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Pyruvate Kinase Function Correlates With Red Blood Cell Properties and Clinical Manifestations in Sickle Cell Disease

M. J. M. Traets[1] | J. F. Bos[1] | S. van der Veen[2] | L. van Pelt[1] | M. J. van Dijk[1] | B. A. van Oirschot[1] | J. R. A. de Wilde[1] | J. J. Jans[3] | W. W. van Solinge[1] | S. E. M. Schols[4] | M. N. Lauw[5] | M. H. Cnossen[6] | E. Nur[7,8] | B. J. Biemond[7] | A. W. Rijneveld[5] | E. J. van Beers[2] | R. van Wijk[1] | M. A. E. Rab[1,5]

[1]Department of Central Diagnostic Laboratory—Research, University Medical Center Utrecht, Utrecht University, Utrecht, the Netherlands | [2]Center for Benign Hematology, Thrombosis and Hemostasis—Van Creveldkliniek, University Medical Center Utrecht, Utrecht, the Netherlands | [3]Section Metabolic Diagnostics, Department of Genetics, University Medical Center Utrecht, Utrecht University, Utrecht, the Netherlands | [4]Department of Hematology, Radboud University Medical Center, Nijmegen, the Netherlands | [5]Department of Hematology, Erasmus MC, University Medical Center, Rotterdam, the Netherlands | [6]Department of Pediatric Hematology and Oncology, Erasmus MC Sophia Children's Hospital, University Medical Center, Rotterdam, the Netherlands | [7]Department of Hematology, Amsterdam University Medical Center, Amsterdam, the Netherlands | [8]Department of Molecular Hematology, Sanquin Research and Landsteiner Laboratory, Amsterdam, the Netherlands

Correspondence: M. A. E. Rab (m.a.e.rab@umcutrecht.nl)

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ABSTRACT

Pyruvate kinase (PK) is a key enzyme involved in the final step of glycolysis, essential to produce adenosine triphosphate (ATP). Relatively decreased red blood cell (RBC) PK activity (reflected by a lower PK/hexokinase [HK] ratio) and PK thermostability (PK activity after exposure to heat) were recently identified as pathophysiological features of sickle cell disease (SCD). In this study, we investigated whether impaired PK function is associated with sickle RBC properties and SCD-related clinical manifestations. This study included 97 non-transfused patients with SCD (88 HbSS, 9 HbS/β0 thalassemia). PK thermostability was correlated with RBC parameters such as reticulocyte count ($r = -0.402$, $p < 0.0001$) and hemoglobin F ($r = 0.394$, $p < 0.0001$), and indicators of impaired functional properties of sickle RBCs, such as the point of sickling ($r = -0.417$, $p < 0.0001$), oxygen affinity ($r = 0.408$, $p < 0.001$) and RBC adhesion to laminin ($r = -0.322$, $p = 0.024$). Additionally, a low PK/HK ratio correlated with decreased PK thermostability ($r = 0.308$, $p = 0.002$), decreased RBC deformability ($r = 0.268$, $p = 0.009$), and elevated 2,3-diphosphoglycerate levels ($r = -0.244$, $p = 0.016$). Multivariate Poisson regression analysis demonstrated that reduced PK thermostability and PK/HK ratio were associated with a higher incidence of SCD-related clinical complications. For every 10-unit decrease in PK thermostability and 1-unit decrease in PK/HK ratio, the incidence of SCD-related clinical complications increased by 11% ($p = 0.012$) and 10% ($p = 0.019$), respectively. Altogether, these findings indicate that impaired PK function is related to compromised sickle RBC properties and SCD-related clinical manifestations. This supports the relevance and underlines the potential of PK activation therapy.

1 | Introduction

Sickle cell disease (SCD) is caused by a mutation in the β-globin gene that leads to the formation of abnormal hemoglobin S (HbS). Upon deoxygenation, red blood cells (RBCs) from patients with SCD sickle due to polymerization and are less deformable. This results in multiple downstream effects, including increased RBC adhesion, microvascular occlusion, and chronic hemolysis [1]. SCD patients experience a broad range of clinical complications, including vaso-occlusive episodes (VOEs) causing acute pain, as well as chronic multi-organ damage, such as cerebral infarction, retinopathy, and nephropathy [1–3]. RBCs rely solely on anaerobic glycolysis to produce adenosine triphosphate (ATP), as they lack a nucleus. ATP is crucial for the RBC to maintain the cell's structure and function as less ATP content in RBCs leads to increased hemolysis [4–7]. Pyruvate kinase (PK) is a key enzyme involved in the final step of glycolysis and responsible for approximately 50% of the cell's ATP production. Studies show that modulation of ATP content enhances RBC health by improving deformability and ATP-dependent antioxidant mechanisms [8, 9]. PK deficiency (PKD), caused by a mutation in the *PKLR* gene, is the most common enzyme deficiency leading to non-spherocytic hemolytic anemia. The mutated PKR decreases PK activity and stability, which leads to ineffective glycolysis, causing insufficient ATP production [4]. Furthermore, the decrease in PK activity is accompanied by elevated levels of 2,3-diphosphoglycerate (2,3-DPG): a glycolytic intermediate upstream of PK, which plays a pivotal role in the regulation of the oxygen affinity of hemoglobin. Patients with sickle cell trait (HbAS) typically do not experience clinical complications. However, when PKD is co-inherited with sickle cell trait, it may induce RBC sickling, resulting in a phenotype resembling SCD [10, 11]. Elevated levels of 2,3-DPG decrease the oxygen affinity of hemoglobin and thus increase the polymerization rate of HbS, thereby facilitating RBC sickling [12]. A decrease in relative PK activity (reflected by a lower PK/hexokinase [HK] ratio) and stability, with the absence of *PKLR* gene mutations, has been recently observed in patients with SCD, establishing these PK properties as a pathophysiological feature [13].

