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ARTICLE

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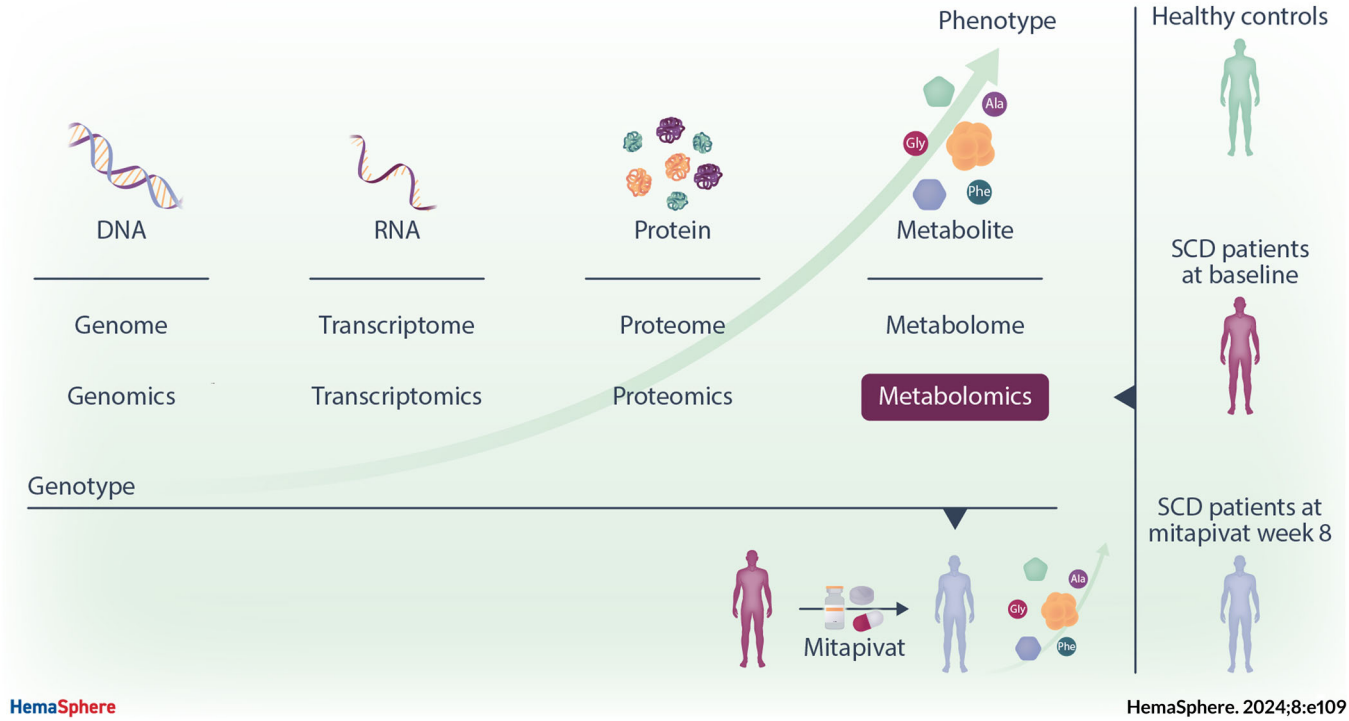
Graphical Abstract



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Correspondence: Eduard J. van Beers (E.J.vanBeers-3@umcutrecht.nl)

Abstract

Mitapivat is an investigational, oral, small-molecule allosteric activator of pyruvate kinase (PK). PK is a regulatory glycolytic enzyme that is key in providing the red blood cell (RBC) with sufficient amounts of adenosine triphosphate (ATP). In sickle cell disease (SCD), decreased 2,3-DPG levels increase the oxygen affinity of hemoglobin, thereby preventing deoxygenation and polymerization of sickle hemoglobin. The PK activator mitapivat has been shown to decrease levels of 2,3-DPG and increase levels of ATP in RBCs in patients with SCD. In this phase 2, investigator-initiated, open-label study (https://www.clinicaltrialsregister.eu/ NL8517; EudraCT 2019-003438-18), untargeted metabolomics was used to explore the overall metabolic effects of 8-week treatment with mitapivat in the dose-finding period. In total, 1773 unique metabolites were identified in dried blood spots of whole blood from ten patients with SCD and 42 healthy controls (HCs). The metabolic phenotype of patients with SCD revealed alterations in 139/1773 (7.8%) metabolites at baseline when compared to HCs (false discovery rate-adjusted $p < 0.05$), including increases of (derivatives of) polyamines, purines, and acyl carnitines. Eight-week treatment with mitapivat in nine patients with SCD altered 85/1773 (4.8%) of the total metabolites and 18/139 (12.9%) of the previously identified altered metabolites in SCD (unadjusted $p < 0.05$). Effects were observed on a broad spectrum of metabolites and were not limited to glycolytic intermediates. Our results show the relevance of metabolic profiling in SCD, not only to unravel potential pathophysiological pathways and biomarkers in multisystem diseases but also to determine the effect of treatment.

INTRODUCTION

Sickle cell disease (SCD) is one of the most common hereditary red blood cell (RBC) disorders, affecting millions of people around the world.[1] SCD is a hemoglobinopathy characterized by chronic hemolytic anemia and vaso-occlusion leading to acute, painful vaso-occlusive episodes and chronic, multiorgan complications.[2] It is a multifactorial and multisystem disease. Factors influencing the complex pathophysiology and heterogeneity include metabolic changes like decreased adenosine triphosphate (ATP) to 2,3-diphosphoglycerate (2,3-DPG)

[1]Center for Benign Hematology, Thrombosis and Hemostasis—Van Creveldkliniek, University Medical Center Utrecht, Utrecht University, Utrecht, The Netherlands
[2]Central Diagnostic Laboratory and Research, University Medical Center Utrecht, Utrecht University, Utrecht, The Netherlands
[3]Section Metabolic Diagnostics, Department of Genetics, University Medical Center Utrecht, Utrecht University, Utrecht, The Netherlands
[4]Department of Hematology, Erasmus MC, University Medical Center Rotterdam, Rotterdam, The Netherlands
[5]Department of Pediatric Hematology, Erasmus MC Sophia Children's Hospital, University Medical Center Rotterdam, Rotterdam, The Netherlands
[6]Department of Hematology, Amsterdam University Medical Center, University of Amsterdam, Amsterdam, The Netherlands
[7]Department of Blood Cell Research, Sanquin Research, Amsterdam, The Netherlands
^Myrthe J. van Dijk, Titine J. J. Ruiter, Eduard J. van Beers, and Richard van Wijk contributed equally to this study.

ratios, decreased levels of reduced glutathione (GSH), and decreased plasma levels of specific amino acids, such as arginine, ornithine, and citrulline.[3–5] Since curative treatment options remain limited, additional disease-modifying therapies apart from hydroxyurea are being developed to reduce the disease burden.[6]

