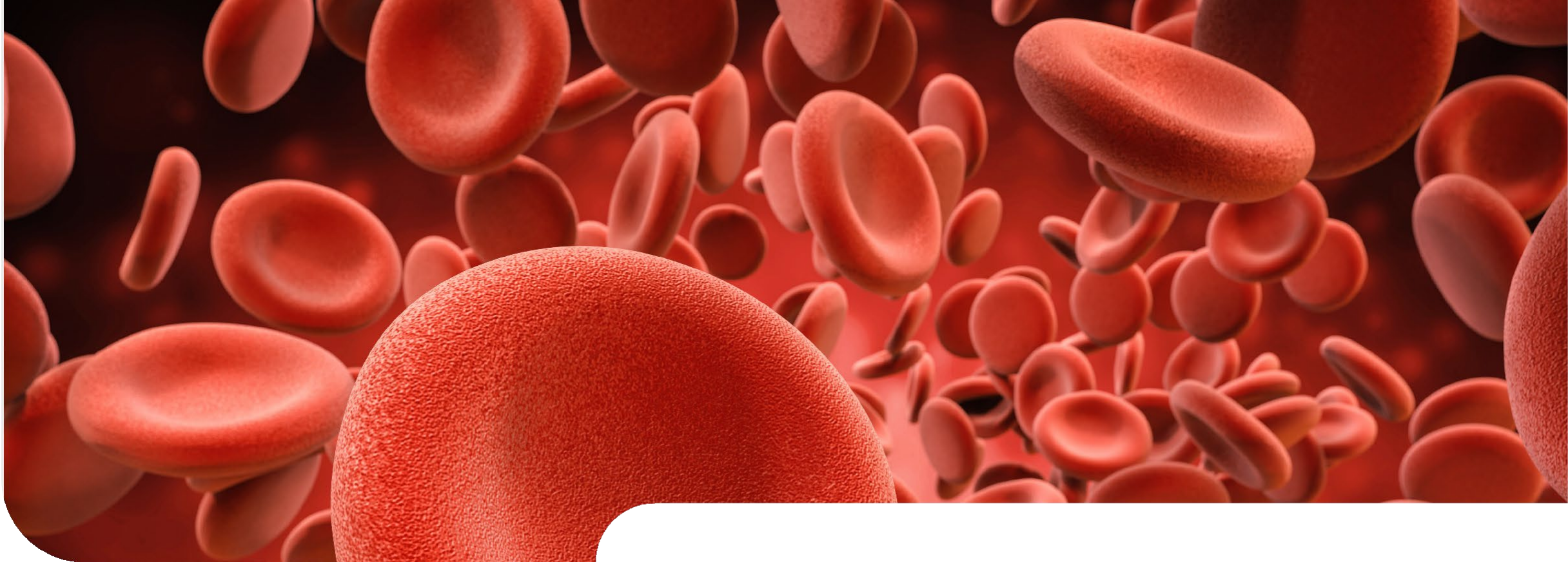

Untargeted Metabolomics on Dried Blood Spots of Patients with Sickle Cell Disease Treated with the Pyruvate Kinase Activator Mitapivat