Currently, activation of PK is being explored as a new therapeutic approach for patients with SCD [14]. Several ongoing clinical trials investigate the safety and efficacy of PK activation by small molecules such as mitapivat (NL8517, NCT04000165, NCT05031780), AG-946 (NCT04536792), and etavopivat (NCT04624659, NCT04987489). Results from clinical trials on mitapivat and etavopivat show an increase in hemoglobin levels, a decrease in hemolytic markers, and an increased ATP/2,3-DPG ratio, which is accompanied by an improvement in oxygen affinity [15–17]. In a phase 2 study of 10 patients on mitapivat, a significant reduction in VOE frequency was observed when combining results of the 8-week dose-finding period and the 1-year fixed-dose extension period compared to baseline (from 1.33 to 0.64 events, $p = 0.049$) [15]. Additionally, a non-significant trend toward a reduction in annualized VOE frequency was observed in the randomized phase 2 study involving 79 patients [18]. A phase 3 study is currently ongoing to further investigate mitapivat's effectiveness (NCT05031780). The results from clinical trials underline that targeting PK is a potentially important approach for the treatment of SCD. In this study, we aim to correlate the PK activity and stability with RBC parameters and functional properties of the sickle RBCs as well as the history of acute and chronic SCD-related clinical manifestations in order to enhance our understanding of the clinical significance of PK activators in the treatment of SCD.

2 | Materials and Methods

2.1 | Study Population

Homozygous HbS (HbSS) and HbS/β0 thalassemia patients, who visited outpatient clinics as part of the Sickle Cell Outcome Research (SCORE) consortium in the Netherlands (Erasmus University Medical Center [UMC], Amsterdam UMC, Utrecht UMC, and Radboud UMC), were eligible to participate. Both children > 3 years and adults were eligible. Patients who received a blood transfusion within 3 months of inclusion were excluded. Patients were allowed to use hydroxyurea treatment or other disease-modifying drugs, such as voxelotor or crizanlizumab. EDTA blood was collected from patients in a steady state condition (i.e., not during a hospital admission and/or VOE, and the dosages of SCD-related drugs remained unchanged for 6 months prior to inclusion). Study procedures were approved by the local medical ethical committees in accordance with the Declaration of Helsinki (protocol number: 17/392). Blood samples from healthy controls were obtained by the Mini Donor Service, a blood donation facility at the University Medical Center Utrecht, the Netherlands (protocol number: 18/774). Blood samples were stored at 4°C, and laboratory measurements (Figure S1) were performed within 24 h.

Medical records were reviewed to obtain information on patient characteristics, current treatment status, and history of SCD-related complications (e.g., VOEs, acute chest syndrome [ACS], cerebrovascular accident [CVA], proliferative retinopathy, osteonecrosis, chronic kidney disease [CKD], leg ulcers, and cholelithiasis) and need for acute exchange transfusion(s) or intensive care unit admission due to a SCD-related complication. VOEs were defined as acute episodes of pain, with no other medically determined cause than a VOE resulting in a visit to a medical facility (e.g., hospital admission, emergency department or outpatient clinic visit). VOEs were only included if they occurred within the last 2 years. All other complications over the patient's lifetime were included. Osteonecrosis was diagnosed based on the combination of physical examination and magnetic resonance imaging results. Patients with a history of cholecystectomy for cholelithiasis were also classified as having cholelithiasis. An increased risk of CKD was defined based on the combination of the urine albumin to creatinine ratio (ACR, > 3.4 mg/mmol) and estimated glomerular filtration rate (eGFR) calculated using the CKD-EPI formula [19]. Figure S2 provided an overview of the complications. The clinical complication rate was defined as the total number of different SCD-related complications, with each complication counting as one point (e.g., three VOEs in the past 24 months would equal point). Acute complications included VOEs, ACS, CVA, the need for acute exchange transfusion, or intensive care unit admission. Chronic complications included proliferative

retinopathy, osteonecrosis, increased risk of CKD, leg ulcers, and cholelithiasis.

2.2 | Hematological Parameters

Routine laboratory parameters (Figure S1A), including complete blood count, were measured using the Abbott Cell-Dyn Sapphire hematology analyzer (Abbott Diagnostics). The percentage of total hypochromic RBCs (RBCs with hemoglobin concentration < 28 g/dL and mean corpuscular volume [MCV] < 120 fL) and total dense RBCs (RBCs with hemoglobin concentration > 41 g/dL MCV < 120 fL) were obtained using the Advia 120/2120 hematology analyzer (Siemens Healthcare diagnostics). High-performance liquid chromatography was performed to analyze the levels of normal hemoglobin (HbA), fetal hemoglobin (HbF), and HbS (Tosoh G8).

2.3 | Pyruvate Kinase Measurements

RBCs were isolated from whole blood using microcrystalline cellulose α-cellulose columns [20]. PK and HK activity and PK thermostability were measured on purified RBCs (Figure S1B,C) [21]. PK thermostability (expressed as % residual PK activity) was performed as described on lysates after 1 h of incubation (53°C) [22]. Protein levels of PKR and PKM2 were determined by Western Blot analysis (Figure S1D) using polyclonal antibodies against PKRL (gifted by the late Dr. Kahn) and monoclonal antibodies against PKM2 (PodiCeps). Actin was used as a loading control (Calbiochem). Alexa Fluor 680-conjugated goat anti-mouse IgG/IgM (Life) and IRDye 800-conjugated goat anti-rabbit IgG (Licor) were used as detection antibodies. THP1 cell lysate (sc-2238) was used as a positive control for PKM2 protein detection, and healthy control RBC lysate was used as a positive control for PKR protein detection. PKR, PKM2, and actin bands were quantified using Odyssey M and Empiria Studio 2.1.

