

# Causal role of L-glutamine in sickle cell disease painful crises: a Mendelian randomization analysis

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## Introduction

Sickle cell disease (SCD) is an autosomal-recessive  $\beta$ -hemoglobinopathy. The disease is caused by a point mutation leading to a Glu $\rightarrow$ Val substitution at the 6<sup>th</sup> codon of the  $\beta$ -globin gene. The complications related to the disease are systemic as they impact multiple organ systems. In 2017, L-glutamine, a metabolite, became the second FDA-approved drug to treat SCD. We undertook this metabolomics experiment to confirm the finding from a randomized control trial (RCT) through mendelian randomization (MR), and identify other molecules that modulate the severity of SCD.

## Background

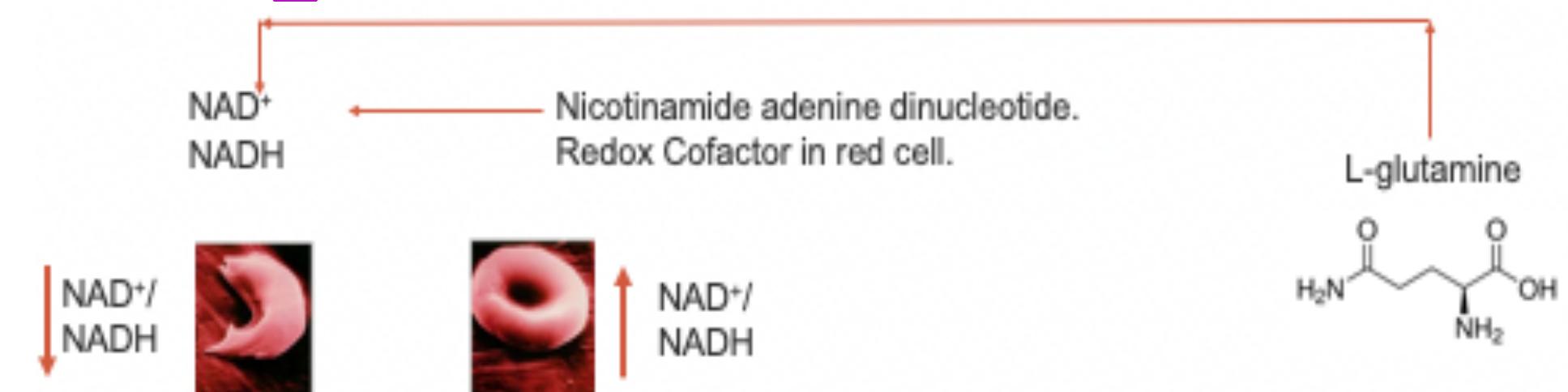


Figure 1. L-glutamine mechanism in SCD: Red blood cells (RBC) from SCD patients have high oxidative stress and a compromised ability to counter free radicals due to a low ratio of the reduction-oxidation (redox) co-factor nicotinamide adenine dinucleotide (NADH):[NAD+] and its reduced form ([NADH]:[NAD+]).

The Endari phase 3 trial included 230 SCA individuals. It resulted in a reduction of vaso-occlusive events (a median of 3 in the L-glutamine group versus 4 in the placebo group;  $P=0.005$ ), a decrease in hospitalizations (2 to 3 in the L-glutamine group compared to the placebo group;  $P=0.005$ ) and an increase time to first painful crisis<sup>1</sup>.