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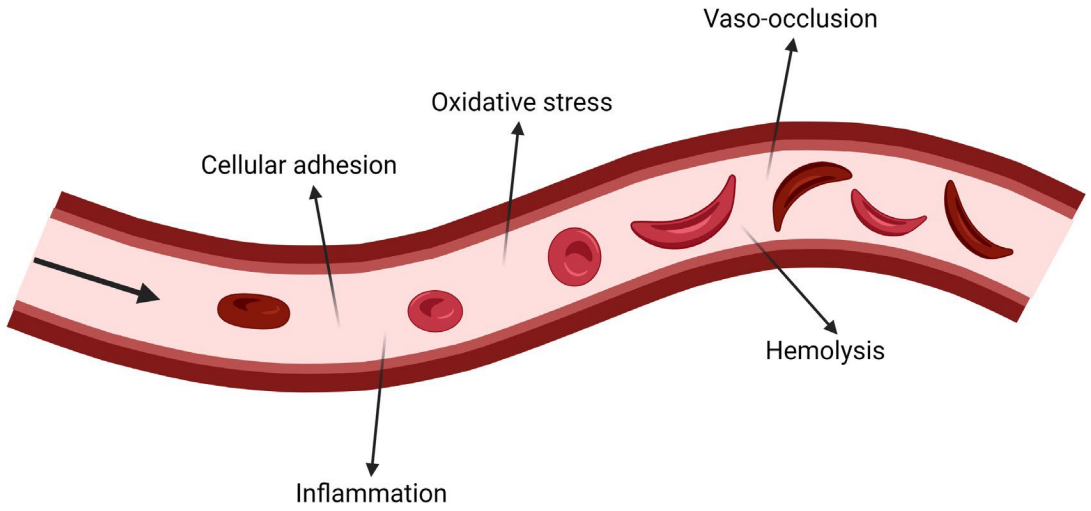
SCORE Sickle Cell Outcome Research

Sickle cell disease

- Sickle cell disease (SCD) is one of the most common inherited red blood cell (RBC) disorders
- SCD is characterized by polymerization of sickle hemoglobin (HbS) upon deoxygenation resulting in acute and chronic, potentially life-threatening complications
- The pathophysiology of SCD is multifactorial and highly complex



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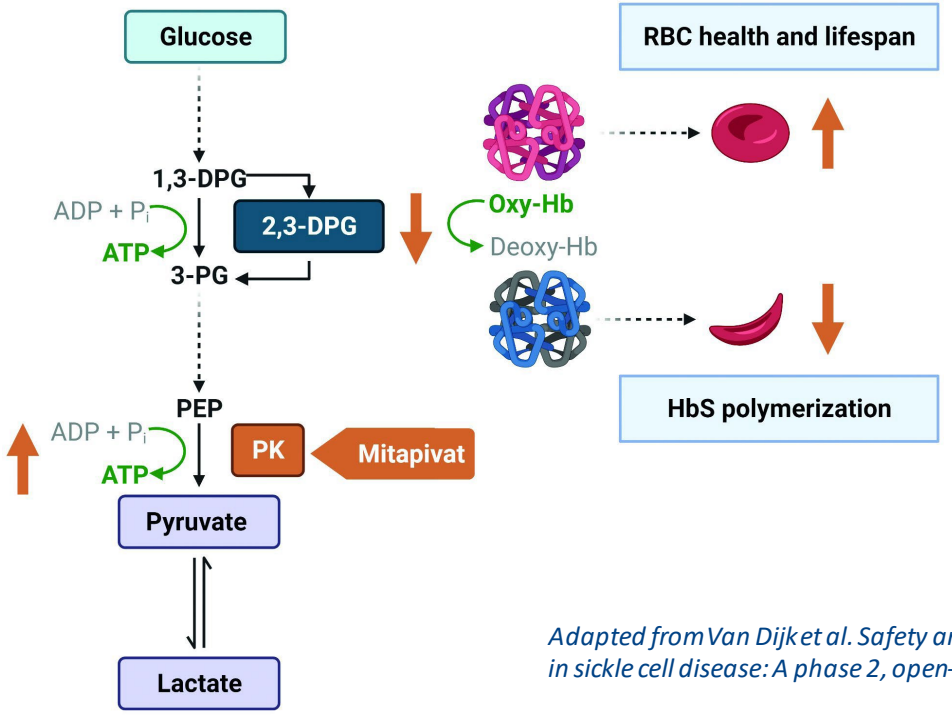
Mitapivat

- Novel anti-sickling agent under development for SCD
- Oral, small molecule, allosteric activator of pyruvate kinase (PK)
- Activation of PK in RBCs could reduce HbS polymerization by:
  - Decreasing [2,3-diphosphoglycerate (2,3-DPG)]
  - Increasing [adenosine triphosphate (ATP)]