2.4 | Oxygen Affinity of Hemoglobin (p50) and 2,3-DPG/ATP Levels

Oxygen affinity (p50) was measured with the Hemox-Analyzer (TCS) and reflects the oxygen tension at which 50% of the measurable reach of hemoglobin is saturated with oxygen (Figure S1D). Levels of 2,3-DPG and ATP were measured using liquid chromatography tandem mass spectrometry after snap-freezing whole blood in liquid nitrogen (Figure S1E) [23]. Metabolite levels were normalized to hematocrit.

2.5 | Oxygen and Osmotic Gradient Ektacytometry

Oxygen and osmotic gradient ektacytometry (oxygenscan and osmoscan, respectively) were performed to measure RBC deformability by the Laser-Optical Rotational Red Cell Analyzer (Lorrca, RR Mechatronics) as previously described [24–26]. Figure S1F shows representative curves of the oxygenscan and osmoscan. Oxygen gradient ektacytometry measures RBC deformability upon deoxygenation. The point of sickling (PoS) is the partial oxygen pressure (pO_2) at a 5% decrease from the EI_{max} reflecting the RBC sickling tendency. The minimum EI (EI_{min}) represents the minimal deformability upon deoxygenation. Osmotic gradient ektacytometry measures RBC deformability, expressed as the elongation index (EI), under an osmotic gradient. Key parameters derived from osmotic gradient ektacytometry are the maximum EI (EI_{max}), reflecting the maximal deformability, and the O_{hyper} (osmolality in the hyper-osmolar region at 50% of the EI_{max}) reflecting the RBCs hydration status.

2.6 | RBC Adhesion to Laminin Assay

RBC adhesion to laminin was measured using a microfluidic device (IBIDI μ-Slide I 0.4 uncoated, Figure S1G). IBIDI slides were incubated for 2 h at 37°C with 6.7 μg/mL laminin (BioLamina). Whole blood (with a standardized concentration of 64×10^6 RBCs/mL) was diluted in N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) buffer (osmolality between 280 and 295 mOsm/kg and final pH of 7.4) with bovine serum albumin (0.5% final concentration) and glucose (5.55 mM final concentration). The sample was perfused at room temperature using a syringe pump (Harvard Apparatus PHD Ultra) at a shear stress of 0.5 dyn/cm^2. After 10 min, the IBIDI slide was washed with HEPES buffer for 2 min, and images were taken at nine different places using the Zeiss Observer Z1 microscope (×20 objective). The number of adherent cells was counted using ImageJ (Fiji).

2.7 | Density Separation

To explore whether RBCs with varying densities exhibit different properties, density separation with Percoll (GE Healthcare) was performed in a subset of patients with SCD [27]. Isolated RBCs were added to the columns containing the three layers with different percentages of Percoll (59%, 70%, and 78%) including HEPES, NaCl, KCl, and NaOH [27]. After centrifugation (15 min, 1665*g*, no brake) the four different RBC fractions were collected and washed two times with sodium chloride 0.9% to remove the Percoll. Fraction 1, on top of the 59% layer, contains RBCs with the lowest density, and is therefore usually enriched with reticulocytes. Fraction 2 was collected on top of the 70% layer, and Fraction 3 on top of the 78% layer. Fraction 4, on the bottom of the 78% layer, contains the most dense RBCs. Afterwards, the fractions were measured with a hematology analyzer as described above, and enzymatic and RBC functional measurements were performed.

2.8 | Statistical Analysis

Statistical analysis was performed in R Studio (v4.4.0) and GraphPad Prism (v10.3.1). Continuous laboratory parameters were presented as the means with standard deviation (SD) or median with interquartile range (IQR), depending on the data distribution. The Shapiro–Wilk normality test was performed to check if the residuals were normally distributed. Continuous values between patients with SCD and the control group were compared using the unpaired t-test or Mann–Whitney test,

depending on the data distribution. Friedman test or mixed effects analysis (in case of random missing values) with respectively Dunn's or Tukey's multiple comparisons test were used to compare results of the RBC density separation. A Spearman rank correlation coefficient was used for the correlation between laboratory parameters and enzyme activity measurements. Multivariate Poisson regression analysis was used for the complication rate (count data) to calculate the rate ratio of PK properties, adjusted for age, sex, and hydroxyurea treatment, in relation to the complication rate. Logistic regression analysis was performed to assess odds ratios (ORs) for complications associated with the PK/HK ratio and PK thermostability, adjusted for age, sex, and hydroxyurea treatment. A $p \leq 0.05$ was considered statistically significant.

3 | Results

Ninety-seven patients with SCD (88 HbSS, 9 HbS/β0 thalassemia; $n=66$ Erasmus UMC, $n=15$ Amsterdam UMC, $n=9$ Utrecht UMC, and $n=7$ Radboud UMC) were enrolled and blood samples were retrieved from 20 healthy controls. Of the SCD patients, 22 were children (median age 13 years [range 6–17]) and 75 were adults (median age 31 years [range 18–58]). Sixty-seven (69.1%) patients were treated with hydroxyurea. Four patients received crizanlizumab, and two patients received voxelotor in addition to hydroxyurea. One patient was treated with voxelotor only. In the past 24 months prior to inclusion, 55 patients with SCD (56.7%) experienced at least one VOE, with a mean of 3.3 VOEs per patient (SD 3.1). ACS occurred at least once in 26 patients (26.8%) during their lifetime, with a mean of 1.4 events (SD 0.5). Further details on patient characteristics, SCD-related treatments, and SCD-related complications are listed in Table S1.