## Methods

### Study design

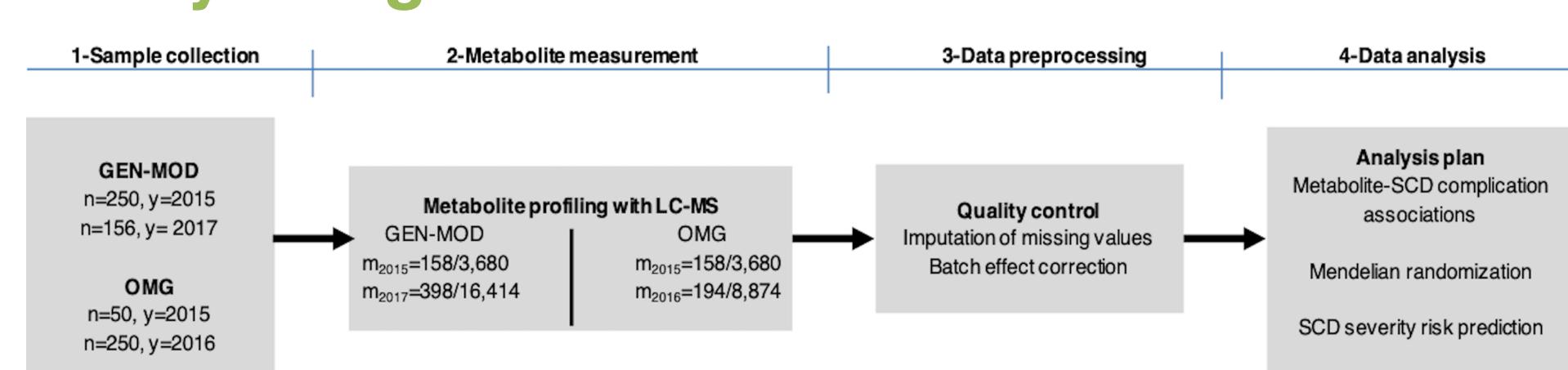


Figure 2. Study design of the metabolomic study in sickle cell disease (SCD) patients. 250 GEN-MOD samples and 50 OMG samples were profiled in 2015, 250 OMG samples were profiled in 2016, and 156 GEN-MOD samples were profiled in 2017. Known/targeted and unknown/untargeted metabolites were measured using liquid-chromatography in tandem with mass spectrometry (LC-MS). Data preprocessing involved standard quality-control procedures, imputation of missing values, batch-effect correction and data scaling. Data analysis included association testing of known metabolites with SCD-related complications, Mendelian randomization, and SCD severity risk prediction using statistical modelling. n, number of patients included in the study; y, year during which metabolites were measured; m, number of targeted/untargeted metabolites measured in each year.

## Results

### 1a. L-glutamine – SCD complications associations

Complications	N	Odds ratio	95% CI	P-value
Painful crises	619	1.1	(0.89-1.2)	0.57
Severity	705	0.98	(0.85-1.1)	0.84
Aseptic necrosis	617	0.99	(0.83-1.2)	0.86
Cholecystectomy	651	1.1	(0.92-1.3)	0.38
Leg ulcer	623	1.1	(0.89-1.3)	0.41
Priapism	448	1.1	(0.89-1.4)	0.34
Retinopathy	524	1.0	(0.84-1.2)	0.99
Renal Parameter	N	Beta	SE	P-value
eGFR	702	-0.082	0.038	0.031
Blood Parameter	N	Beta	SE	P-value
Bilirubin	585	0.10	0.041	0.010
Hematocrit	697	-0.09	0.038	0.016
Hemoglobin	685	-0.11	0.038	0.0031
LDH	579	0.057	0.042	0.17
MCH	626	0.070	0.040	0.06
MCV	697	0.08	0.038	0.03
RBC	698	-0.089	0.030	0.0028
Reticulocyte	666	0.097	0.039	0.01

Table 1. Associations between L-glutamine plasma levels and sickle cell disease (SCD)-related complications and other clinically relevant phenotypes. In participants from the GEN-MOD and OMG cohorts, we tested the association between L-glutamine levels measured in plasma and SCD-related complications or clinically relevant blood-based biomarkers. Dichotomous phenotypes were analyzed using logistic regression while correcting for age, sex, hemoglobin (HU) usage, SCD genotypes, and cohort affiliation. Quantitative phenotypes were corrected for age, sex, HU usage, SCD genotype and cohort affiliation. They were inverse normal-transformed before being tested for association using linear regression. Odds ratio and effect sizes (Beta) are given per standard deviation change in L-glutamine plasma levels. LDH, lactate dehydrogenase; RBC, red blood cell; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; eGFR, estimated glomerular filtration rate; LDH, lactate dehydrogenase; CI, confidence interval; SE, standard error.