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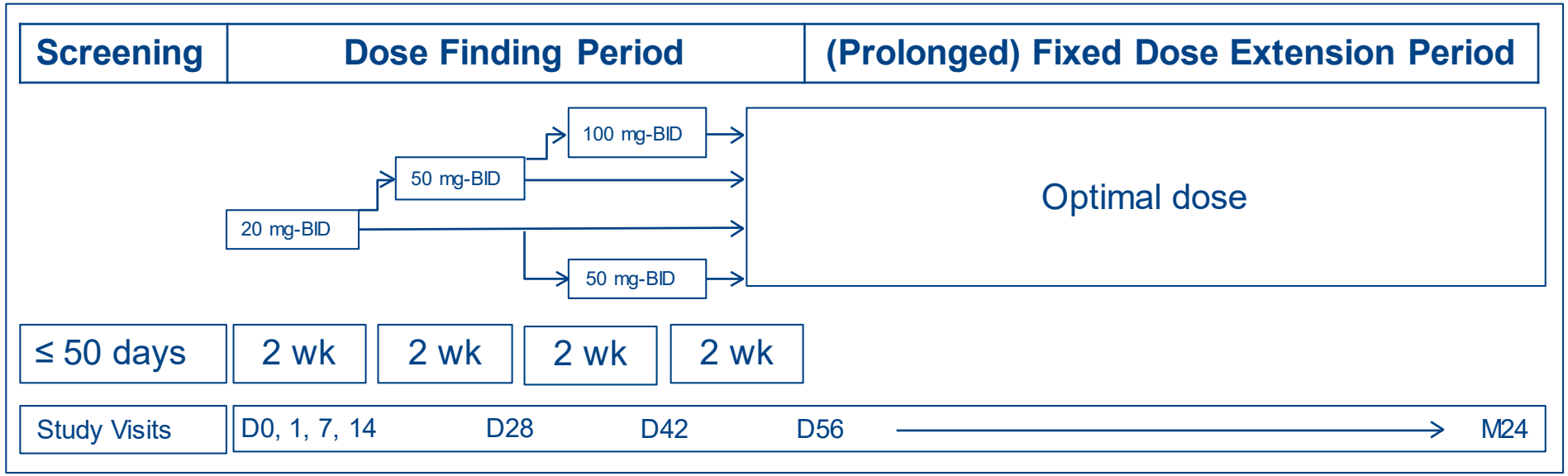
Adapted from Van Dijk et al. Safety and efficacy of mitapivat, an oral pyruvate kinase activator, in sickle cell disease: A phase 2, open-label study. Am J Hematol. 2022;97(7):E226-E229.

This slide contains investigational products not yet approved by regulatory authorities