One such novel potential disease-modifying therapy under development in SCD is mitapivat, an oral, small-molecule allosteric activator of pyruvate kinase (PK).[7] Recently, mitapivat was approved for treating hemolytic anemia in adults with PK deficiency. PK is a regulatory enzyme in glycolysis that converts phosphoenolpyruvate to pyruvate, yielding net ATP. In addition to increasing ATP levels, PK activation reduces glycolytic intermediates including 2,3-DPG in RBCs, thereby increasing the oxygen-affinity of hemoglobin (Hb).[8–10] Consistent with PK activation, mitapivat treatment in patients with SCD improved the ATP/2,3-DPG ratio. Previously reported results of clinical studies in SCD established proof of concept of mitapivat to reduce RBC sickling upon deoxygenation, increase Hb levels, and decrease markers of hemolysis.[8,9]

Metabolomics is a newly emerging "omics" technique that represents the large-scale, unbiased analysis of small molecules (<1500 Da), also known as metabolites, within biological cells, fluids, tissues, or organisms.[10] Metabolomics is downstream from (epi) genomics, transcriptomics, and proteomics at the bottom of the "omics" cascade, and therefore more closely related to the actual cellular phenotype than any other "omics" approach.[11] As opposed to targeted metabolomics, which studies specific metabolites that are known to be relevant, untargeted metabolomics is a hypothesis-generating discovery strategy that is used to simultaneously measure a large number of metabolites to better understand disease pathophysiology. In recent decades, untargeted metabolomics was applied for the identification of biomarkers, studying dysregulated cellular pathways, and determining metabolic responses to therapeutic interventions in various patient groups.[11–14]

Here, using an untargeted metabolomics approach, we studied the metabolic effects of mitapivat in the ESTIMATE study. This is an open-label, investigator-initiated, phase 2 study in which patients with SCD received multiple increasing doses of mitapivat in the first eight weeks of the dose-finding period. We used dried blood spots (DBS) of whole blood and demonstrated distinct metabolic profiles in patients with SCD compared to healthy controls (HCs). Furthermore, we report on the metabolic changes in patients with SCD treated with mitapivat. Lastly, our study shows the general potential of our untargeted metabolomics approach in exploring metabolic blood responses to novel therapies.

METHODS

Samples

In the ESTIMATE study, an open-label, investigator-initiated, phase 2 study, patients aged 16 years or older diagnosed with SCD (genotype HbSS, HbS/β^0-thalassemia or HbS/β^+-thalassemia) were treated with mitapivat. Eligibility criteria, study objectives, and methodology were previously reported.[8] The study was approved by the Medical Research Ethics Committee Utrecht, The Netherlands (protocol number 20-220, Netherlands Trial Register NL8517, EudraCT 2019-003438-18). All patients provided written informed consent before enrollment and all procedures were conducted in accordance with the Declaration of Helsinki. In the first 8-week dose-finding period of the study, patients took mitapivat in multiple ascending doses: from 20 mg twice daily in weeks 1 and 2, to 50 mg twice daily in weeks 3 and 4, to 100 mg twice daily from week 4 to week 8, unless dose-limiting side effects occurred. Routine laboratory tests including hematological parameters were performed at all visits. For the exploratory objective of untargeted metabolomics in DBS, whole blood from patients with SCD was collected at baseline and treatment week 8. Whole blood from pseudonymous HCs collected by our ethical-approved institutional blood donor service (protocol no. 18/774) served as control. Immediately after collecting whole blood in ethylenediaminetetraacetic acid tubes, samples were stored on ice and 50 µL aliquots of whole blood were spotted on a filter paper (Whatman Grade CF-12). All papers were left to dry in a dark place for at least 4 hours at room temperature and subsequently stored at −80°C in a foil bag with a desiccant package for further analysis. PK and hexokinase (HK) activity and PK thermostability measurements were performed at low (suboptimal) and high (V_{max}) substrate conditions (final phosphoenolpyruvate concentration 0.5 and 5 mM, respectively) on lysates of RBCs. RBCs were purified using columns with cellulose (1:1 w/w α-cellulose and cellulose type 50 [Sigma-Aldrich] in NaCl 0.9%) as described previously.[15–18] The PK/HK ratio was used to correct for the effect of RBC age-related enzymatic activity.[19]

Metabolic profiling

Sample preparation, direct-infusion high-resolution mass spectrometry (DI-HRMS) using a TriVersa NanoMate system (Advion) mounted onto the interface of a Q Exactive high-resolution mass spectrometer (Thermo Scientific) and data processing were performed as previously reported.[20,21] In short, we used a methanol-based sample extraction of 3 mm DBS (~3.1 µL whole blood) for DI-HRMS measurements. The scan range was 70–600 mass-to-charge ratio (m/z). Mass peak intensities for metabolite annotation were averaged over technical duplicates. In addition, as there is no chromatographic separation, DI-HRMS does not separate isomers and mass peak intensities therefore consist of summed intensities of these isomers. Metabolite annotation was performed using a peak calling bioinformatics pipeline developed in R programming software (version 3.6.1) based on the human metabolome database (pipeline is available on: https://github.com/UMCUGenetics/DIMS).[22]

Data analysis

To compare the metabolic profiles of the patients and HCs, annotated mass peak intensities were converted to Z-scores. These scores were calculated by the following formula:

$$Z - score = \frac{(\text{Mass peak intensity of patient or HC sample} - \text{Mean mass peak intensities of HC samples})}{\text{Standard deviation mass peak intensities of HC samples}}.$$

Data analyses of the calculated Z-scores were conducted in MetaboAnalyst (version 5.0) without further data filtering or normalization.[23] To assess the variation and separation between groups, multivariate analyses were performed to reduce the dimensionality of the data by the unsupervised principal component analysis (PCA) and supervised partial least square discriminant analysis (PLS-DA) in MetaboAnalyst. Heatmaps were created showing the most significant metabolites identified by two-sample or paired t-tests with equal group variance for Z-scores from patients with SCD at baseline versus HCs and Z-scores from patients with SCD at baseline versus treatment week 8 with mitapivat, respectively.

Additional analyses were performed in IBM SPSS Statistics (version 27.0.0.0) or GraphPad Prism (version 9.3.0; 463). We used two-sample *t*-tests (or nonparametric Mann–Whitney test) to compare patients at baseline to HCs, and paired *t*-tests (or nonparametric Wilcoxon matched-pairs signed rank test) to compare patients at baseline to treatment week 8. We analyzed hematological parameters and *Z*-scores of unique metabolites of known RBC-related pathways, such as glycolysis and the pentose phosphate pathway (PPP).[24] The predict method of the mixOmics package in R-software (version 4.3.1) was used to determine the position of patients with SCD at treatment week 8 based on the metabolites that separate patients with SCD at baseline from HCs in the PLS-DA. False discovery rate (FDR)-adjusted *p*-values were reported for unpaired data (i.e., healthy controls and patient baseline) to correct for multiple testing. Unadjusted *p*-values were reported for paired data (i.e., patient baseline and treatment week 8 *Z*-scores) and analyses in Graphpad Prism. FDR-adjusted *p*-values and unadjusted *p*-values were considered statistically significant when $p < 0.05$.

