was evident, as highlighted by heat map representations – especially when focusing on the top 50 metabolites/lipids (Figure 2A) or proteins (Figure 2B) by linear discriminant analyses (Supplementary Table 1). Similar results were obtained by time-series ANOVA, when focusing on the patients for whom all time points were available). Such trends are highlighted herein by line plot representations of selected top responding omics results (Supplementary Figure 1.C) and are further illustrated by the heat map representation of time series measurements across all samples at all visits (without the filter for the top significant features (Supplementary Figure 1.D).

Pathway analyses of combined multi-omics data identified multiple sub-networks of metabolites/proteins involved in glycolysis (PKR – KPYR) representing one of the nodes with the highest betweenness centrality (Figure 2.C). Additional pathways significantly affected by mitapivat included proteins of mitochondrial origin and carboxylic acids of the Krebs cycle, amino acid catabolism, especially glutaminolysis and glutathione synthesis and tryptophan/kynurenine metabolism, nucleoside metabolism and proteasome components.

Mitapivat treatment significantly impacts the levels of mitochondrial proteins

The most consistent and important finding in the proteome is the significant depletion of proteins of mitochondrial origin in LDRBCs immediately after 2 weeks of mitapivat treatment at V2 (Figure 3.A). Several components of mitochondrial electron transport chain (e.g., ATPB) or other key cytosolic enzymes (e.g., mitochondrial malate dehydrogenase – MDHM) with roles in apoptosis (e.g., cytochrome c – CYTC) all were rapidly depleted within 2 weeks of initiating mitapivat and remained low in most patients for the whole duration of the study (Figure 3.B). This effect appears to be lost for a subset of mitochondrial proteins (especially components of complex V ATP synthase; Krebs cycle enzymes isocitrate dehydrogenase and fumarate
hydratase, aspartate aminotransferase and mitochondrial elongation factors) by visit 12, but this could be due to the small sample size (Figure 3.A). Of note, these results could not be explained by changes in reticulocyte counts, since there were minimal changes in reticulocyte levels throughout the duration of the study (Supplementary Table 1; Supplementary Figure 2.A). Correlation of omics data to CBC results did not highlight a significant association between proteins of mitochondrial origin and reticulocyte counts, platelet or white blood cell counts (Supplementary Figure 2.B). Only the levels of MDH cytosolic (MDHC) and mitochondrial (MDHM) subunits were negatively and positively correlated with reticulocyte counts, though they ranked 315 and 317, respectively, in the list of omics correlates to this CBC parameter (Supplementary Figure 2.B). Despite the drop in mitochondrial proteins immediately after the first visits, the levels of corresponding carboxylic acids were transiently elevated between visits 5-6 after 24 weeks of mitapivat treatment (Supplementary Figure 3.A). As an internal validation of the omics results and caveat in the interpretation of the data, it is worth noting that thrombospondin 1 (TBSP1) and platelet factor 4 (PLF4) ranked amongst the top three positive correlate to platelet counts (Supplementary Figure 2.B).

Mitapivat significantly promotes PKR (KPYR) activity and boosts DPG consumption and ATP production in human SCD RBCs in vivo

Mechanistically, mitapivat was designed to stabilize the active conformation of PKR, thus boosting late glycolysis, with concomitant consumption of DPG and generation of ATP (Figure 4.A). Consistent with the proposed mechanism of mitapivat, our results confirmed the elevation of glycolytic metabolites upstream to DPG (hexose phosphate – isomers, fructose bisphosphate and glyceraldehyde 3-phosphate), concomitant with the reduction of DPG, phosphoglyceric (isomers) and phosphoenolpyruvate downstream to DPG. Of note, while our mass spectrometry (MS)-based approach does not distinguish between the 1,3- and 2,3-DPG isomers, the latter being by far the most abundant one in mature RBCs, MS results were in strong agreement with standard clinical measurements of 2,3-DPG via enzymatic assays (see correlation analyses below). The end products of glycolysis in RBCs, pyruvate and lactate were both significantly elevated after visits 2-3 (2-3 months interval after initiation of mitapivat treatment – Figure 4.B). Some of these changes are consistent with the elevation in the levels of multiple glycolytic enzymes, especially after visit 10 (72 weeks of treatment), suggestive of additional phenomena
focusing glycolysis beyond PKR activation. Of note, the levels of KPYR (PKR – Figure 4.C) were not found to increase with treatment, with minor albeit significant decreases after the first visit, with levels that remained constant after that time point. No significant changes were observed in other PK isoforms (KPYM – pyruvate kinase M), with elevation in other glycolytic enzymes (glyceraldehyde 3-phosphate dehydrogenase, bisphosphoglycerate mutase, phosphoglycerate kinase – Figure 4.C; but not hexokinase - HK1 or KPYR/HK1 ratios – Supplementary Figure 1.C) after visit 11. Also of note, the MS-based metabolic measurements of increased ATP (Figure 1.C) and decreased DPG (Figure 4.B) were independently validated via standard CLIA-certified clinical chemistry assays (see correlation analyses below). Elevation of lactate and consumption of DPG was further consistent with the enzymatic assay-based detection of PKR activity.