### Results (continued)

#### 1b. L-glutamine – Mendelian randomization

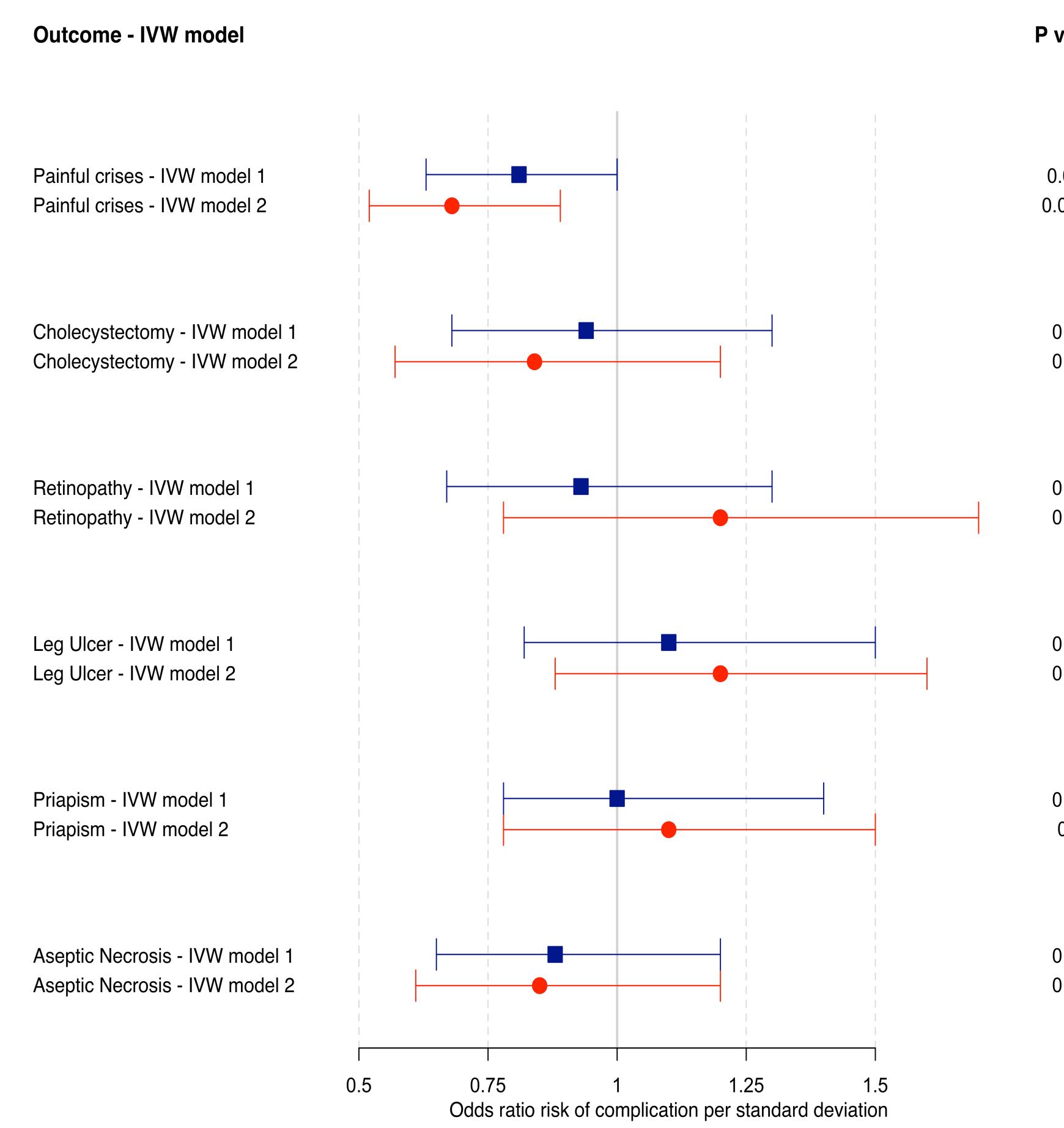


Figure 3. Mendelian randomization (MR) analysis of L-glutamine with sickle cell disease (SCD) painful crises. Forest plot of MR evaluating the causal relationship between L-glutamine levels and painful crises in SCD patients. Effect sizes and standard errors of the top 51 variants associated with L-glutamine were retrieved from large European mGWAS. Associations statistics between these 51 variants and SCD complications were calculated in the large prospective and well-characterized CSSCD. In model 1, we considered all 51 SNPs as instruments, whereas model 2 only included 27 variants not associated with other metabolites. The MR effect size estimates and 95% confidence intervals were calculated using the inverse variance-weighted (IVW) random effect method.