RESULTS

Baseline characteristics, hematological parameters, and PK results

Baseline DBS samples of whole blood from ten patients with SCD enrolled in the ESTIMATE study and 42 HCs, the number that was taken along in research assays for quality analysis, were obtained for untargeted metabolomics analyses. Baseline characteristics are summarized in Supporting Information S1: Table 1. Notably, 6/10 (60%) patients with SCD had concomitant hydroxyurea therapy, provided the dose was stable for ≥3 months before inclusion and also during this study period. One patient with SCD was lost to follow-up shortly after the first dosing. Therefore, nine patients with SCD were included to evaluate metabolic alterations between baseline and treatment week 8 with mitapivat.[8] Notably, one patient (SCD8) received prophylactic RBC transfusion therapy of two units because of an elective cholecystectomy on treatment day 34. This patient also needed a dose reduction from 100 to 50 mg twice daily starting from treatment week 6 because of an increase in transaminase levels. The other eight patients took 100 mg twice daily at treatment week 8. Median (interquartile range [IQR]) compliance based on pill count was 97.7% (95.3%–100.0%) during this period. Significant changes in routine hematological parameters were observed following treatment with mitapivat including increased Hb level, hematocrit, and RBC count, and decreased reticulocyte and white blood cell count (Supporting Information S1: Table 2). The PK/HK ratio and PK thermostability (V_{max} conditions) were clearly reduced in patients with SCD. Both PK and HK activities decreased to a similar extent in line with the decreased reticulocyte count after 8-week treatment with mitapivat compared to baseline. As a result, the PK/HK ratio remained unchanged (Supporting Information S1: Figure 1).

Distinct metabolic profiles of patients with SCD at baseline compared to healthy controls

Analysis of mass spectrometry peak data obtained from DBS samples of SCD patients at baseline and HCs resulted in 4071 metabolite annotations. Internal standards, lipids, and isomers were removed for final data analysis, resulting in 1773 unique metabolites. Unsupervised PCA revealed evident visual separation of metabolic profiles of SCD patients at baseline and HCs (Figure 1A). Supervised PLS-DA, taking into account the group label, confirmed robust separation of SCD patients at baseline from HCs (Figure 1B). Both analyses indicate a distinct metabolic profile for the investigated groups, with close clustering of HCs and a higher level of heterogeneity in patients with SCD. Metabolites contributing most to the separation of patients and HCs on PLS-DA, as reflected by high variable importance in projection (VIP) scores, include multiple polyamines (N1-acetylspermidine, N1-acetylspermine, spermidine, spermine), (derivatives of) purines (7-methylguanine, hypoxanthine, inosine), and acyl carnitines (propionylcarnitine, L-acetylcarnitine, linoelaidyl carnitine, L-palmitoylcarnitine) (Figure 1C and Supporting Information S1: Table 3). Several metabolites with high VIP scores were also identified as significantly different between patients with SCD and HCs by two-sample *t*-tests. The metabolic phenotype of patients with SCD revealed significant alterations in *Z*-scores of 139/1773 (7.8%) metabolites when compared to HCs (FDR-adjusted $p < 0.05$). The 25 most significant metabolites again included increases of (derivatives of) polyamines, purines, and acylcarnitines, as well as increases of fatty acid-derived mediators of inflammation (leukotriene A4) and decrease in creatinine (Figure 1D and Supporting Information S1: Table 3).

Metabolic alterations upon treatment with mitapivat in patients with SCD

PCA of untargeted metabolomics data from DBS samples of nine patients with SCD showed close clustering of each individual without clear separation of groups, indicating that the metabolome of each individual did not significantly change upon 8-week treatment with mitapivat (Figure 2A). Contrary to PCA, PLS-DA showed a clear separation between baseline and treatment week 8 samples (Figure 2B). The 25 metabolites with the highest VIP scores, responsible for the separation on PLS-DA, include acyl carnitines (propionylcarnitine, butenylcarnitine, oleoylcarnitine), purine and pyrimidine nucleotides (inosinic acid and ureidosuccinate), and niacinamide (Figure 2C Supporting Information S1: Table 4). The 8-week treatment with mitapivat significantly altered 85/1773 (4.8%) metabolite *Z*-scores compared to patients at baseline as identified by paired *t*-tests (unadjusted $p < 0.05$). Hierarchical clustering of the 25 most significantly different *Z*-scores revealed increases in lactate and ATP (Figure 2D Supporting Information S1: Table 4). Additionally, several intermediates of pyrimidine metabolism (beta-alanine, thymidine 3′,5′-cyclyc monophosphate, ureidosuccinate), amino acids and derivatives (N-phenylacetyl pyroglutamate, thiocysteine, glutamylvaline and S-phenylmercapturate) increased upon treatment, whereas levels of glycerate (oxidation product of glycerol), purine derivates (6-succinoaminopurine), and bilirubin decreased. Furthermore, several acyl carnitines were elevated in patients with SCD at baseline, of which propionylcarnitine decreased upon treatment, whereas other acyl carnitines increased compared to baseline (tetradecanoylcarnitine and malonylcarnitine).

Additionally, we analyzed how the changes in the metabolome of patients with SCD upon 8-week treatment with mitapivat related to the metabolome of the patients at baseline compared to the metabolome of HCs. We, therefore, plotted the 8-week treatment patient samples onto the PLS-DA comparing patients with SCD at baseline to HCs, which showed that patients were closer to HCs upon 8-week treatment with mitapivat (Figure 3A). Additionally, we analyzed the effects of 8-week mitapivat treatment on the previously described 139 significant different *Z*-scores of metabolites (FDR-adjusted $p < 0.05$) between patients with SCD at baseline and HCs. Paired *t*-tests showed significant alterations in 18/139 (12.9%) *Z*-scores between baseline and treatment week 8 (unadjusted $p < 0.05$), although