_Beyond glycolysis: mitapivat treatment significantly reduces sickle RBC acyl-carnitines, induces transient increases in pentose phosphate pathway and amino acid levels_

The metabolic pathways that were most significantly affected by mitapivat other than glycolysis were acyl-carnitine and free fatty acid metabolism (Figure 5). The levels of almost all free fatty acids decreased over the course of the treatment, while almost all acyl-carnitines transiently increased between visits 2 and 6, to decrease again afterwards, suggesting a stabilization of the Lands Cycle pathway of damaged membrane lipid remodeling (Figure 5.A-C). This consideration is in part supported by the lipidomics data, showing transient elevation of oxylipins and bile acids within the same time window (Supplementary Figure 3.B). Of note, all very-long chain (especially C20 series) acyl-carnitines showed a strong positive correlation with RBC counts, suggesting an association between RBC numbers (but not size – MCV and hemoglobin concentration) with the acyl-carnitine pool throughout the study (Supplementary Figure 2.B).

Consistent with a transient membrane remodeling phenotype at visits 2-3, altered phosphoproteomics profiles were observed – especially for structural proteins band 3 (SLC4A1) and ankyrin (ANK1), as well as functional proteins (HBA1, HBG, HBB in the heat map in Figure 5.D, HBB P.S73 is shown in Figure 5.E), transporters (the monocarboxylate transporter SLC16A1; the excitatory amino acid transporter SLC1A3) and the modulatory proteins adducin 1 and 2 (Figure 5.D). However, trends diverged for different residues, with transient elevation in phospho-Y347 of SLC4A1 corresponding to decreases in the levels of the neighboring phospho-
S349 residue (Figure 5.E). Similar observations held true for ANK1, with elevated phospho-S1666 accompanied by parallel decreases in phospho-S1671 (Figure 5.E).

In the same time window (visit 2-6 – i.e., until 6 months from treatment), all nucleotides and, even more strikingly, all free amino acids (especially glutamine, arginine, methionine) increased before decreasing again to levels comparable to pre-treatment levels by visit 10 – suggestive perhaps of altered intake from the bloodstream or increased proteolysis to remove damaged protein components, consistent with the elevation in proteasomal proteins and the increased availability of ATP to fuel energy-dependent proteasomal activity (Supplementary Figure 3.D).

In this view, it is interesting to note that after visit 2 a remarkable elevation in glutaminolysis is accompanied by a delayed elevation in the levels of the key antioxidant reduced glutathione (GSH) only by visit 4 (Supplementary Figure 3.E). Of note, kynurenine levels correlated with mitochondrial proteins, showing a depletion after visit 2, an increase at V3 and V4 followed by a decrease and then an increase again at visit 10 in a subset of patients (driving increases of the median of the line plot in Supplementary Figure 3.E).

Changes to the acyl-carnitine, free fatty acid and oxylipin pools were accompanied by widespread changes in the lipidome in almost all classes (Supplementary Figure 4.A). Indeed, all classes but diacylglycerols increased transiently after visit 2 and decreased afterwards (similar to the trends described above for the amino acids). Of all these changes, the most notable was the transient elevation of lysosphospholipids (especially lysophosphatidylcholines– LPCs) and sphingomyelins (especially SM – Supplementary Figure 4).