#### 2b. 3-ureidopropionate and eGFR – Mendelian randomization

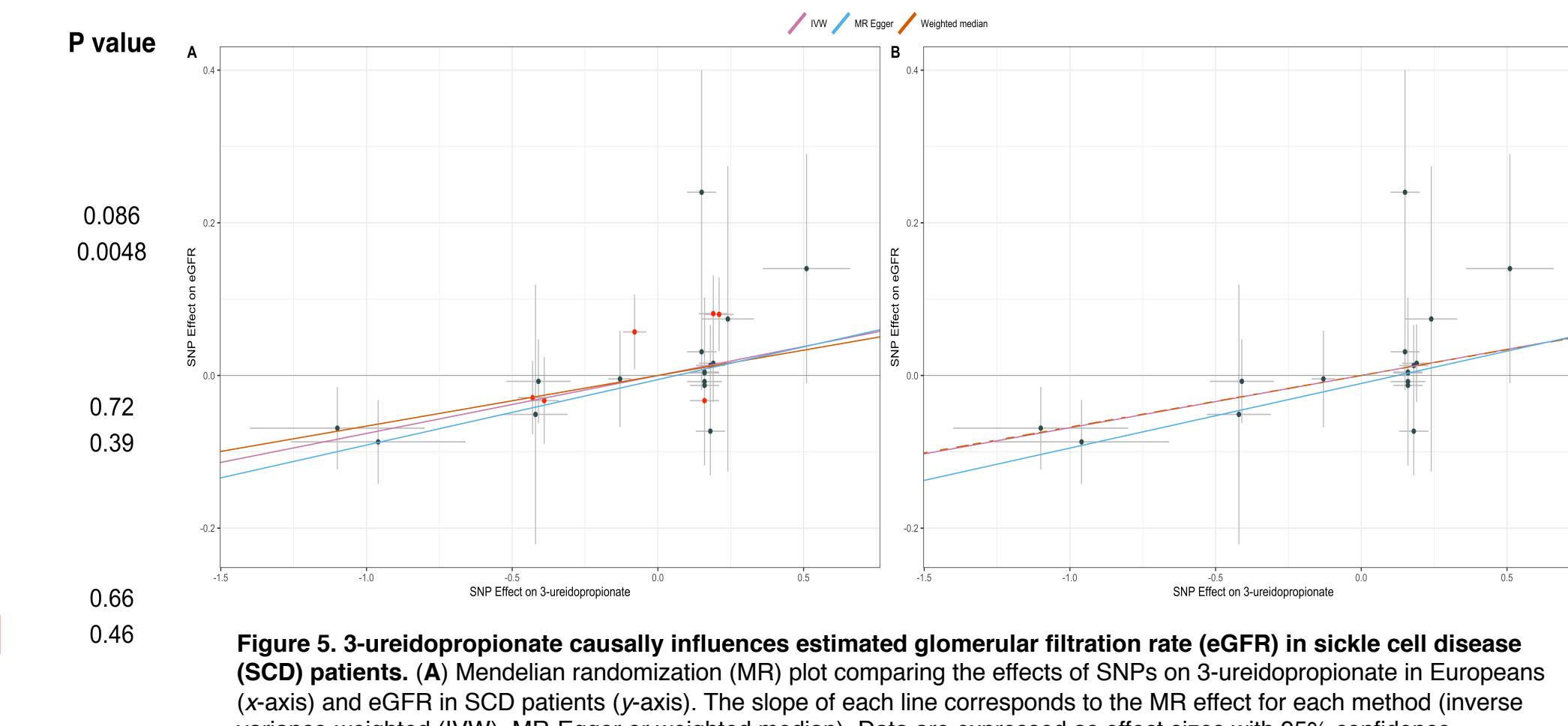


Figure 5. 3-ureidopropionate causally influences estimated glomerular filtration rate (eGFR) in sickle cell disease (SCD) patients. (A) Mendelian randomization (MR) plot comparing the effects of SNPs on 3-ureidopropionate in Europeans (x-axis) and eGFR in SCD patients (y-axis). The slope of each line corresponds to the MR effect for each method (inverse variance-weighted (IVW), MR-Egger or weighted median). Data are expressed as effect sizes with 95% confidence intervals. SNPs in red are pleiotropic. (B) Same as A, except that we removed pleiotropic variants

#### 2c. 3-ureidopropionate PRS association with eGFR

	eGFR (CSSCD)	eGFR (GEN-MOD)
PTS <sub>22SNPs</sub>	0.069, P=0.044, N=859	-0.098, P=0.56, N=399
PTS <sub>16SNPs</sub>	0.082, P=0.016, N=859	-0.080, P=0.12, N=399

Table 3. 3-ureidopropionate polygenic trait score association with eGFR. Association results between polygenic trait scores (PTS) calculated using 22 3-ureidopropionate-associated SNPs (PTS<sub>22SNPs</sub>) or after excluding pleiotropic variants (PTS<sub>16SNPs</sub>). For the PTS, the effect size is per PTS standard deviation units. Results are presented in the format: effect size (beta), P-value, sample size