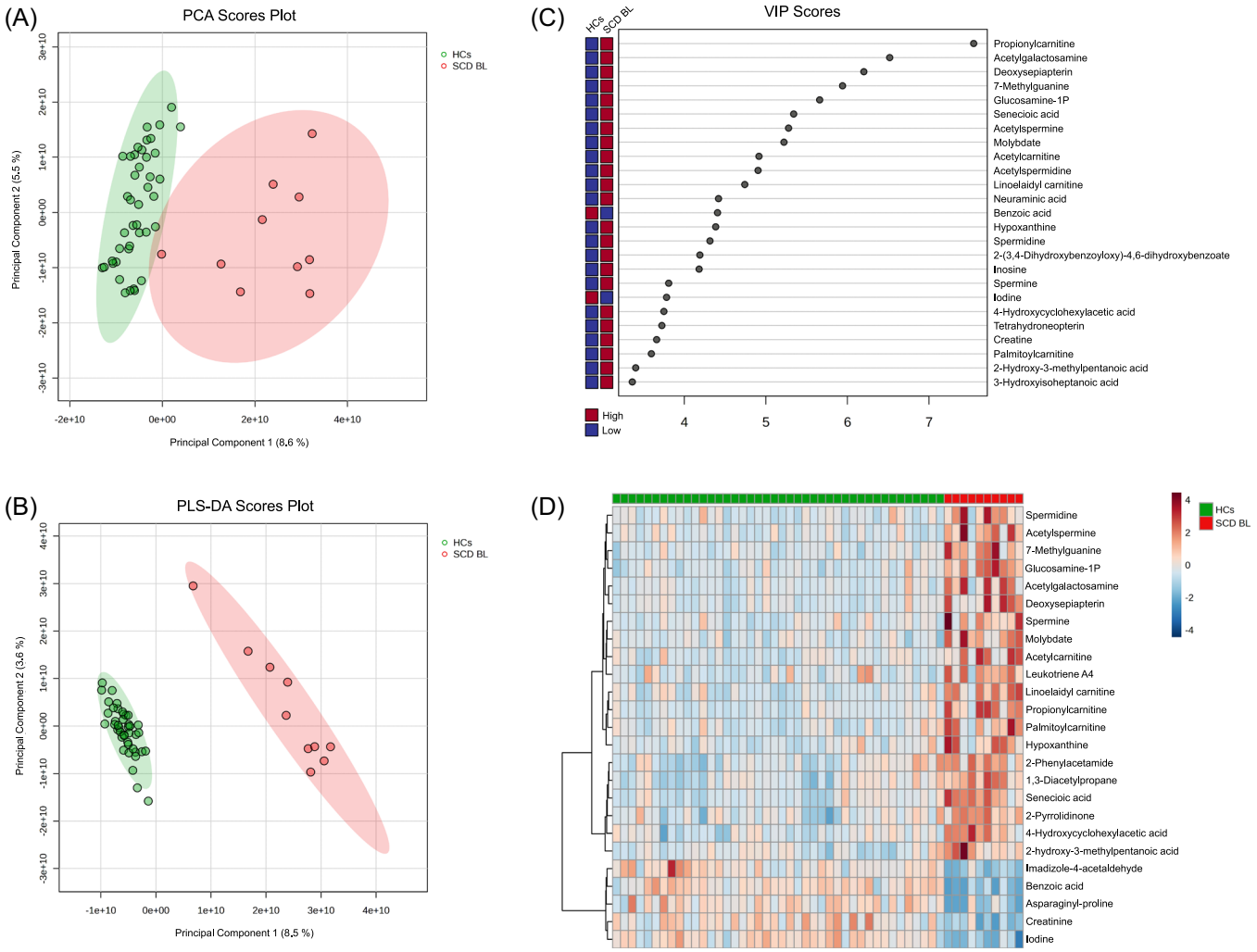

FIGURE 1 Metabolic profile in dried blood spots of whole blood from patients with sickle cell disease (SCD) and healthy controls (HCs). (A) Principal component analysis of patients with SCD at baseline (SCD BL, red) and HCs (green) displayed with 95% confidence regions. (B) Partial least square discriminant analysis (PLS-DA) of SCD BL (red) and HCs (green) displayed with 95% confidence regions. (C) Variable importance in projection (VIP)-scores demonstrating the 25 metabolites that contribute most to the separation of SCD BL and HCs on PLS-DA. (D) Heatmap of the 25 most significant metabolites identified by two-sample *t*-tests (false discovery rate [FDR] $p < 0.01E{-}06$). The heatmap was created without clustering and autoscaling of metabolites. A comprehensive overview of *p*-values and isomers is displayed in Supporting Information S1: Table 3.

again none reached an FDR-adjusted $p < 0.05$ (Figure 3B and Supporting Information S1: Table 5). Interestingly, 15/18 (83.3%) metabolites had a *Z*-score closer to zero (i.e., closer to the *Z*-scores of HCs) (Figure 3B and Supporting Information S1: Table 5). Altogether, our findings indicate that mitapivat affects glycolysis as well as other metabolic pathways, thereby partly restoring the metabolic profile in patients with SCD.

Focused metabolic pathway analysis

Based on the proof of the mechanism of mitapivat treatment in SCD,[8,9] we evaluated changes in metabolites of the glycolytic pathway and the PPP. A significant increase in glyceraldehyde 3-phosphate, malate, and adenosine monophosphate was found in patients with SCD at baseline compared to HCs, whereas ATP was significantly decreased (Figure 4A). Upon 8-week treatment with mitapivat, a significant increase in end-products of glycolysis, that is, pyruvate, lactate, and ATP, was observed (Figure 4A), with lactate and ATP being among the top 25 significantly different metabolites (Figure 2D). Regarding the PPP and its respective metabolites, we observed a decrease in 6-phosphate gluconolactone and an increase in pentose phosphate levels in patient baseline samples compared to HCs, and both did not change upon mitapivat treatment (Figure 4A).

Next, we evaluated the effects of 8-week mitapivat treatment on other metabolic pathways that are affected in SCD. First, analyses of ascorbate and glutathione metabolism showed a significant increase in glutamine, glutamate, cystine, 5-oxoproline, and 2,3-diketogulonate in patient baseline samples compared to HCs, whereas a significant decrease in γ-glutamylcysteine and reduced glutathione were observed. No significant changes in these metabolites were observed upon 8-week treatment with mitapivat (Figure 4B). Second, alterations in arginine and polyamine metabolism were observed in patient baseline samples compared to HCs with significantly higher *Z*-scores for creatine, fumarate, spermidine, and spermine, and lower *Z*-scores for arginine, creatinine, ornithine,