Omics correlates to mass spec-detected mitapivat levels in HbSS RBCs

After cataloguing the changes in the metabolome, lipidome and proteome over the course of the study, we then correlated omics findings to functional measurements, based on 2 groups of data for all 15 patients: all timepoints V1-V12 (n=150 samples) and for timepoints V1 to V6 (n=90 samples). Mass spec-based measurements of mitapivat levels in RBCs confirmed a strong positive association between mitapivat levels and glycolysis, with lactate and ATP ranking amongst the most significant positive correlates, and DPG and phosphoenolpyruvate as the top negative correlates – confirming the specificity of the treatment (Figure 6.A-C). Mitapivat levels were positively associated with the most abundant acyl-carnitines, especially palmitoyl (AC
16:0), stearoyl (AC 18:0) and oleyl (AC 18:1), confirming a potential link between membrane lipid remodeling and mitapivat (Figure 6.A-B). Similarly, mitapivat levels were negatively associated with free fatty acids and succinate. At the protein level, mitapivat was strongly negatively associated with multiple mitochondrial proteins, especially mitochondrial malate dehydrogenase (MDHM, Figure 6.D-E). On the other hand, we observed a positive association with key RBC antioxidant enzymes like peroxiredoxin 2 (PRDX2, Figure 6.F).

Unsupervised analyses confirm a significant association between mitapivat levels, PKR activity, DPG consumption and ATP levels

Clinical measurements of DPG corrected for hematocrit are strongly negatively associated with omics-measured mitapivat and ATP levels (Figure 7.A) while ATP corrected for hematocrit is positively associated with omics measured PKR activity and mitapivat levels (Figure 7.B). Clinical measurements of PKR activity also positively correlated with elevation of almost all acyl-carnitines and mitapivat (Figure 7.C). Serving as internal control for the quality of the proteomics data, KPYR (PKR protein) levels measured by mass spectrometry were identified as the top overall positive correlate to PKR levels measured in the clinical arm of the study (Figure 7.D). Decrease in p50 and increase in t50 are both anti-sickling effects; increased HbS oxygen affinity (decreased p50) indicates less very low affinity polymer in red cells, while increased t50 indicates longer delay times, allowing more cells to escape the microcirculation before HbS polymerizes and makes RBCs less flexible. Elevation in the levels and activity of KPYR mass-spec measurement was positively associated to increases in p50 (along with succinate, as well as spermidine and putrescine - Figure 7E, polyamines whose levels were positively correlated to reticulocyte counts – Supplementary Figure 2.B) and negatively associated with sickling time (t50) (Figure 7F), suggestive of functional implications of these omics changes upon mitapivat treatment.

Discussion

Clinical studies have shown that RBCs in patients with SCD have elevated DPG and a functional deficiency of ATP, which has also been observed in more recent metabolomic studies. \(^9\)-\(^11\) Recently, we reported\(^13\) that mitapivat was well-tolerated in this cohort, with beneficial effects observed with respect of several hematological parameters: above all the mean hemoglobin at 24
wks was reported to increase significantly from baseline (mean: 1.38 g/dL, SD: 0.88 g/dL; p<0.0001), with minor changes in fetal hemoglobin percentages. Here we describe results from the first multi-omics analysis of RBCs from patients with SCD being treated with mitapivat therapy for up to 2 years. We confirm the specificity of mitapivat, a PK activator, using a combination of metabolomics, proteomics, lipidomics and correlation to clinical measurements of DPG and ATP levels, PK protein and activity levels, HbS oxygen affinity (p50), and sickling kinetics (t50). We leverage state of the art high-throughput approaches, – which we had recently used to investigate the metabolome of subjects with sickle cell trait and SCD, and patients with pyruvate kinase deficiency. Our measurements confirmed that the increased RBC ATP levels are sustained during a time window of 2 years in this study. Through a combination of multi-omics approaches we show that elevated ATP levels are indeed associated with elevation in reduced glutathione pools (glutathione synthesis is an ATP-dependent process) and, above all, activation of the Lands cycle – a pathway that relies on acyl-carnitine pools to restore oxidatively damaged lipids. These findings are interesting in that they align with similar observations from metabolomics studies on dried blood spots from SCD patients on mitapivat from the SCORE trial. In this context, it is worth noting that our data suggest the presence of a transition period in which increased ATP availability is associated with depletion of free carnitine, transient increases in lysophospholipids and declines in free fatty acids, and alteration of ATP-dependent membrane protein phosphorylation profiles (e.g., SLC4A1, ANK1), suggestive of ongoing membrane lipid remodeling within the (<120 day) lifespan of the sickle RBCs originally exposed to the drug at the beginning of the treatment. Our results also suggest that replenishing depleted carnitine pools via exogenous supplementation (dietary or – better delivered – intravenously administered) of L-carnitine could be a testable intervention to complement the mitapivat regimen under evaluation in this study, at least at initiation of treatment within the first 6 months-time period. It appears plausible that the initial lipidomics profile is a readout of existing circulating irreversibly damaged sickle RBCs that will need to be completely removed from the bloodstream before a full representation of the benefits of increased ATP for mature erythrocytes derived from de novo erythropoiesis when already on mitapivat could be manifested. In this regard, it is worth noting that very-long chain acyl-carnitine levels were associated with total RBC counts, but not reticulocyte, platelet or white blood cell counts, nor to other RBC-related parameters (MCV, MCH, HCT, etc). An alternative
An explanation consistent with the data is that an extension in RBC lifespans upon mitapivat treatment would explain the generally lower carnitine pools, as carnitine reservoirs are consumed as RBCs age in circulation.35