3.1 | Pyruvate Kinase Activity and Stability Are Compromised in RBCs From Patients With SCD

Patients with SCD showed an increased absolute PK (median of 13.2 [IQR 11.1–15.0] vs. 7.7 [IQR 6.7–8.5]) and HK activity (median 2.4 [IQR 1.9–2.7] vs. 0.8 [IQR 0.7–0.9]) compared to healthy controls (Table 1). This increase can most likely be attributed to reticulocytosis (median 225.0×10^9/L in SCD patients compared to 59.8×10^9/L in healthy controls), since PK and HK are both RBC age dependent [28]. When evaluating PK activity to that of HK activity, a decreased PK/HK ratio was observed (median of 5.7 [IQR 5.0–6.4] in SCD patients compared to a median of 9.4 [IQR 8.5–10.2] in healthy controls), indicating that the PK activity is decreased in light of the mean RBC age. Median PK thermostability was 74.8% (IQR 66.6–81.5) in patients with SCD compared to 92.2% (IQR 88.7–95.7) in healthy controls. These results confirm our previous findings of decreased relative PK activity and compromised PK thermostability in patients with SCD [13]. When stratifying patients based on hydroxyurea treatment, those who were treated with hydroxyurea demonstrated significantly higher absolute PK activity (Table S2). However, no significant differences were observed in the PK/HK ratio and PK thermostability between SCD patients treated with hydroxyurea and those who were not.

PKR protein was detected in all patients with SCD, while PKM2 was either absent or expressed at very low levels (Figure S3). PKR protein levels (assessed as the normalized PKR-to-actin ratio) in SCD patients showed a significant positive, though weak, correlation with PK activity ($r=0.25$, $p=0.02$) and PK HK ratio ($r=0.24$, $p=0.03$), not with PK thermostability ($r=0.11$, $p=0.303$).

3.2 | RBCs From SCD Patients Have a Lower Oxygen Affinity, Reduced Deformability, and Increased Dehydration and Adhesion Compared to RBCs From Healthy Controls

SCD patients have significantly increased ATP and 2,3-DPG levels (Table 1). Median ATP levels were 7.7% higher compared to healthy controls (916.5 [IQR 836.5–1033.5] vs. 850.6 [790.9–910.2]), whereas median 2,3-DPG levels were 42.2% higher compared to healthy controls (3460 [3275–3782] vs. 2434 [2316–2676]), resulting in a decreased ATP/2,3-DPG ratio in SCD patients (median 0.27 [IQR 0.23–0.29] vs. 0.34 [0.27–0.40]). In line with this increase in 2,3-DPG, increased p50 values were observed in patients with SCD reflecting a lower oxygen affinity: median 25.7 (IQR 24.3–27.4) versus 24.2 (IQR 23.5–25.1) in controls. RBCs from patients with SCD showed decreased deformability in both oxygenscan and osmoscan analyses (Table 1 and Figure S4), with lower EI_{min} in the oxygenscan (median 0.04 [IQR 0.02–0.10] vs. 0.62 [0.61–0.62], $p<0.001$) and lower EI_{max} in the osmoscan (median 0.51 [IQR 0.48–0.54] vs. 0.59 [0.58–0.59], $p<0.001$) compared to RBCs of healthy controls. Upon deoxygenation, the median PoS was 40.70 mmHg (IQR 33.6–47.8). Furthermore, RBCs from patients with SCD are more dehydrated, as indicated by a significantly decreased O_{hyper} (median 380.0 [IQR 368.0–393.0] vs. 444.5 [IQR 441.0–453.3], $p<0.001$). Additionally, these RBCs exhibit increased adhesion to laminin (Table 1): 12.0 RBCs (IQR 9.0–18.9) in SCD versus 5.4 RBCs (SD 2.8) in healthy controls, $p<0.0001$.

3.3 | Pyruvate Kinase Thermostability and PK/HK Ratio Are Associated With RBC Properties

When examining PK thermostability in the correlation analysis (Figure 1, Figure S5), we observed that patients with SCD who had decreased PK thermostability exhibited significantly higher absolute reticulocyte counts ($r=-0.402$, $p<0.0001$) and % dense RBCs ($r=0.368$, $p=0.0005$), lower hemoglobin ($r=0.262$, $p=0.010$) and HbF ($r=0.394$, $p<0.0001$) levels, and increased RBC adhesion to laminin ($r=-0.322$, $p=0.024$). Further, an inverse correlation of PK thermostability with p50 ($r=-0.408$, $p<0.001$) and PoS ($r=-0.417$, $p<0.0001$), and a positive correlation with EI_{max} ($r=0.344$, $p=0.001$) and EI_{min} ($r=0.449$, $p<0.0001$) was observed. These results imply that patients with decreased PK thermostability had less deformable RBCs, which sickled at a higher oxygen tension. This suggests that an increase in PK thermostability is beneficial for sickle RBCs. We found no significant correlation between PK thermostability and 2,3-DPG ($r=-0.155$, $p=0.131$). Further, no correlation of O_{hyper} (marker of RBC dehydration) with PK thermostability ($r=0.093$, $p=0.373$) was observed, which suggests that RBCs with reduced PK thermostability are not more dehydrated, as assessed by osmotic gradient ektacytometry.

TABLE 1 | Laboratory characteristics of SCD patients compared to healthy controls.