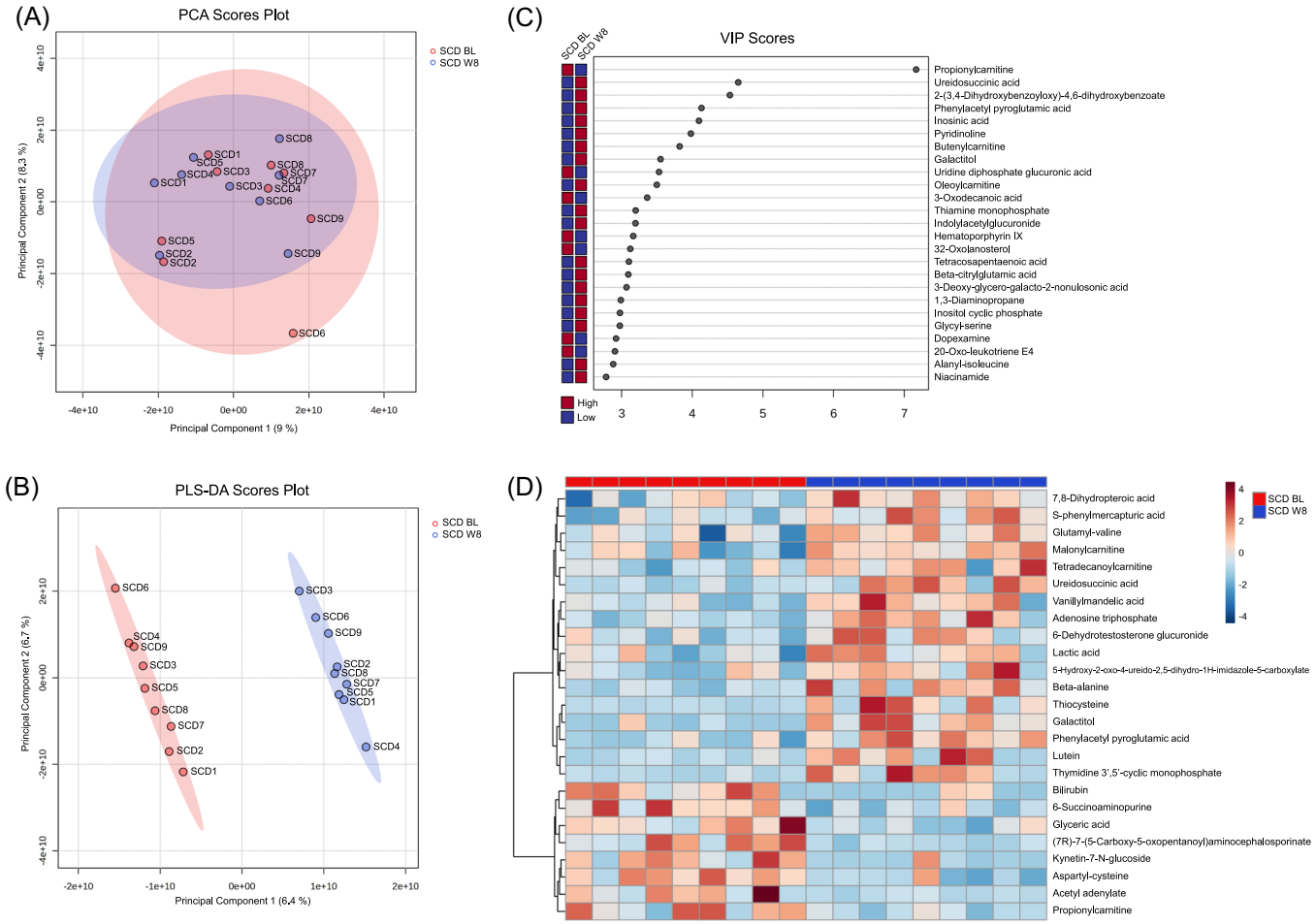

FIGURE 2 Metabolic profile in dried blood spots of whole blood from patients with sickle cell disease (SCD) at baseline and treatment week 8 with mitapivat. (A) Principal component analysis of patients with SCD at baseline (SCD BL, red) compared to treatment week 8 with mitapivat (SCD W8, blue) displayed with 95% confidence regions. Sample names are displayed and each number corresponds to an individual patient. (B) Partial least square discriminant analysis (PLS-DA) of SCD BL (red) compared to SCD W8 (blue) displayed with 95% confidence regions. Sample names are displayed and each number corresponds to an individual patient. (C) VIP-scores demonstrating the 25 metabolites that contribute most to the separation of SCD BL compared to SCD W8 on PLS-DA. (D) Heatmap of the 25 most significant metabolites identified by paired *t*-tests (unadjusted $p < 0.02$). The heatmap was created without clustering and autoscaling of metabolites. A comprehensive overview of *p*-values and isomers is displayed in Supporting Information S1: Table 4.

citrulline, and aspartate. None of these metabolites significantly changed upon 8-week treatment with mitapivat (Figure 4C). Lastly, investigation of carnitine and phospholipid metabolism showed a significant increase in carnitine, acetylcarnitine, and choline, and a reduction in the carnitine precursor lysine, in which no effect was observed upon 8-week treatment with mitapivat (Figure 4D).

DISCUSSION

In this study, we performed untargeted metabolomics in DBS of whole blood from patients with SCD treated with the oral PK activator mitapivat. To our knowledge, this is the first study in patients with SCD that evaluates more than a thousand unique metabolites upon PK activation treatment. We report on a metabolic fingerprint in SCD patients at baseline compared to HCs and provide a comprehensive overview of widespread metabolic changes in patients with SCD upon 8-week treatment with mitapivat. Our data indicates that mitapivat affects (end-products of) glycolysis, acyl carnitines, and derivatives of nucleotide metabolism. Our findings provide detailed insight into the complex metabolic effects of PK activator therapy in SCD and may contribute to the identification of potential biomarkers in the future.

While mitapivat was initially designed to treat hemolytic anemia in patients with PK deficiency, we showed that patients with SCD also have reduced PK enzyme activity and stability, which is consistent with the literature.[5] Previous metabolomics studies describe slightly increased levels of glycolytic intermediates upstream of PK in RBCs from patients with SCD.[4,25,26] Taken together, these findings provide a rationale for the treatment of SCD with mitapivat. We did not observe significant differences between patients and HCs in glycolytic and PPP intermediates, except for glyceraldehyde-3-phosphate, 6-phosphate gluconolactone, and pentose phosphate (and/or metabolites with the same respective mass to charge ratio, legend Figure 4). Perhaps our method lacks some sensitivity to detect minor differences, especially in a small cohort of patients with a heterogeneous disease. We did however observe a significant decrease in ATP and an increase in adenosine monophosphate, which