One of the most striking and unexpected findings was the decrease in RBC mitochondrial proteins within 2 weeks of exposure to mitapivat treatment. Although some immature reticulocytes could be retained in the leuko-depleted RBC cells, we utilized the same technique for leuko-depletion utilized in a previous study36. Furthermore, the mitapivat treatment was not associated with significant changes in reticulocyte counts in this study, and mitochondrial protein levels were also not significantly associated with reticulocyte counts (with the exception of MDH isoforms). Of note, we previously showed that mature RBCs accounted for the major source of the mitochondria DNA (detected by PCR-based assay) in the leuko-depleted RBCs. Multiple groups have reported that mature sickle red cells abnormally retain mitochondria that contribute to sickle inflammatory pathology in various ways – as a source of cell-free mitochondria DNA that acts as a damage-associated molecular patterns, generation of reactive oxygen species36-38. Functionally, we had previously suggested a link between the accumulation of mitochondrial metabolites like succinate and the stabilization of transcription factors like the hypoxia-inducible factor 1alpha and its downstream targets like the pro-inflammatory cytokine interleukin 1β.39 Similarly, succinate levels are a predictor of cardiovascular function and exercise intolerance both in murine models and patients with SCD.26 Here it is interesting to observe that early decreases of proteins of potential mitochondrial origin in the mature erythrocytes were associated to transient elevation and then decreases of carboxylic acids like succinate. In this regard, we and others previously associated elevation of carboxylic acids, as well as kynurenine levels to activation of cGAS-STING-interferon signaling not just upon viral infection, but also upon age- or disease-related elevation of circulating mitochondrial DNA and RNA.40-42 In the context of SCD, we have recently associated RBC and plasma kynurenine levels to poor cardiorenal function and outlook in this patient population,9-11 as well as to elevation in basal levels of hemolysis and osmotic fragility in older healthy blood donors with higher body mass indices.43 Transient elevation of kynurenine in the earliest time visits during the trial are accompanied by ultimate declines below the initial, pre-treatment levels, suggesting a potential beneficial effect of mitapivat on this pathway.
There are several points to note in this study. First of all, here we performed a longitudinal study in SCD patients, which is more powerful than cross-sectional studies that include healthy controls, as it affords to directly monitor the impact of mitapivat in a patient-specific fashion, while controlling for factors like the complexity of a disease like SCD. However, similar omics studies are necessary to understand the impact of mitapivat in healthy controls, or to determine whether mitapivat-associated changes promote a phenotypic change towards a “healthy RBC” omics profile. While samples were buffy coat depleted and filtered to remove residual platelets and leukocytes, it is not possible to exclude cell extrinsic effects on the RBC metabolome, which is indeed influenced by metabolite uptake from plasma, as is the case for kynurenine.

Altogether, muti-omics investigation confirmed the direct benefits of activating PK in SCD, i.e., increasing ATP and decreasing DPG, clearly correlating with functional measurements of oxygen affinity (p50) and sickling kinetics (t50). The increased ATP, however, has additional beneficial effects - one of these being an almost immediate reduction in protein and metabolic markers of retention of mitochondria in the mature RBCs. Other beneficial effects shown in changes in the metabolomics-lipid-proteomics profile provide insights on a potential series of pathways that warrants further mechanistic testing in the future. Our data also suggests that – on top of glycolytic metabolites - mitochondrial proteins could be a useful biomarker for monitoring response of biochemical efficacy of activating PK as a therapeutic approach in sickle cell disease.
References


Figure Legends

Figure 1 Altersations of the metabolome in sickle red blood cells (RBCs) from patients on mitapivat

In A, an overview of the clinical study. Fifteen sickle cell patients (SS genotype) were enrolled in this clinical trial, with all 15 patients being treated for up to 6 months, 14 for a whole year and 6 up to 2 years (visit 12). RBC samples underwent multi-omics characterization (A). In B-C, linear model analysis of metabolomics+lipidomics data or proteomics data identified molecules associated with the treatments, either unadjusted (x axis) or adjusted by patient-specific responses (y axis). Highlighted metabolites (B) or proteins (C) represent the variables with the highest weights across linear discriminant analysis 1 (LDA1).