	SCD patients ($n=97$)	Healthy controls ($n=20$)	p
RBC parameters			
Hemoglobin (g/dL)	9.1 (8.0–10.2)	13.8 (13.4–14.7)	< 0.001
RBC count (10^{12}/L)	3.0 (2.5–3.5)	4.7 (4.5–4.9)	< 0.001
Hematocrit (%)	27.3 (23.7–30.6)	41.6 (40.1–42.9)	< 0.001
MCV (fL)	89.4 (78.1–99.3)	88.9 (87.2–92.0)	0.871
MCHC (g/dL)	34.0 (32.9–35.1)	33.6 (32.9–34.2)	0.164
Reticulocyte count (10^9/L)	225.0 (156.0–309.0)	59.8 (51.5–75.1)	< 0.001
Hypochromic RBCs (%)[a]	3.5 (2.2–6.2)	0.2 (0.1–0.4)	< 0.001
Dense RBCs (%)[a]	4.4 (2.9–6.5)	0.7 (0.4–1.1)	< 0.001
Hemoglobin F (%)	9.4 (5.9–18.3)	0.6 (0.5–0.7)	< 0.001
Hemoglobin S (%)	77.1 (68.8–81.6)	0.0 (0.0–0.0)	< 0.001
Enzymatic properties			
PK activity (U/gHb)	13.2 (11.1–15.0)	7.7 (6.7–8.5)	< 0.001
HK activity (U/gHb)	2.4 (1.9–2.7)	0.8 (0.7–0.9)	< 0.001
PK/HK ratio	5.7 (5.0–6.4)	9.4 (8.5–10.2)	< 0.001
PK thermostability (% residual activity)	74.8 (66.6–81.5)	92.2 (88.7–95.7)	< 0.001
Metabolic properties			
ATP (mg/L RBCs)	916.5 (836.5–1033.5)	850.6 (790.9–910.2)	0.009
2,3-DPG (mg/L RBCs)	3460 (3275–3782)	2434 (2316–2676)	< 0.001
ATP/2,3-DPG ratio	0.27 (0.23–0.29)	0.34 (0.27–0.40)	< 0.001
Functional RBC parameters			
p50 (mmHg)[b]	25.7 (24.3–27.4)	24.2 (23.5–25.1)	0.001
EI_{min} (EI)[c]	0.04 (0.02–0.10)	0.62 (0.61–0.62)	< 0.001
EI_{max} (EI)[c]	0.51 (0.48–0.54)	0.59 (0.58–0.59)	< 0.001
Point of sickling (mmHg)	40.7 (33.6–47.8)	NA	NA
Ohyper (mOsm/kg)	380.0 (368.0–393.0)	444.5 (441.0–453.3)	< 0.001
RBC adhesion to laminin (n)[d]	12.0 (9.0–18.9)	4.9 (3.8–5.3)	< 0.001

Note: Numbers represent median (IQR).
Abbreviations: 2,3-DPG, 2,3-diphosphoglycerate; ATP, adenosine triphosphate; HK, hexokinase; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; PK, pyruvate kinase; RBC, red blood cell.
[a]Percentage of hypochromic and dense RBCs is missing in 12 (12.4%) of the SCD patients and 3 (15.0%) of the healthy controls.
[b]p50 is missing in 20 (20.6%) of the SCD patients.
[c]EI_{min} is derived from the oxygenscan, and the EI_{max} is derived from osmoscan.
[d]RBC adhesion to laminin is missing in 48 (49.5%) of the SCD patients and 6 (30.0%) of the healthy controls.

Since PK and HK activity are correlated with red cell age (correlation PK and HK activity and reticulocytes: $r=0.452$ and $r=0.505$, respectively, both $p<0.0001$), we investigated whether the PK/HK ratio was correlated with RBC properties. Only significant correlations with PK thermostability ($r=0.308$, $p=0.002$), 2,3-DPG ($r=-0.244$, $p=0.016$) and EI_{max} ($r=0.268$, $p=0.009$) were observed, indicating that patients with a higher PK/HK ratio had increased PK thermostability and RBC deformability and lower 2,3-DPG levels. No significant correlation was observed between the PK/HK ratio and hemoglobin levels ($r=0.167$, $p=0.101$) or reticulocyte count ($r=-0.169$, $p=0.098$).

3.4 | Increase in RBC Density Is Associated With a Decrease in Enzyme Activity

Since PK activity and PK thermostability are differentially correlated with reticulocyte count and dense RBCs (Figure S5), we performed RBC density separation of blood samples from five HbSS patients to explore these findings in more detail (Figure 2). Figure 2A displays the density separation columns of five patients with HbSS showing a heterogeneous RBC distribution among patients. The increase in RBC density is reflected by an increase in mean corpuscular hemoglobin