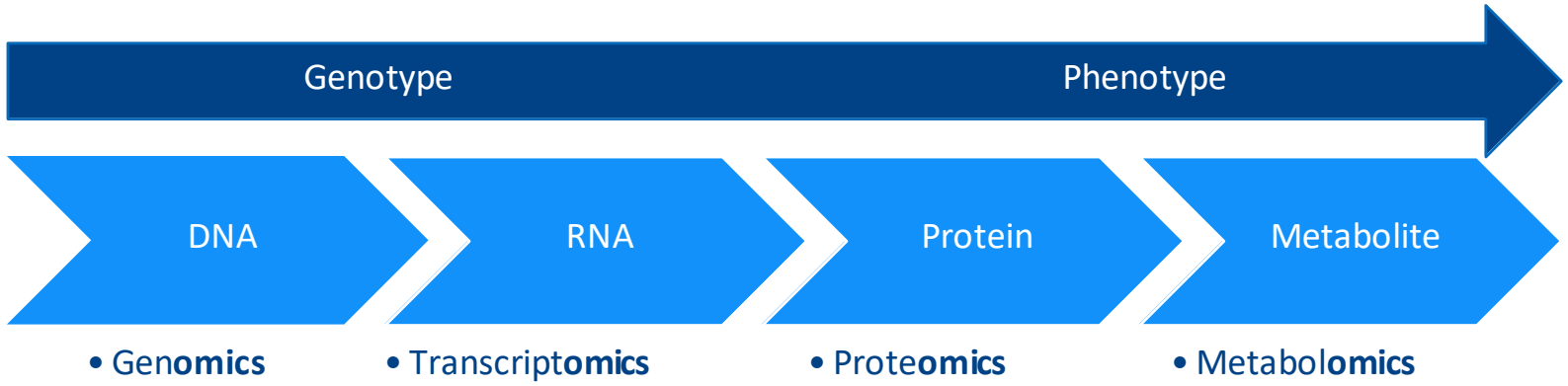
The ESTIMATE study

- An investigator-initiated, phase 2, open-label study *(EudraCT 2019-003438-18)*
- Patients ≥16 years with SCD treated with mitapivat
- Primary objective: to assess the safety and efficacy (proof of concept)
- One of the exploratory objectives: to evaluate the metabolome
- Interim analysis: baseline vs treatment week 8 at the end of the Dose Finding Period



Untargeted metabolomics

- The large-scale, unbiased, analytic study of **all metabolites**
  - e.g. biologically active small molecules (m/z = 70–600)
  - i.e. sugars, organic acids, amino acids, peptides, fatty acids, nucleotides
- To identify and characterize the complete metabolome (metabolic fingerprint): "hypothesis-generating discovery strategy"[1]
- Can be used to compare groups (e.g. cases vs controls, treated vs untreated)



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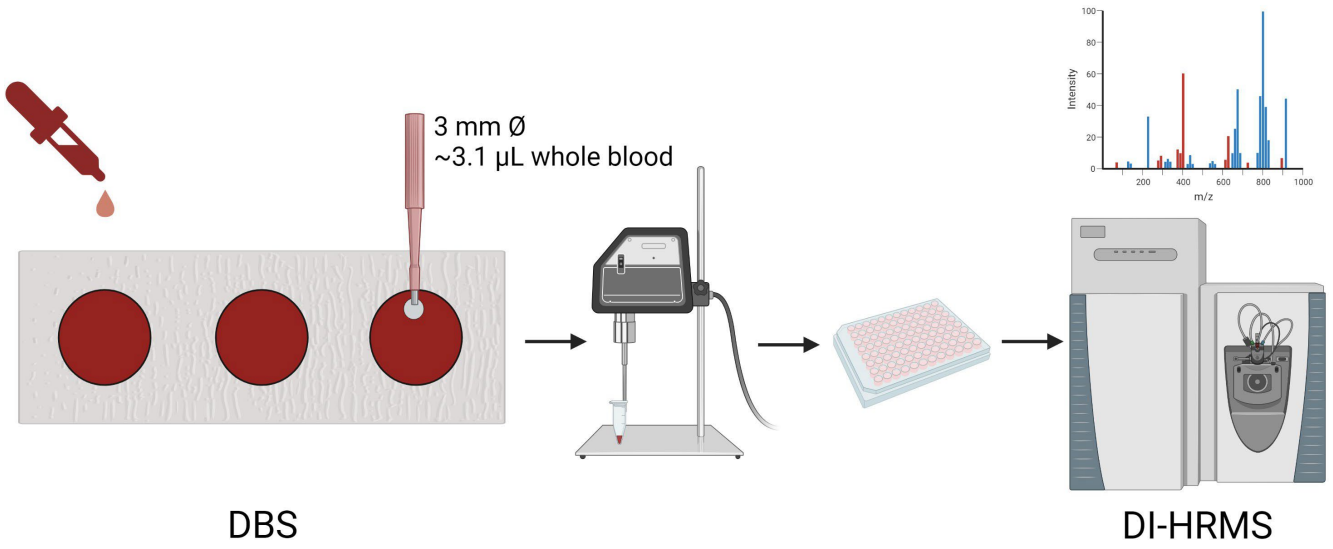
Methods