In D, line plots of mitapivat, ATP and carnitine – the very drug being administered, along with the levels of the top metabolites impacted by the treatment. In light blue, median metabolite levels across all samples, while range intervals are shown in light grey. Data are shown as peak area abundance (arbitrary unit on the y axis), while the x axis represents visits 1-12. In E, LDA identified two major components (LDA1 and 2 – x and y, respectively) discriminating samples across visits (z axis).

Figure 2 Heat map and network analysis of top metabolites and lipids or proteins affected by mitapivat treatment in sickle red blood cells. In A and B, the top 50 metabolites/lipids and proteins (based on linear discriminant analyses) impacted by mitapivat treatment are shown as a function of time (visits), respectively. A full list of these features is provided in Supplementary Table 1. Merged data from these analyses were uploaded to Omicsnet to perform combined pathway analyses (C).

Figure 3 Impact of mitapivat treatment on RBC residual mitochondrial proteins. In A, heat map of median values of peak areas for proteins identified despite the gene ontology classification as proteins of mitochondrial origin or localization. In B, selected line plots for the most significantly impacted members of this group through the course of the study.

Figure 4 Impact of mitapivat on sickle RBC glycolysis. In A, an overview of glycolysis, with highlighted the reaction catalyzed by pyruvate kinase - PKR (Uniprot name KPYR) – the target of mitapivat. In B and C, line plots for mass spectrometry-based measurements of peak areas of glycolytic metabolites and enzymes during the course of the study.

Figure 5 Sickle RBC Membrane remodeling after mitapivat treatment. Acyl-carnitines and free fatty acids are significantly impacted by mitapivat treatment (A-B). In A, heat map of the median values of each metabolite in these pathways across all subject for up to 2 years of treatment (visit 12). In B, a highlight of acyl-carnitine depletion during the course of the treatment, especially free and saturated fatty acid-conjugated acyl-carnitines (pathway overview in C). In D, longitudinal phosphoproteomics analyses suggest a transient increase in protein phosphorylation at visits 2-3. Significant changes in band 3 (SLC4A1), hemoglobin beta (HBB) and ankyrin (ANK1) were observed, with diverging trends at different S/T/Y residues (E).

Figure 6 Omics correlates to mass spec-detected mitapivat in sickle RBCs. Metabolite (A-C) and protein correlates (D-F - Spearman) to mitapivat levels in RBCs from SCD patients during a 2 year period treatment (visits 1-12; n=6) or just within the first 6 months (n=15). Volcano plots indicate Spearman correlations (x axis) and -log10 of related p-values. In C-F, line plots for selected metabolites and proteins, with Mitapivat levels (independent variable) shown on the y axis upon 90 degree rotation of the original graph for ease of visualization.

Figure 7 Multi-omics correlates to functional readouts on RBCs from SCD patients on mitapivat for 2 years. Correlates (Spearman) are shown for 2,3-DPG and ATP (CLIA-
measured) upon normalization to hematocrit (A-B); PKR activity and levels (C-D), p50 and sickling time - T50 (E-F).
Mitochondrial Proteins

Visits

- Isoform 3 of Oligoribonuclease
- Fumarate hydratase
- 10 kDa heat shock protein
- Dihydrolipoyl dehydrogenase
- Isoform 2 of Stomatin-like protein 2
- Cytochrome b–c1 complex subunit 1
- ATP synthase subunit f
- ATP–binding cassette sub-family B member 10
- Malate dehydrogenase
- ATP synthase subunit beta
- ATP synthase F(0)
- Isoform 2 of Superoxide Dismutase [Mn]
- Cytochrome b–c1 complex subunit 2
- Isoform 3 of ATP synthase subunit f
- Phosphate carrier protein
- Isocitrate dehydrogenase
- Isoform 2 of Aspartate aminotransferase
- Elongation factor Tu
- Isoform 2 of Electron transfer flavoprotein subunit alpha
- Isoform 2 of Ferrochelatase
- Oxygen–dependent coproporphyrinogen-III oxidase
- 60 kDa heat shock protein
- ATP synthase subunit alpha
- NAD(P) transhydrogenase
- Stress–70 protein