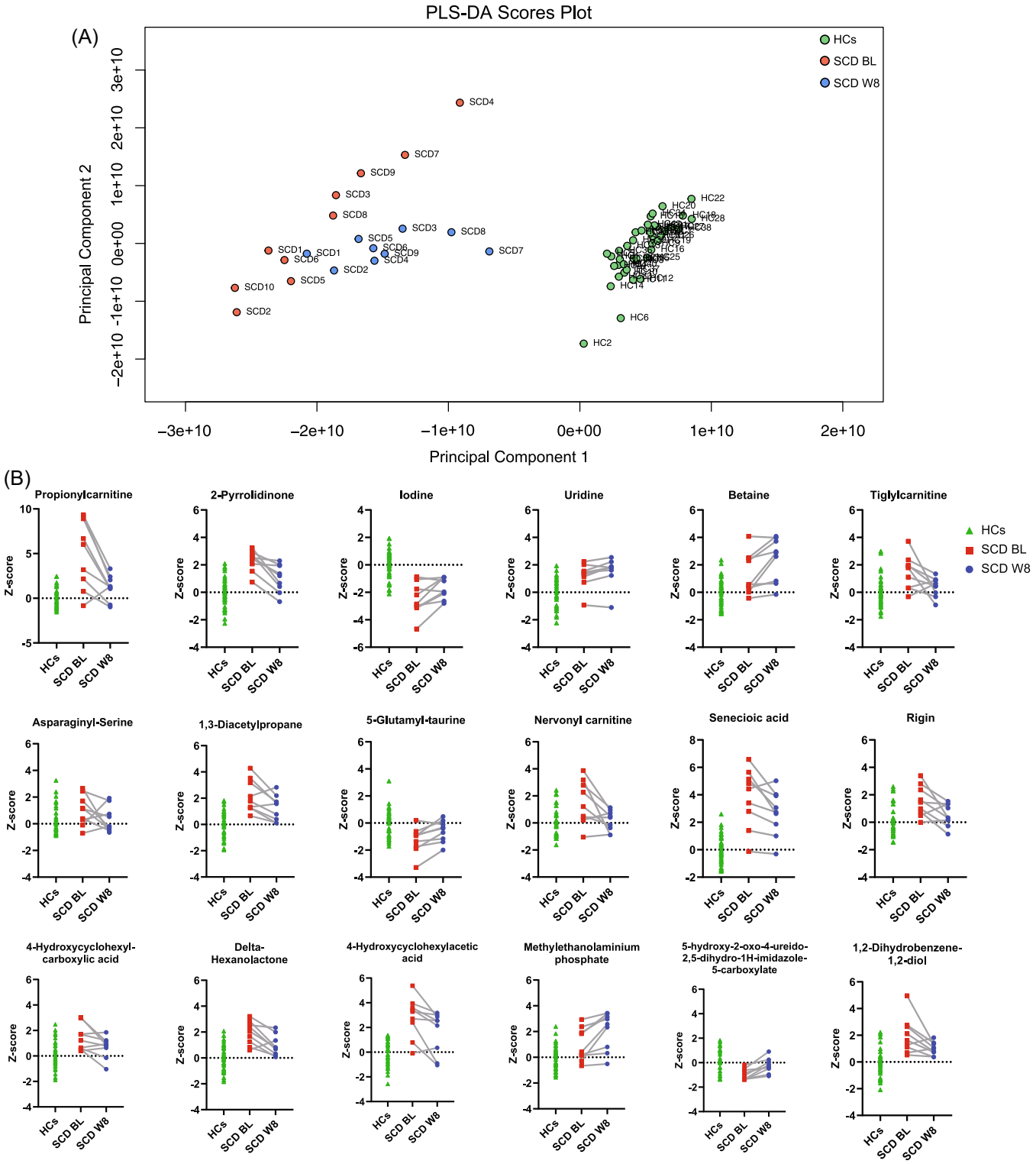

FIGURE 3 Effects of 8-week treatment with mitapivat on the sickle cell disease (SCD) metabolome. (A) Partial least square discriminant analysis of patients with SCD at baseline (SCD BL, red) and healthy controls (HCs, green) on which patients with SCD upon 8-week treatment with mitapivat (SCD W8, blue) were plotted. Sample names are displayed and each number corresponds to an individual patient. (B) A selection of *Z*-scores of metabolites was analyzed in the patient cohort because these metabolites were significantly different between HCs (green) and SCD BL (red) (FDR-adjusted $p < 0.05$). Paired *t*-tests showed significant alterations in 18/139 (12.9%) *Z*-scores at SCD W8 (blue) compared to SCD BL (unadjusted $p < 0.05$), which are shown in this figure. Except for *N*-methylethanolaminium phosphate, betaine, and uridine, all *Z*-scores changed toward zero, which is based on the mean of the *Z*-scores of HCs. A comprehensive overview of *p*-values and isomers is displayed in Supporting Information S1: Table 5.

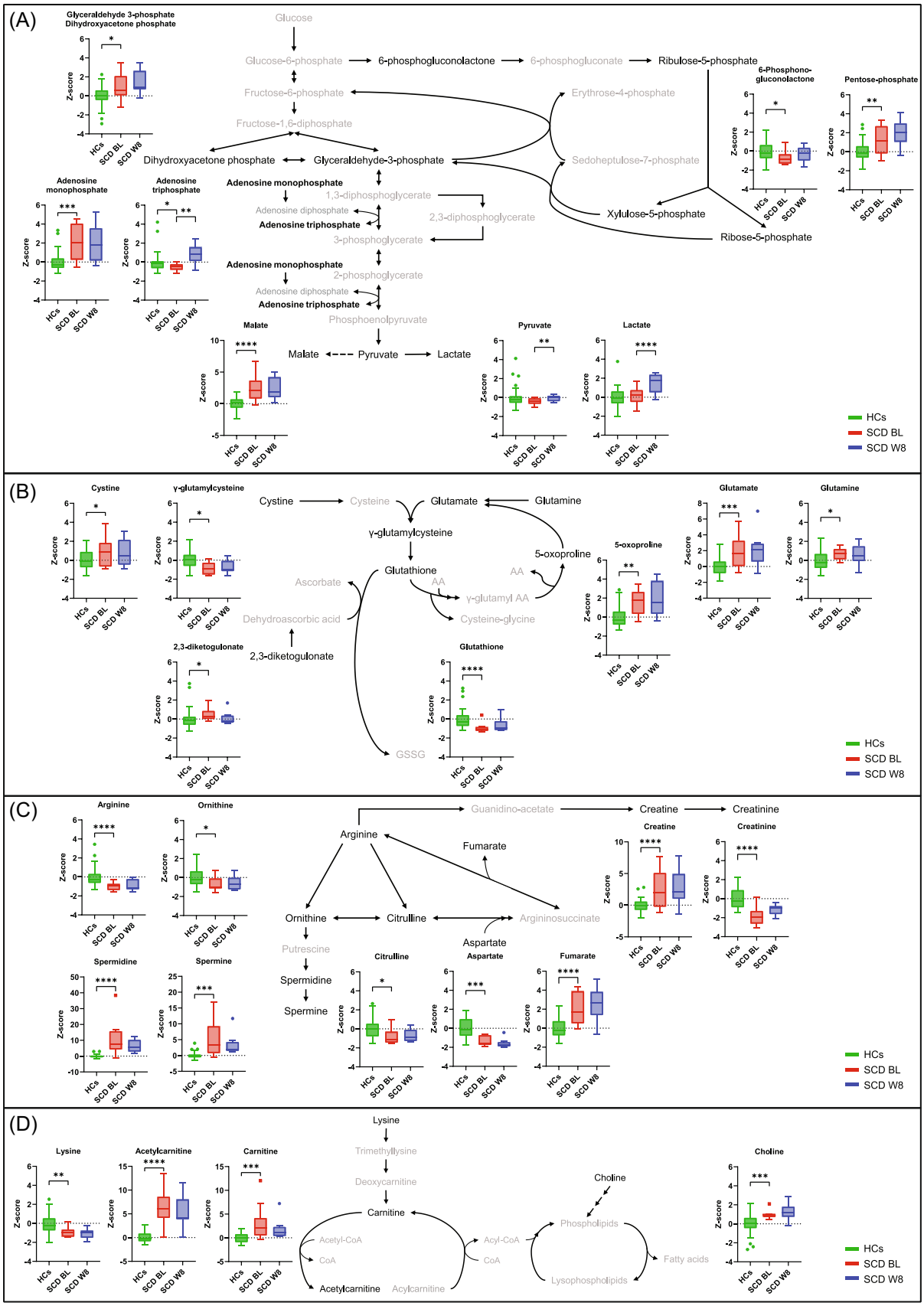

FIGURE 4 (See caption on next page).