Data generation

- Dried blood spots (DBS)
  - 3 x 50 µL whole blood collected in EDTA anticoagulant tubes

- Direct-infusion high-resolution mass spectrometry (DI-HRMS)
  - Simultaneous assessment of thousands of metabolites[2,3]

- Workflow:



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Methods

Data annotation, processing and analysis

- Metabolite annotation results in ~1900 distinct metabolites/isomers (HMDB v5.0)
- Z-scores without further data filtering or normalization in a final dataset

- Comparison of metabolic profiles:
  1. Patients with SCD and healthy controls (HCs)
  2. Patients' baseline and treatment week 8 data
- Statistics: use of MetaboAnalyst (v5.0) and Graphpad Prism (v9.3.0)
  - (Paired) T-tests
  - Multivariate analyses
    - Heatmaps, principal component analysis (PCA), partial least squares-discriminant analysis (PLS-DA)

Results: patients with SCD vs HCs

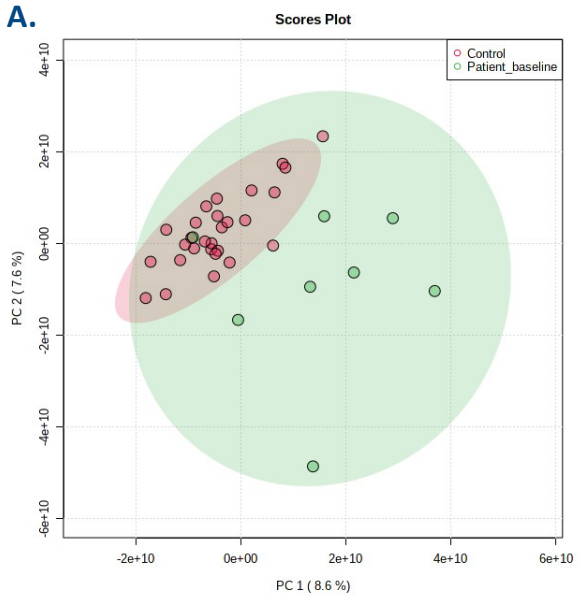
- 1907 metabolites/isomers identified in DBS of 9 patients with SCD and 29 HCs

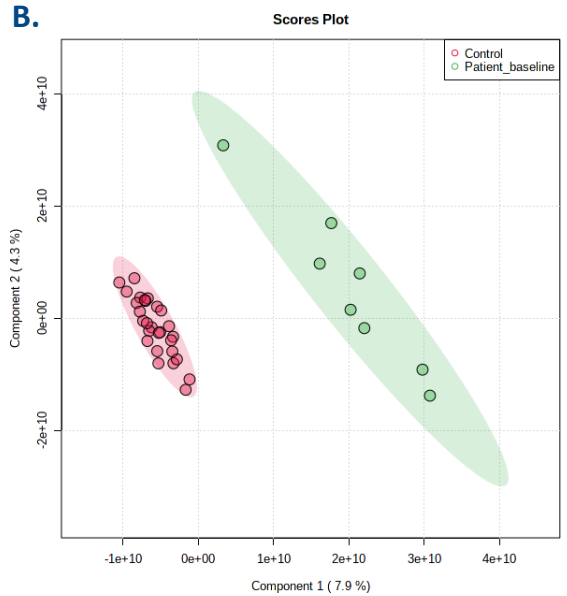
Baseline characteristics	Patients with SCD (n=9)	HCs (n=29)
SCD genotype, n (%)	HbSS: 7 (78) HbS/β^0: 1 (11) HbS/β^+: 1 (11)	N/A
Age, years, median (range)	22 (16-59)	38 (25-65)
Gender, female [n (%)]	6 (67%)	21 (72%)
Hb, g/dL, mean (SD)	9.1 (1.1)	14.6 (1.4)
RETC, % of RBCs, mean (SD)	9.6 (4.8)	1.4 (0.5)
WBC, 10^9/L, , mean (SD)	8.6 (2.7)	5.6 (1.1)
PLT, 10^9/L, mean (SD)	462 (143)	276 (58)