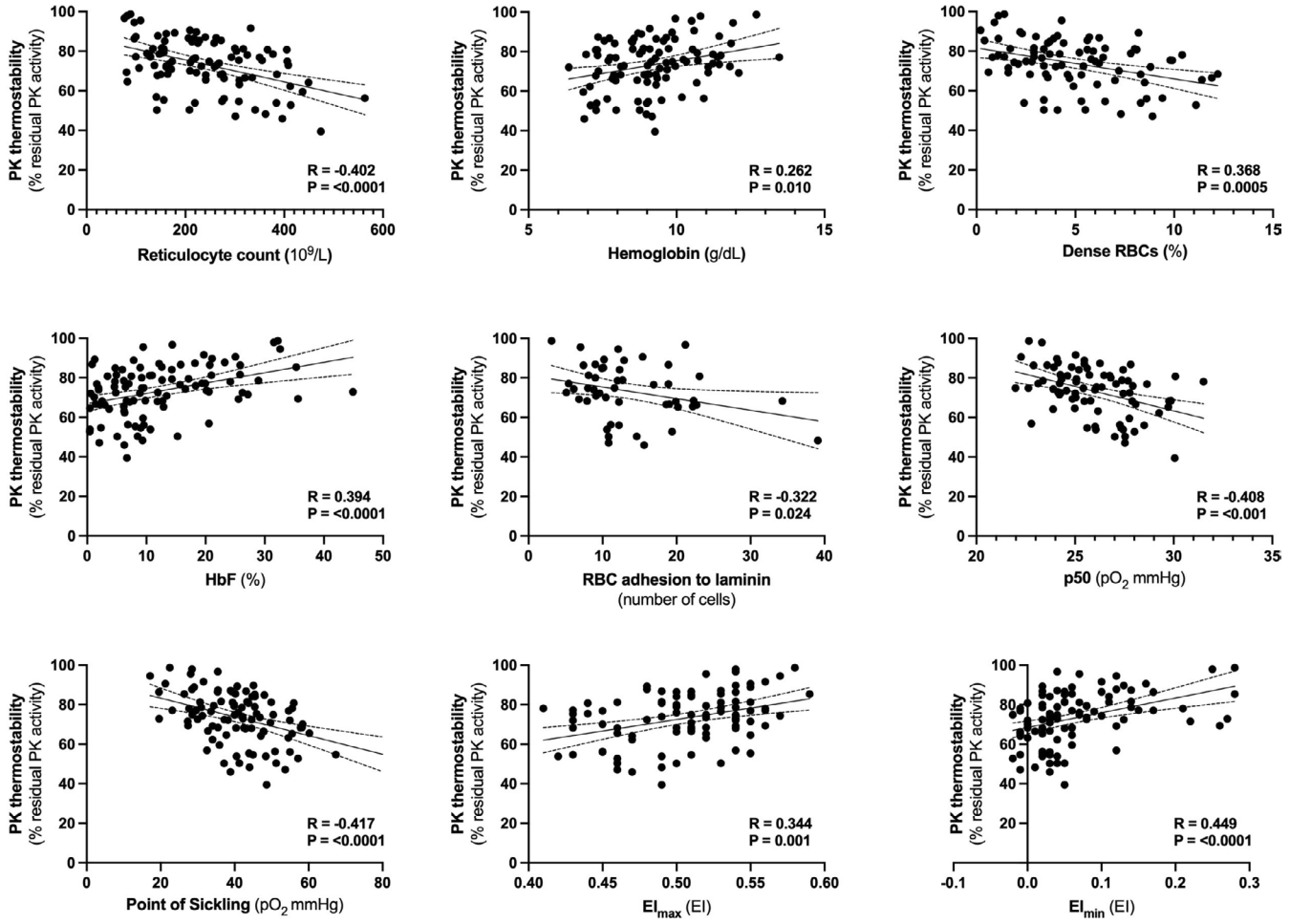

FIGURE 1 | Linear regression with Spearman correlation coefficient of PK thermostability measurements and laboratory parameters. PK thermostability, expressed as the residual PK activity after 60 min of incubation at 53°C, is significantly correlated with reticulocyte count, hemoglobin, dense RBCs, and hemoglobin F (HbF). Additionally, patients with low PK thermostability showed increased RBC adhesion to laminin, reduced oxygen affinity (reflected by a higher p50), a tendency to sickle at higher oxygen tensions upon deoxygenation (reflected by a higher point of sickling) and decreased deformability (reflected by lower EI_{max} and EI_{min} values).

concentration (MCHC) (Figure 2B) and a decrease in reticulocyte levels (Figure 2C). In line with the decrease in reticulocytes, we observed a significant decrease in PK and HK activity (Figure 2D,E). The PK/HK ratio was significantly higher in Fraction 4 compared to the total RBCs, primarily because HK decreased more substantially than PK with increasing density (Figure 2F). PK thermostability was not significantly different compared to the total of all fractions (Figure 2G). Further, the densest RBCs (Fraction 4) exhibited the most strongly reduced deformability (EI) and highest PoS, indicating that they sickle earlier upon deoxygenation (Figure 2H,I).

3.5 | Patients With SCD and Impaired Pyruvate Kinase Function Have More Clinical Complications

To identify whether PK thermostability and PK/HK ratio are related to clinical severity, a clinical complication rate was determined. Results of multivariate Poisson regression analysis (Table 2) showed a significant relationship between PK thermostability and total complication rate (rate ratio = 0.89, $p = 0.012$) indicating that for every 10-unit increase in PK thermostability, the complication rate decreases by 11%. Furthermore, this analysis showed that for every unit increase in PK/HK ratio, the total complication rate decreases by 10% (rate ratio = 0.90, $p = 0.019$). When complications were categorized into acute and chronic, significant associations were observed between PK thermostability and the chronic complication rate (rate ratio 0.84, $p = 0.014$) and PK/HK ratio and the acute complication rate (rate ratio 0.85, $p = 0.016$).

Logistic regression analysis demonstrated that a 10-unit increase in PK thermostability was associated with a 61% decrease in the adjusted odds of experiencing more than one complication ($p \leq 0.001$), while a 1-unit increase in PK/HK ratio reduced these odds by 30% ($p = 0.030$). Specifically, each 10-unit increase in PK thermostability lowered the adjusted odds of experiencing more than one chronic complication by 41% ($p = 0.007$) and each 1-unit increase in PK/HK ratio lowered the adjusted odds of experiencing more than one acute complication by 40% ($p = 0.011$). Additionally, the adjusted odds of having two or more VOEs or cholelithiasis decreased by about 29% ($p = 0.048$) and 53% ($p = 0.001$), respectively, with a 10-unit increase in PK thermostability. For each unit increase in PK/HK ratio, the adjusted