## 3. SCD Severity - Prediction

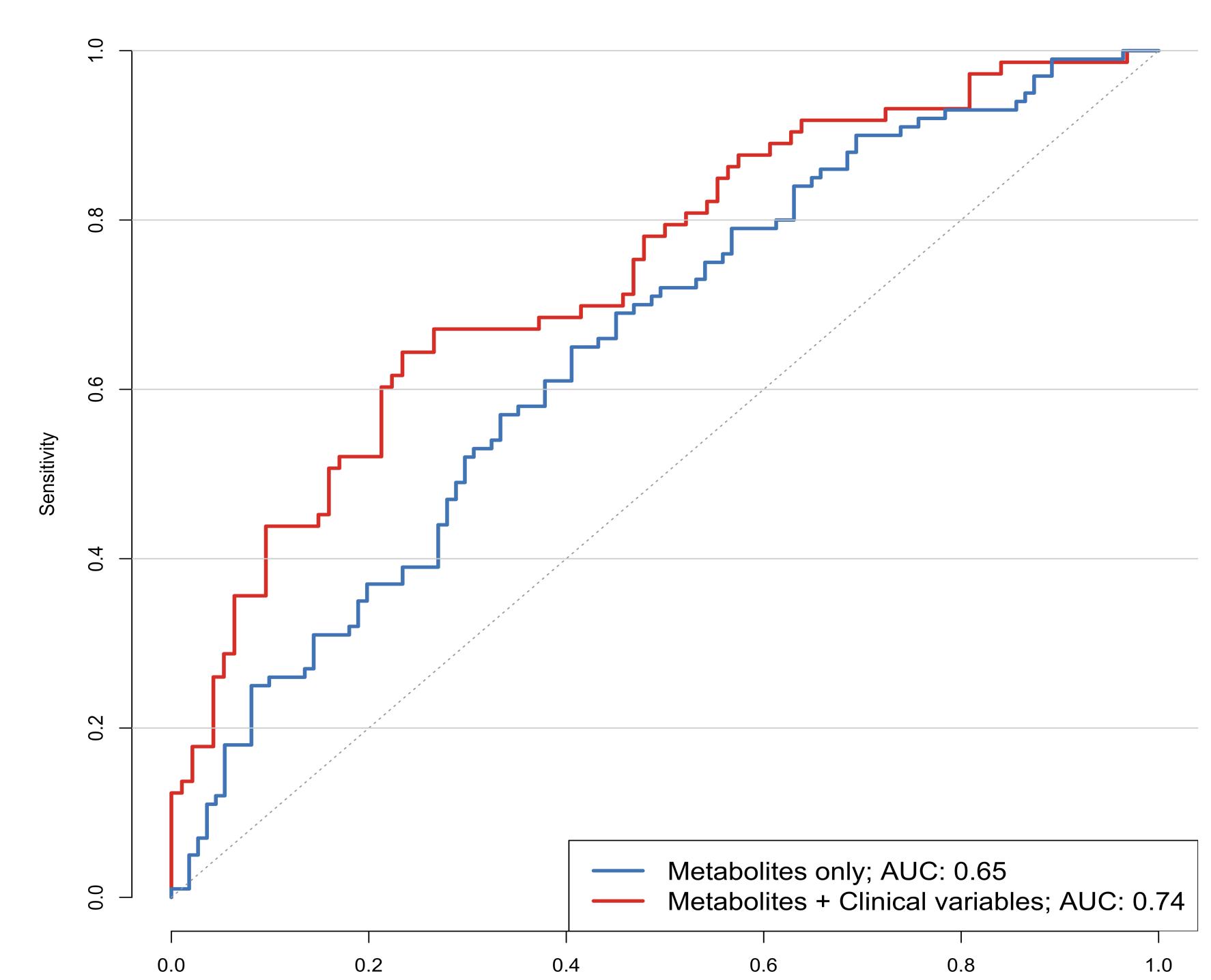


Figure 6. Receiver Operating Characteristic (ROC) analysis of metabolites and hematological traits predicting SCD Severity. Optimized ROC curves and corresponding areas under the ROC curve (AUC) for metabolites only (blue), and for metabolites together with hematological traits (red). We assessed 129 known metabolites and 6 clinical variables (PBC count, mean corpuscular volume (MCV), white blood cell count (WBC), hemoglobin levels (Hb), fetal hemoglobin percentage (HbF) and reticulocyte percentage (Retic)). We built logistic regression models in the training set using both backward and forward elimination algorithms implemented in the function stepAIC in the MASS package. Predictors selection/elimination was performed until the Akaike information criterion (AIC) showed no improvement<sup>2</sup>.

## Conclusions

While the mutation was discovered back in 1956, and about 300,000 babies are born each year with SCD, a restricted number of treatment options exist, and predicting severe individuals remains a challenge. To address these challenges, we performed the largest metabolomic study in SCD patients, measuring 129 known metabolites in 705 participants:

- We identified a promising causal relationship between L-glutamine levels and painful crises, consistent with recent results from a phase 3 clinical trial
- We highlighted 3-ureidopropionate, an intermediate in the metabolism of uracil, as a potential positive modulator of eGFR
- Our predictive models highlighted metabolites, such as creatinine, and cotinine. Both are well established predictors of disease morbidity and mortality