Graphs showing changes over visits for ATPB, MDHM, and CYTC.
SUPPLEMENTARY MATERIAL

Functional and multi-omics signatures of mitapivat efficacy upon activation of pyruvate kinase in red blood cells from patients with sickle cell disease

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The Supplementary Table contains all raw data and elaborations.
SUPPLEMENTARY METHODS - EXTENDED

Study design and Preparation of Blood Samples

This study evaluated one of the exploratory endpoints in an open label Phase 1/2 study (NCT04610866), i.e., the long-term safety and tolerability of mitapivat. This study was approved by the National Heart, Lung, and Blood Institute Institutional Review Board and was performed in accordance with the Declaration of Helsinki\(^1\). Blood samples for ex-vivo studies were obtained from 15 patients with HbSS enrolled in the study. Patients were all adult (age >18 years) with confirmed SCD (HbSS) and baseline hemoglobin (Hb) 7.1 - 10.5 g/dL, with no recent blood transfusions, erythropoietin therapy, or changes in SCD-specific therapies including hydroxyurea (HU) and L-glutamine.\(^2\) All patients started mitapivat at 50 mg twice daily (BID), escalating after 4 weeks (wks) to 100 mg BID; dose adjustments were performed for safety and tolerability, per Principal Investigator (PI) discretion. At the time of data cutoff (March 23, 2023), all 15 patients completed the core period of 24 wks (visit 6, V6), 14 patients completed 48 wks (V8), 10 patients completed 72 wks (V10) and 6 pts completed 92 wks (V12). RBCs obtained from fresh whole blood in EDTA at baseline (V1, prior to drug initiation) and longitudinal time points over the course of the study, were leuko-depleted (LDRBC), flash-frozen in a mixture of ethanol and dry ice, and kept frozen at -80°C until analysis. Clinical measurements of DPG and ATP (both corrected for hematocrit to obtain intra-RBC concentrations), PKR protein and activity levels, oxygen affinity (p50) and sickling kinetics (t50) were accompanied by multi-omics (metabolomics, lipidomics, and proteomics) characterization of the LDRBCs. In total, 150 (6x12, 4x10, 4x8 and 1x6) timepoint samples were analyzed (Fig 1A). Sickling kinetics were measured by counting the fraction of sickled red cells as a function of time in a 384 well-plate using a machine learning method while slowly deoxygenating cells with nitrogen to 5% oxygen in the oxygen pressure- and temperature-controlled humidified chamber of a Biotek “Lionheart FX” automated microscope system (Agilent Technologies)\(^3\). The t50 is the time at which 50% of the cells are sickled. Oxygen dissociation curves were measured with a Hemox Analyzer (TCS Scientific Corp). The p50 is the partial pressure of oxygen at which hemoglobin is 50% saturated with oxygen. After centrifuging 6 ml whole blood at 800g for 10 minutes at room temperature, the plasma was removed and the RBC pellets were resuspended by adding 3 ml PBS. To obtain leuko-depleted RBCs (LDRBCs), the resuspended RBCs were subjected to leukodepletion process using NEO High-Efficiency Leukocyte Reduction Filter for Red Blood Cells (Haemonetics, PA USA).
The collected LDRBCs were then freshly frozen in a mixture of ethanol and dry ice and kept frozen at -80°C until analysis. PKR activity was measured as described. Briefly PK-R activity was measured by a coupled enzyme system with lactate dehydrogenase (LDH) in which the pyruvate produced by PK-R was reduced to lactate with the concomitant oxidation of NADH to NAD. Reaction progress was followed by a change in the oxidation state of the cofactor spectrophotometrically at 340 nm. PK-R protein levels were determined by Mesoscale Assay (MesoScale Discovery) goat anti-PKLR antibody (Aviva) and mouse anti-PKLR antibody (Abcam). SULFO-TAG goat anti-mouse (MesoScale Discovery) was used as detection antibody. Protein level was determined by normalizing light intensity of the SULFOTAG electrochemiluminescence to lysate protein concentration.