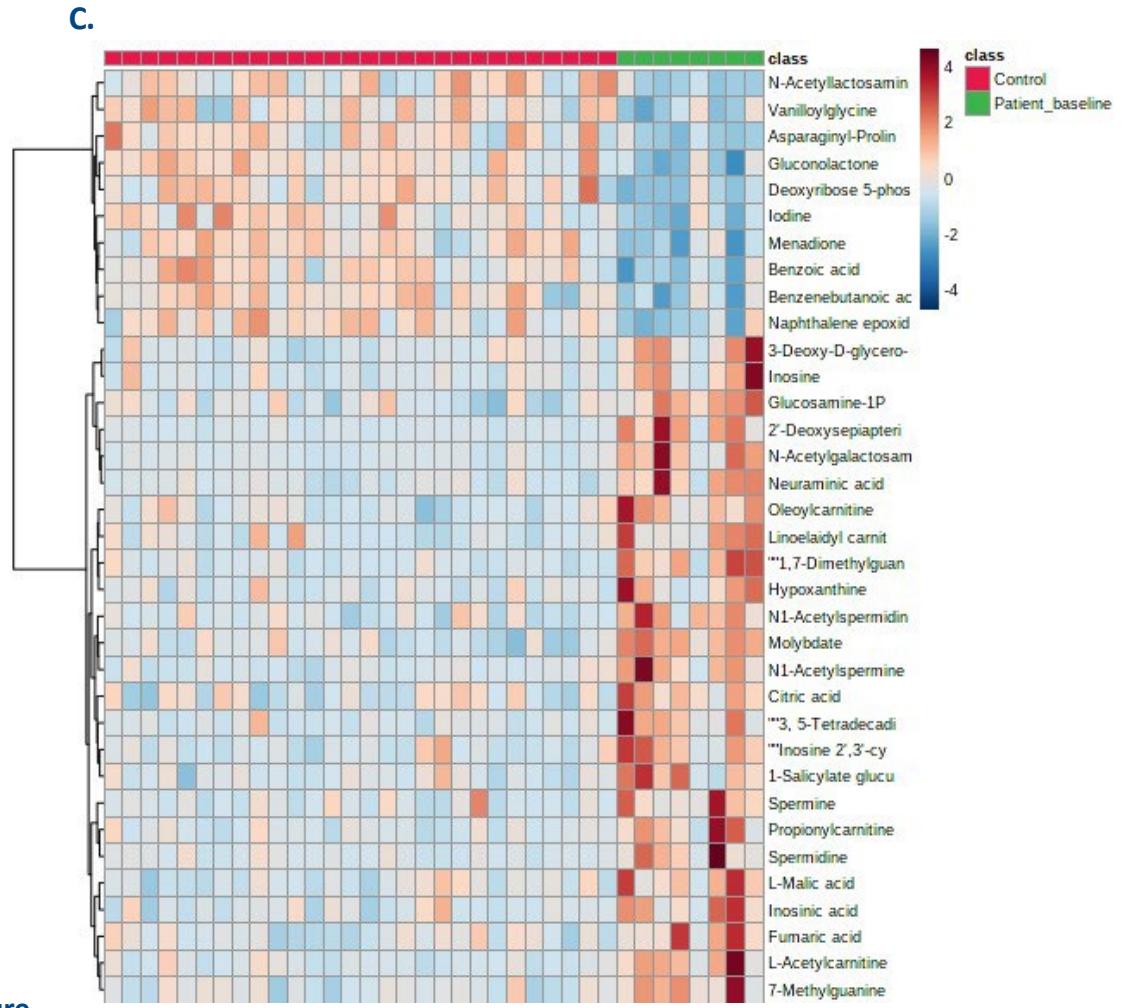
- 8/9 (89%) patients and 28/29 (97%) HCs included in final analyses based on quality control and outlier detection