FIGURE 4 Effect of mitapivat on metabolites in red cell-related metabolic pathways. Schematic representation of (A) glycolysis and the pentose phosphate pathway (PPP) in red blood cells (RBCs), (B) glutathione and ascorbate metabolism, (C) arginine and polyamine metabolism, and (D) carnitine metabolism. *Z*-scores of metabolites (and isomers) that were significantly different between healthy controls (HCs, $N = 42$) and patients with sickle cell disease (SCD) at baseline (BL) or between patients with SCD at BL and 8-week treatment (W8) with mitapivat ($N = 9$ pairs) were written in black and plotted in a boxplot with Tukey whiskers. Two-sample *t*-tests (or nonparametric Mann–Whitney test) or paired *t*-tests (or nonparametric Wilcoxon matched-pairs signed rank test) were used when appropriate. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$ (unadjusted). Isomers include (A) glyceraldehyde 3-phosphate: dihydroxyacetone phosphate; adenosine monophosphate: 2′-deoxyguanosine 5′-monophosphate, 3′-AMP, adenosine 2′-phosphate, 2-hydroxy-dAMP; adenosine triphosphate: dGTP, 2-hydroxy-dATP; 6-phosphogluconolactone: 2-keto-3-deoxy-6-phosphogluconate; ribulose-5-phosphate: xylulose 5-phosphate, ribose 5-phosphate, ribose 1-phosphate, D-Xylulose 1-phosphate, D-arabinose 5-phosphate, beta-L-arabinose 1-phosphate; pyruvate: malonic semialdehyde; lactate: hydroxypropionate, glyceraldehyde, dihydroxyacetone, methoxyacetate. (B) 2,3-diketogulonate: diketogulonate, citrate, isocitrate, D-threo-isocitrate; 5-oxoproline: pyroglutamate, pyrrolidonecarboxylate, pyrroline hydroxycarboxylate, *N*-acryloylglycine, 1-pyrroline-4-hydroxy-2-carboxylate; glutamate: *N*-methyl-D-aspartate, *N*-acetylserine, L-4-hydroxyglutamate semialdehyde; glutamine: ureidoisobutyrate, alanylglycine. (C) Citrulline: argininate; aspartate: iminodiacetate; fumarate: maleate; creatine: beta-guanidinopropionate. (D) Lysine: (3S)-3,6-diaminohexanoate.

likely reflects the reduced PK activity and/or stability. In agreement with previous studies, no significant differences in metabolites downstream of PK were detected.[4,25,26] Importantly, upon 8-week treatment with mitapivat, ATP levels increased significantly, as well as the levels of pyruvate and lactate, consistent with the mechanism of action of mitapivat. Increased ATP levels are presumed to be beneficial in SCD, as ATP is needed to maintain RBC membrane integrity, deformability, and antioxidant capacity.[7] We previously showed that mitapivat decreased levels of the red cell-specific metabolite 2,3-DPG.[8] Here, using an untargeted metabolomics approach without chromatographic separation of metabolites, we were not able to reproduce these results. Regarding the PPP, we did not observe significant differences upon 8-week treatment with mitapivat. To elucidate the effect of PK activation on glycolysis and PPP dynamics in detail, further study is needed, for example, using glucose tracing. Of note, our approach showed reduced levels of bilirubin upon 8-week treatment with mitapivat. Mitapivat is described as a weak inducer of uridine diphosphate glucuronosyltransferase family 1 member A1 (UGT1A1),[27] which is involved in conjugating bilirubin, and thereby its clearance. It could also indicate a reduction in hemolysis, which is consistent with our previously reported routine laboratory markers of hemolysis.[8] Altogether, these findings support that mitapivat treatment can be beneficial for SCD patients.

In addition to glucose metabolism, we found several metabolites of polyamine metabolism to be significantly altered in SCD patients compared to HCs. Normally, nitric oxide (NO) synthase converts arginine to citrulline and NO, an important signaling molecule that regulates vascular tone. In SCD, intravascular hemolysis releases arginase, which degrades arginine to ornithine instead, thereby impairing NO homeostasis and causing vascular complications.[28] As ornithine is the precursor for polyamines (putrescine, spermidine, and spermine), this mechanism might contribute to altered polyamine metabolism in SCD. Of note, we did not observe significant alterations in arginine and polyamine metabolism upon 8-week treatment with mitapivat.

Next to polyamine metabolism, we observed significant changes in nucleotide metabolism. Mature RBCs are unable to synthesize purine nucleotides de novo and are therefore dependent on salvage pathways to replenish their purine nucleoside phosphate pools.[29] We observed increases in intermediates of the purine salvage pathway in patients with SCD at baseline, including inosine and hypoxanthine, as also observed in another metabolomics study.[25] Ribose-phosphates, which were increased in SCD patients at baseline, are a product of phosphorolysis of inosine and guanine. These ribose-phosphates can enter glycolysis via glyceraldehyde-3-phosphate, which was also elevated in SCD patients at baseline. Furthermore, hypoxanthine is converted into xanthine and uric acid (which was significantly increased in patients with SCD at baseline compared to HCs; data not shown) during purine catabolism, thereby producing large amounts of reactive oxygen species. As purine metabolism activity is increased in RBCs, this may contribute to hemolytic vasculopathy in SCD.[30] Upon 8-week treatment with mitapivat, the decrease of a specific purine derivate (6-succinoaminopurine) was among the top 25 most significant alterations, as well as the increase in levels of ATP, indicating that mitapivat affects purine metabolism. As opposed to purine nucleotides, pyrimidine nucleotides are not synthesized nor salvaged and remain only in trace quantities in the cell upon maturation.[31] The observed increases of pyrimidine catabolism intermediates (beta-alanine, thymidine 3′,5′-cyclyc monophosphate, and ureidosuccinate) upon 8-week treatment with mitapivat could indicate improvements in RBC maturation.