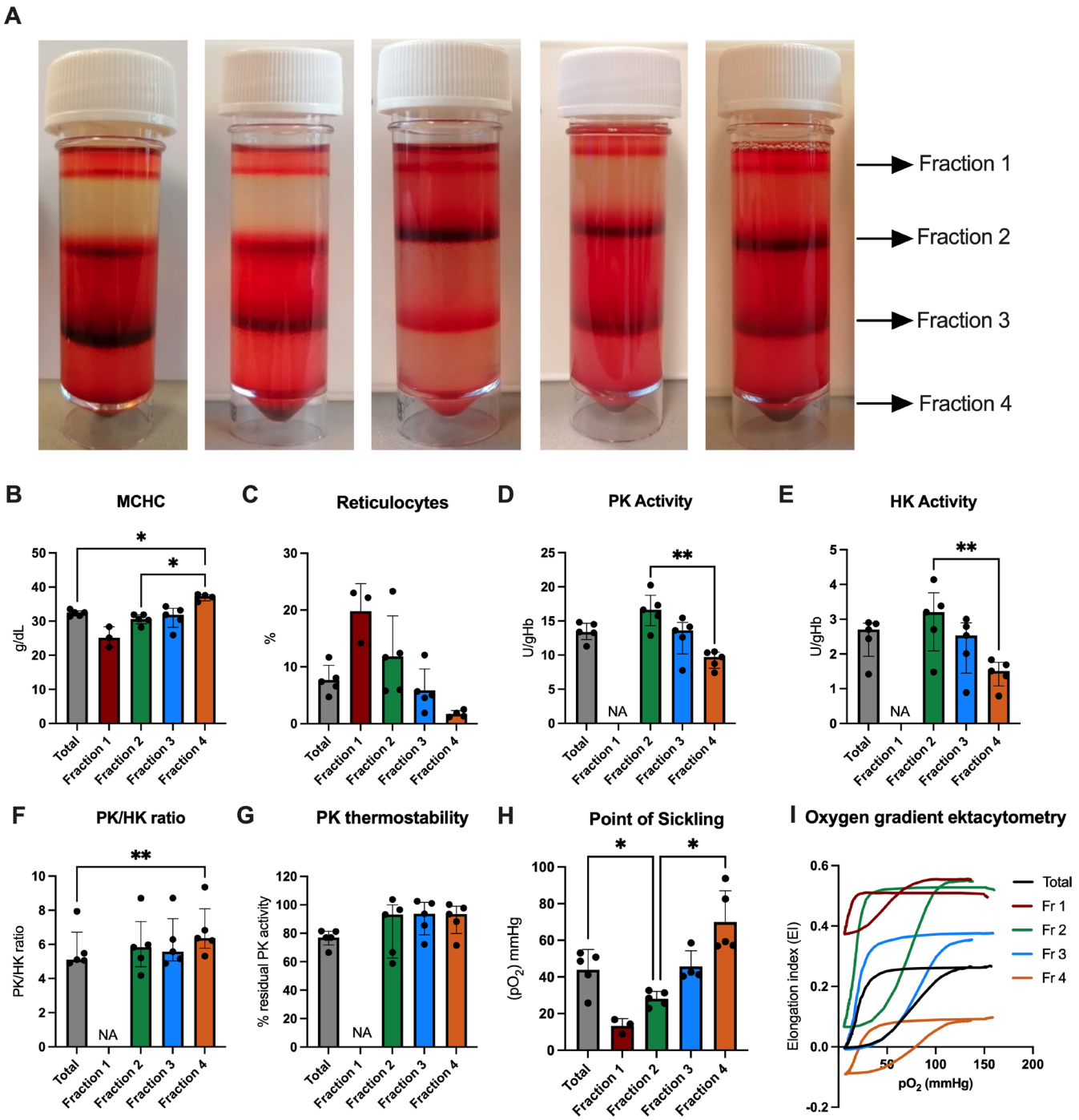

FIGURE 2 | Enzymatic measurements and oxygen gradient ektacytometry curves of RBCs after density separation. Bars represent median values with interquartile range. Only significant comparisons were shown. All other comparisons between fractions were nonsignificant. Enzyme activity measurements in Fraction 1 were not feasible due to an insufficient volume. (A) Percoll gradient density separation of five different patients with HbSS. Fraction 1 contains RBCs with the lowest density, and Fraction 4 contains RBCs with the highest density. The distribution of fractions differs between patients. (B) The mean corpuscular hemoglobin concentration (MCHC) increase reflects the increase in density. (C) Reticulocyte levels were the highest in the low dense fraction; however, these results were not statistically significant. (D, E) Enzymatic pyruvate kinase (PK) and hexokinase (HK) activity decreases with increasing density. (F) PK/HK ratio is significantly higher in Fraction 4 compared to the PK/HK ratio of the total RBCs. (G) PK thermostability is not significantly different compared to the total of all fractions. (H, I) RBCs with the highest density have the lowest deformability and sickle earlier upon deoxygenation. [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 2 | Univariate and multivariate Poisson and logistic regression analysis show significant effect of PK thermostability and PK/HK ratio on clinical complications in SCD.