High throughput metabolomics: Metabolomics analyses were performed as previously described. Leuko-depleted RBC (LDRBC) samples were thawed on ice and a 10 µL aliquot treated with 90 µL of ice cold 5:3:2 MeOH:MeCN:water (v/v/v) then vortexed for 30 min at 4 °C. Supernatants were clarified by centrifugation (10 min, 12,000 g, 4 °C). The resulting metabolite extracts were analyzed (10 µL per injection) by ultra-high-pressure liquid chromatography coupled to mass spectrometry (UHPLC-MS — Vanquish and Q Exactive, Thermo). Metabolites were resolved on a Phenomenex Kinetex C18 column (2.1 x 150 mm, 1.7 µm) at 45 °C using a 5-minute gradient method in positive and negative ion modes (separate runs) over the scan range 65-975 m/z exactly as previously described. Oxylipins were resolved on a Waters ACQUITY UPLC BEH C18 column (2.1 x 100 mm, 1.7 µm) at 60 °C using mobile phase (A) of 20:80:0.02 MeCN:water:formic acid (FA) and a mobile phase (B) of 20:80:0.02 MeCN:isopropanol:FA. For negative mode analysis the chromatographic gradient was as follows: 0.35 mL/min flowrate, 0% B 0-0.5 min, 25% B at 1 min, 40% B at 2.5min, 55% B at 2.6min, 70% B at 4.5 min, 100% B at 4.6-6 min, 0% B at 6.1-7 min. The Q Exactive MS was operated in negative ion mode, scanning in Full MS mode (2 μscans) from 150 to 1500 m/z at 70,000 resolution, with 4 kV spray voltage, 45 sheath gas, 15 auxiliary gas. Following data acquisition, raw files were converted to .mzXML using RawConverter, then metabolites assigned and peaks integrated using Maven (Princeton University) in conjunction with the KEGG database and an in-house standard library. Quality control was assessed as using technical replicates run at beginning, end, and middle of each sequence as previously described.
Untargeted lipidomics: Total lipids were extracted as previously described: 10 μL of LDRBCs were mixed with 90 μL of cold methanol. Samples were then briefly vortexed and incubated at -20 °C for 30 minutes. Following incubation, samples were centrifuged at 12,700 RPM for 10 minutes at 4 °C and 80 μL of supernatant was transferred to a new tube for analysis. Lipid extracts were analyzed (10 uL per injection) on a Thermo Vanquish UHPLC/Q Exactive MS system using a 5 min lipidomics gradient and a Kinetex C18 column (30 x 2.1 mm, 1.7 μm, Phenomenex) held at 50 °C. Mobile phase A: 25:75 MeCN:water with 5 mM ammonium acetate; Mobile phase B: 90:10 isopropanol:MeCN with 5 mM ammonium acetate. The gradient and flow rate were as follows: 0.3 mL/min of 10% B at 0 min, 0.3 mL/min of 95% B at 3 min, 0.3 mL/min of 95% B at 4.2 min, 0.45 mL/min 10% B at 4.3 min, 0.4 mL/min of 10% B at 4.9 min, and 0.3 mL/min of 10% B at 5 min. Samples were run in positive and negative ion modes (both ESI, separate runs) at 125 to 1500 m/z and 70,000 resolution, 4 kV spray voltage, 45 sheath gas, 25 auxiliary gas. The MS was run in data-dependent acquisition mode (ddMS²) with top10 fragmentation. Raw MS data files were searched using LipidSearch v 5.0 (Thermo).