In addition, we observed a significant increase in carnitine levels in patients with SCD compared to HCs. Carnitines are used in mature RBCs to counter lipid peroxidation. The acyl-CoA/acyl-carnitine system, also known as the Lands cycle, facilitates the replacement of oxidized fatty acid moieties from membrane phospholipids via phospholipase A2, which releases a fatty acid from a phospholipid, and lysophospholipid acyltransferases, which incorporate a fatty acid into the phospholipid.[32,33] The carnitine system is imbalanced in SCD and promotes sickling, inflammation, and tissue damage.[34] Upon 8-week treatment with mitapivat, we observed a significant decrease in propionylcarnitine, as well as an increase in other acylcarnitines, whereas levels of carnitine and acetylcarnitine did not change. ATP is required for acyl-CoA synthesis in the reacylation step of the Lands cycle,[33] which might demonstrate a link between mitapivat treatment and the Lands cycle. Further studies are needed to elucidate the exact mechanism by which mitapivat affects carnitine metabolism.

The metabolic profile was obtained from whole blood and not from purified RBCs or plasma. A broad spectrum of metabolites could thereby be evaluated, which is especially helpful in multisystem diseases like SCD and treatments with multisystem effects like mitapivat.[2] A potential limitation of this technique is that differences in RBC metabolites are mitigated by the presence of white blood cells, platelets, and plasma. It could also be possible that metabolites decrease in plasma, and as a result increase intracellularly, or just the other way around, thereby eliminating an effect on the whole blood level. Recently, metabolic effects of treatment with mitapivat in SCD were studied at the RBC level, showing beneficial effects on glycolysis and activation of the Lands cycle.[35] This is in line with our findings. Hb levels, hematocrit, and RBC count are lower in patients with SCD, whereas reticulocyte, white blood cell, and platelet counts are higher compared to HCs. We did not correct for this nor the change in blood composition including RBC subpopulations upon treatment with mitapivat. This potentially causes metabolites to be significantly elevated or reduced that are unrelated to SCD pathophysiology. For example, observed increases in citrate, fumarate, and malate, which

are intermediates of the Krebs cycle, could be attributed to increased levels of white blood cells and reticulocytes in the baseline SCD samples. However, it also supports the observation by Moriconi et al. that mature RBCs of sickle cell patients retain mitochondria,[36] possibly due to ineffective maturation of reticulocytes.[37] Other confounders are the use of concomitant drugs, including hydroxyurea, potential differences in diet, and the use of non-race-matched HCs to which metabolites are normalized. Increasing our sample size, subgroup analysis (e.g., SCD genotype, with or without concomitant hydroxyurea therapy), and extending the time of treatment could provide further insights into the effects of mitapivat on RBC metabolism. Nevertheless, many observed differences between HCs and SCD patients at baseline have been previously reported in RBC content and plasma, indicating that the effects of some of these confounders can be considered minimal.[4,25,26,38]

DI-HRMS is a hypothesis-generating method for untargeted metabolomics that is superior to chromatographic separation in being nonselective and very sensitive to identifying many mass peaks.[20] However, an observed mass peak is relative and not quantitative and can account for multiple metabolite annotations because isomers are not separated. For example, betaine and valine have the same *m/z* value, as do 2,3-diketogulonate and citrate. Therefore, the peak intensity is the sum of these metabolites, complicating the interpretation of the data. Despite this limitation, 1773 unique metabolites were identified, which is considerably more than the general hundreds of metabolites identified using liquid chromatography and/or gas chromatography MS-based metabolomics methods in SCD.[39–41]

To conclude, we demonstrate that our metabolomics approach can be instrumental in understanding and evaluating metabolic blood responses of patients with SCD to therapies such as mitapivat. Mitapivat partly restored the metabolic blood profile and caused metabolic effects beyond glycolysis. Our results provide promising leads for further study into the pathophysiological and therapeutic mechanisms that determine patient's responses to therapeutic monitoring and possible treatment-related side effects. Future and larger studies are needed to assign specific metabolites as biomarkers for individualized therapy in this multisystem disease. Lastly, this comprehensive characterization of metabolic changes in SCD might serve as a tool to further develop and improve novel drugs.

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AUTHOR CONTRIBUTIONS

Judith J. M. Jans, Eduard J. van Beers, and Richard van Wijk directed the research; Myrthe J. van Dijk, Titine J. J. Ruiter, Minke A. E. Rab, Brigitte A. van Oirschot, Judith J. M. Jans, Eduard J. van Beers, and Richard van Wijk designed the research and developed methodology; Myrthe J. van Dijk, Cleo Derichs, Anita W. Rijneveld, Marjon H. Cnossen, Erfan Nur, Bart J. Biemond, Marije Bartels, and Eduard J. van Beers recruited the patients and provided patient data; Myrthe J. van Dijk, Titine J. J. Ruiter, Sigrid van der Veen, Brigitte A. van Oirschot, and Jennifer Bos performed the research and acquired data; Myrthe J. van Dijk, Titine J. J. Ruiter, Judith J. M. Jans, Eduard J. van Beers, and Richard van Wijk analyzed and interpreted data; Myrthe J. van Dijk and Titine J. J. Ruiter wrote the manuscript; Sigrid van der Veen, Wouter W. van Solinge, Roger E. G. Schutgens, Judith J. M. Jans, Eduard J. van Beers, and Richard van Wijk reviewed the manuscript critically. All authors revised the manuscript and approved the submitted version.

CONFLICT OF INTEREST STATEMENT

Minke A. E. Rab and Richard van Wijk received research funding from Axcella Health Inc. and Pfizer. Eduard J. van Beers and Richard van Wijk are consultants for Pfizer. Minke A. E. Rab, Eduard J. van Beers, and Richard van Wijk received research funding and are consultants for Agios Pharmaceuticals Inc. M.H.C's institution has received investigator-initiated research and travel grants as well as speaker fees over the years from the Netherlands Organisation for Scientific Research (NWO) and Netherlands National Research Agenda (NWA), the Netherlands Organization for Health Research and Development (ZonMw), the Dutch Innovatiefonds Zorgverzekeraars, Baxter/Baxalta/Shire/Takeda, Pfizer, Bayer Schering Pharma, CSL Behring, Sobi Biogen, Novo Nordisk, Novartis, and Nordic Pharma, and for serving as a steering board member for Roche, Bayer, and Novartis for which fees go to the Erasmus MC as an institution. Erfan Nur receives research funding from Novartis and Emmaus and participates in the advisory board of Novartis. Bart J. Biemond received research funding from Sanquin, Global Blood Therapeutics/Pfizer, and Novartis and participated in advisory boards of Novartis, Bristol-Myers Squibb/Celgene, Global Blood Therapeutics/Pfizer, Novo Nordisk, and CSL Behring. The remaining authors declare no competing financial interests.

DATA AVAILABILITY STATEMENT

For original data and protocol, please contact the corresponding author. Data will be shared as allowed by the General Data Protection Regulation and European Union privacy laws, depending on the location of the new data controller/processor and applicable national law.

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ORCID

Titine J. J. Ruiter http://orcid.org/0009-0008-3656-7718
Roger E. G. Schutgens http://orcid.org/0000-0002-2762-6033
Eduard J. van Beers https://orcid.org/0000-0002-3934-7189

SUPPORTING INFORMATION

Additional supporting information can be found in the online version of this article.

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