Results: patients with SCD vs HC

- Multivariate analyses yielded distinct metabolic profiles
- 55/1907 (2.9%) metabolites were significantly different between patients and HCs, e.g.:
  - Increase of acyl carnitines, (derivatives of) polyamines, purines and pyrimidines
  - Decrease of carbohydrates and benzenoids







Figure

A. Principal component analysis (PCA)

B. Partial least square discriminant analysis (PLS-DA)

C. Heatmap of top 35 metabolites identified by t-test (FDR-adjusted p-value <0.023) created using Euclidean distance with autoscaling of features

Results: baseline vs treatment week 8

- 2/1907 metabolites were significantly increased after 8 weeks of treatment with mitapivat vs baseline (FDR-adjusted p-value <0.05):
  - Butenylcarnitine, an acyl carnitine
  - Inosinic acid, or inosine-5-monophosphate (5'-IMP), a purine nucleotide

Patients with SCD	Baseline (n=9)	Treatment week 8 (n=8)	p-value*
Hb, g/dL, mean (SD)	9.1 (1.1)	10.6 (1.0)	<0.001
RETC, % of RBCs, mean (SD)	9.6 (4.8)	4.2 (1.6)	<0.001
WBC, 10^9/L, , mean (SD)	8.6 (2.7)	6.8 (2.1)	<0.05
PLT, 10^9/L, mean (SD)	462 (143)	490 (237)	ns

**The paired sample t-test or Wilcoxon signed-rank test were used when appropriate for n=8 pairs with baseline and treatment week 8 results*

Results: baseline vs treatment week 8

- 8/55 (14.5%) identified distinct metabolites between patients and HCs showed a significant increase after 8 weeks of treatment with mitapivat vs baseline in patients

Metabolite	Baseline	Treatment week 8	Unadjusted p-value
Vanilloylglycine	$-1.54x10^9$ ($1.16 x10^9$)	$0.08x10^9$ ($0.61x10^9$)	0.005
Oleoylcarnitine	$1.70x10^9$ ($1.49x10^9$)	$4.60x10^9$ ($2.40x10^9$)	0.008
Orotidine	$1.23x10^9$ ($1.10x10^9$)	$4.20x10^9$ ($2.58x10^9$)	0.011
Creatinine	$-1.29x10^9$ ($0.82x10^9$)	$0.05 x10^9$ ($0.59x10^9$)	0.015
Asparaginyl-Proline	$-1.61x10^9$ ($0.67x10^9$)	$-1.07x10^9$ ($0.81 x10^9$)	0.021
L-Palmitoylcarnitine	$2.45x10^9$ ($2.49x10^9$)	$5.13x10^9$ ($3.79x10^9$)	0.025
N-Acetyl-L-phenylalanine	$-1.52x10^9$ ($0.52x10^9$)	$-0.66x10^9$ ($0.79x10^9$)	0.026
2-Amino-3-phosphonopropionic acid	$-1.02x10^9$ ($1.02x10^9$)	$-0.25x10^9$ ($1.37x10^9$)	0.046

Mean (± SD) of Z-scores are shown

Conclusion

- **Patients with SCD showed a distinct metabolic profile compared to HCs**
  - RBC-related pathways?
    - Acyl carnitines would be involved in the turnover and repair of RBC membranes[4]
    - Polyamines have been found to possibly stabilize the RBC membrane[5]
    - Purinergic signaling pathways mediate hypoxic metabolic reprogramming[6]
- **In the patient cohort, 8 weeks of treatment with mitapivat showed, again, changes in an acyl carnitine and purine nucleotide compared to baseline**
  - Reflecting cellular response? Changes in blood components?
- Promising starting point to further unravel the underlying pathophysiology of SCD and to characterize the cellular effects of PK activators and other therapies in SCD

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Discussion