Global proteomics: Proteomics analyses were performed as described. A volume of 10 μL of LDRBCs were lysed in 90 μL of distilled water; 5 μL of lysed LDRBCs were mixed with 45 μL of 5% SDS and then vortexed. Samples were reduced with 10 mM DTT at 55 °C for 30 min, cooled to room temperature, and then alkylated with 25 mM iodoacetamide in the dark for 30 min. Next, a final concentration of 1.2% phosphoric acid and then six volumes of binding buffer (90% methanol; 100 mM triethylammonium bicarbonate, TEAB; pH 7.1) were added to each sample. After gentle mixing, the protein solution was loaded to a S-Trap 96-well plate, spun at 1500 x g for 2 min, and the flow-through collected and reloaded onto the 96-well plate. This step was repeated three times, and then the 96-well plate was washed with 200 μL of binding buffer 3 times. Finally, 1 μg of sequencing-grade trypsin (Promega) and 125 μL of digestion buffer (50 mM TEAB) were added onto the filter and left to incubate at 37 °C for 6 h. To elute peptides, 100 μL of three different buffers were applied in sequence: (i) 50 mM TEAB, (ii) 0.2% formic acid (FA), and (iii) 50% acetonitrile with 0.2% FA. The peptide solutions were pooled, lyophilized, and resuspended in 500 μL of 0.1 % FA.
Each sample was loaded onto individual Evotips for desalting and then washed with 200 μL 0.1% FA followed by the addition of 100 μL storage solvent (0.1% FA) to keep the Evotips wet until analysis. The Evosep One system (Evosep, Odense, Denmark) was used to separate peptides on a Pepsep column, (150 um inter diameter, 15 cm) packed with ReproSil C18 1.9 μm, 120A resin. The system was coupled to a timsTOF Pro mass spectrometer (Bruker Daltonics, Bremen, Germany) via a nano-electrospray ion source (Captive Spray, Bruker Daltonics). The mass spectrometer was operated in PASEF mode. The ramp time was set to 100 ms and 10 PASEF MS/MS scans per topN acquisition cycle were acquired. MS and MS/MS spectra were recorded from m/z 100 to 1700. The ion mobility was scanned from 0.7 to 1.50 Vs/cm2. Precursors for data-dependent acquisition were isolated within ± 1 Th and fragmented with an ion mobility-dependent collision energy, which was linearly increased from 20 to 59 eV in positive mode. Low-abundance precursor ions with an intensity above a threshold of 500 counts but below a target value of 20000 counts were repeatedly scheduled and otherwise dynamically excluded for 0.4 min.

Database Searching and Protein Identification: MS/MS spectra were extracted from raw data files and converted into .mgf files using MS Convert (ProteoWizard, v. 3.0). Peptide spectral matching was performed with Mascot (v. 2.5) against the Uniprot human database. Mass tolerances were +/- 15 ppm for parent ions, and +/- 0.4 Da for fragment ions. Trypsin specificity was used, allowing for 1 missed cleavage. Protein N-terminal acetylation, isopeptide bond formation with loss of ammonia (K), peptide N-terminal pyroglutamic acid formation, phosphorylation (S,T,Y), were set as variable modifications with Cys carbamidomethylation set as a fixed modification. Scaffold (v 4.8, Proteome Software, Portland, OR, USA) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least two identified unique peptides.

Statistics and visualization: Statistical analysis was conducted using MetaboAnalyst v 5.0 and RStudio (v.4.2.3) with the following normalization to visit 0 levels and autoscale normalization (i.e., data were mean-centered and divided by the standard deviation of each variable). Line plots and volcano plots of correlations (Spearman) were generated upon analysis of the raw data via
Rstudio. Network analysis and pathway analysis were performed in OmicsNet v 2.0 using as input the significantly altered metabolites and proteins (FDR-adjusted $p < 0.05$). Biorender (https://www.biorender.com/) was used to generate summary vignettes.

**SUPPLEMENTARY REFERENCES**


Supplementary Figure 1: Alterations of the metabolome in SCD RBCs from patients on mitapivat

In A, uMAP of the results from the metabolomics analyses of RBCs from patients treated with the pyruvate kinase activator mitapivat for up to 2 years (visits 1 through 12). In B, line plots show mitapivat levels in RBCs in each one of the 15 patients over time (gray regions indicate the ranges for all patients combined over time. In C, depletion of short chain propanoyl- and butyryl carnitine are observed in SCD patients on mitapivat, while early decreases and late increases in the levels of phosphofructokinase (PFKL) are observed in this population over the time period tested in this study. In D, heat map of all the metabolic changes during the course of the study.
**Supplementary Figure 2 Omics and Complete Blood Counts (CBC)** In A, line plots show trends for CBC parameters across visits (median ± ranges for all patients). In B, correlation of omics correlates to CBC parameters. X and Y axes represent rho (Spearman) and significance (-log10(p-values)) from correlation analyses.
Supplementary Figure 3 Heat map of carboxylic acids, pentose phosphate pathway (PPP) and glutathione (A), nucleic acids (B), bile acids and oxylipins (C), amino acids and polyamines (D) in RBCs from SCD patients undergoing mitapivat treatment for up to 2 years. In E, line plot of selected metabolites from these pathways.
Supplementary Figure 4 Lipidomics analyses of RBCs from SCD patients during a 2 year period treatment with mitapivat. In A, line plots show the trends for the major lipid classes. In B, heat maps of the phospholipids, related lysophospholipids, sphingomyelins, ceramides, diacyl- and triacylglycerols.