### 2a. Metabolites – SCD complications associations

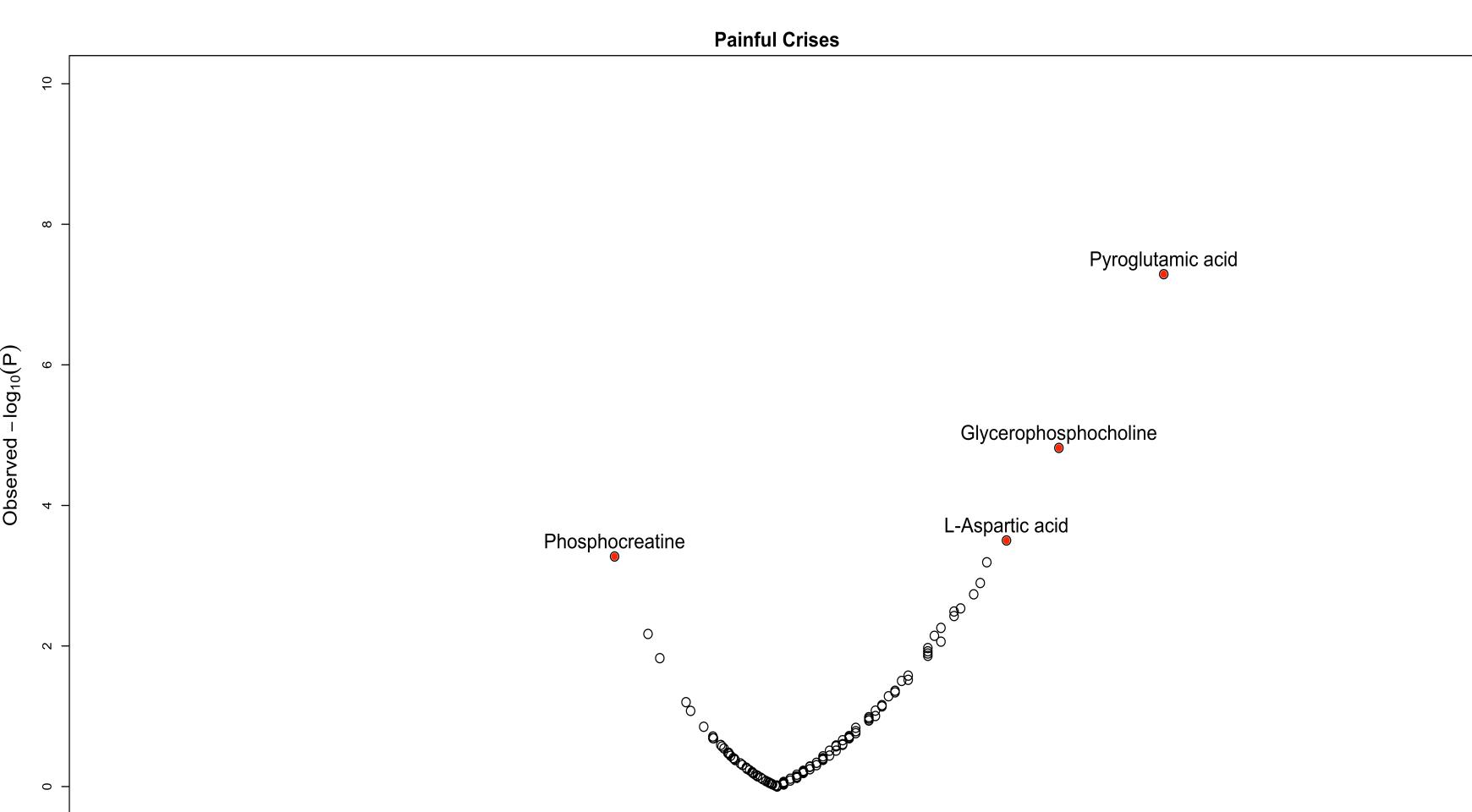


Figure 4. Known metabolites associated with painful crises and estimated glomerular filtration rate (eGFR). We tested 129 metabolites against clinical complications by logistic regression (linear regression for quantitative eGFR). On the x-axis, we report effect sizes (effect sizes for eGFR) in metabolite standard deviation units, whereas the y-axis presents the observed analytical P-values. Red circles highlight metabolites with  $P_{\text{perm}} < 0.05$  calculated using 100,000 permutations. In total, we found 4 metabolites for painful crises, 1 metabolite for aseptic necrosis, 1 metabolite with cholecystectomy, 2 metabolites for priapism, 2 metabolites for leg ulcers, 4 metabolites for retinopathy and 57 metabolites for eGFR

## References

- Nishihara Y, Miller ST, Kanter J, et al. A Phase 3 Trial of L-Glutamine in Sickle Cell Disease. *N Engl J Med*. 2018;379(3):226-235.
- W. N. Venables BDR. Modern Applied Statistics with S: Springer; 2002.