PK thermostability, for every 10-unit increase						
	Univariate Poisson's regression			**Multivariate Poisson's regression[a]**		
	RR	**95% CI**	***p***	**RR**	**95% CI**	***p***
Total complication rate	0.85	(0.77–0.94)	**0.001**	0.89	(0.81–0.97)	**0.012**
Acute complication rate	0.89	(0.78–1.02)	0.081	0.92	(0.80–1.05)	0.194
Chronic complication rate	0.80	(0.70–0.93)	**0.003**	0.84	(0.73–0.97)	**0.014**
	Univariate logistic regression			**Multivariate logistic regression[a]**		
	OR	**95% CI**	***p***	**OR**	**95% CI**	***p***
> 1 any complication	0.45	(0.28–0.67)	**< 0.001**	0.39	(0.22–0.64)	**< 0.001**
> 1 acute complications	0.78	(0.58–1.09)	0.147	0.81	(0.56–1.14)	0.226
> 1 chronic complication	0.58	(0.39–0.82)	**0.004**	0.59	(0.39–0.86)	**0.007**
VOE ≥ 2 < 24 months	0.69	(0.49–0.96)	**0.033**	0.71	(0.49–0.99)	**0.048**
ACS	0.88	(0.62–1.25)	0.463	0.91	(0.63–1.31)	0.622
CVA	0.64	(0.37–1.09)	0.100	0.72	(0.41–1.23)	0.239
Acute exchange transfusion	0.71	(0.49–1.00)	0.057	0.73	(0.50–1.05)	0.096
Intensive care unit admission	1.04	(0.65–1.71)	0.875	1.11	(0.68–1.85)	0.683
Proliferative retinopathy	0.83	(0.56–1.22)	0.342	0.92	(0.61–1.39)	0.686
Osteonecrosis	0.76	(0.50–1.14)	0.180	0.78	(0.51–1.16)	0.219
Increased risk CKD	0.76	(0.52–1.10)	0.150	0.82	(0.55–1.23)	0.343
Leg ulcer	0.93	(0.43–2.11)	0.844	0.91	(0.44–1.93)	0.795
Cholelithiasis	0.49	(0.31–0.74)	**0.001**	0.47	(0.29–0.73)	**0.001**
PK/HK ratio, for every unit increase						
	Univariate Poisson's regression			**Multivariate Poisson's regression[a]**		
	RR	**95% CI**	***p***	**RR**	**95% CI**	***p***
Total complication rate	0.91	(0.83–1.00)	0.050	0.90	(0.82–0.98)	**0.019**
Acute complication rate	0.86	(0.75–0.97)	**0.020**	0.85	(0.75–0.97)	**0.016**
Chronic complication rate	0.95	(0.83–1.08)	0.445	0.92	(0.81–1.04)	0.216
	Univariate logistic regression			**Multivariate logistic regression[a]**		
	OR	**95% CI**	***p***	**OR**	**95% CI**	***p***
> 1 any complication	0.82	(0.62–1.08	0.165	0.70	(0.50–0.96)	**0.030**
> 1 acute complications	0.64	(0.43–0.90)	**0.018**	0.60	(0.39–0.86)	**0.011**
> 1 chronic complication	1.00	(0.75–1.31)	0.999	0.93	(0.68–1.23)	0.602
VOE ≥ 2 < 24 months	0.66	(0.45–0.91)	**0.021**	0.64	(0.43–0.89)	**0.015**
ACS	0.94	(0.68–1.25)	0.690	0.92	(0.66–1.24)	0.610
CVA	0.47	(0.22–0.88)	**0.029**	0.47	(0.21–0.90)	**0.042**
Acute exchange transfusion	0.88	(0.62–1.19)	0.435	0.86	(0.60–1.16)	0.353
Intensive care unit admission	0.80	(0.48–1.22)	0.360	0.79	(0.47–1.20)	0.335
Proliferative retinopathy	1.25	(0.92–1.71)	0.142	1.21	(0.87–1.71)	0.248

(Continues)

TABLE 2 | (Continued)

	Univariate logistic regression			Multivariate logistic regression[a]		
	OR	**95% CI**	***p***	**OR**	**95% CI**	***p***
Osteonecrosis	0.93	(0.63–1.30)	0.711	0.91	(0.61–1.27)	0.597
Increased risk CKD	0.86	(0.60–1.16)	0.362	0.79	(0.54–1.10)	0.197
Leg ulcer	0.56	(0.20–1.23)	0.209	0.47	(0.15–1.10)	0.135
Cholelithiasis	0.78	(0.55–1.07)	0.137	0.75	(0.51–1.04)	0.105

Note: Bold values represent statistical significance ($p \leq 0.05$).
[a]In the multivariate regression analysis, the variables were adjusted for age, sex, and hydroxyurea treatment. Total complication rate is a combination of all acute and chronic complications. Acute complication rate includes vaso-occlusive episodes, acute chest syndrome, cerebrovascular accident, the need for acute exchange transfusion, and intensive care unit admission. Chronic complication rate includes proliferative retinopathy, osteonecrosis, increased risk of chronic kidney disease, leg ulcer, and cholelithiasis. Rate ratios (RR) and odds ratios (OR) were calculated for every 10-unit increase in PK thermostability and every unit increase in pyruvate kinase/hexokinase (PK/HK) ratio.

odds of experiencing two or more VOEs or a CVA decreased by 36% ($p = 0.015$) and 53% ($p = 0.042$), respectively.

4 | Discussion

In this study, we demonstrated decreased PK thermostability and decreased relative PK activity (PK/HK ratio) in patients with SCD. Furthermore, PK thermostability and PK/HK ratio were related to sickle RBC properties and clinical manifestations of SCD. Decreased PK thermostability is associated with an increase in reticulocyte count as well as impaired functional properties of RBCs, such as a decrease in RBC deformability and oxygen affinity, and an increase in RBC adhesion. The PK/HK ratio shows a negative association with 2,3-DPG levels. This suggests that a modest reduction in PK activity, which is downstream of 2,3-DPG in glycolysis, compared to HK activity, which is upstream of 2,3-DPG, results in increased levels of 2,3-DPG. We also showed that a decreased PK thermostability and PK/HK ratio are associated with the occurrence of SCD-related complications such as VOEs [29]. These results imply that decreased PK thermostability and PK/HK ratio are pathophysiological features of SCD that are related to clinical severity. Hence, these findings are of importance in light of PK activator therapies that are currently under investigation in clinical trials.

An elevated ATP content was observed in sickle RBCs compared to healthy controls. This is likely attributed to reticulocytosis, accompanied by increased absolute PK activity and oxidative phosphorylation in the mitochondria for ATP production [30]. The increased PK and HK activity levels in patients with SCD are both attributed to younger RBC age [31], as confirmed by our density separation data (Figure 2). When adjusted for RBC age, SCD patients exhibit a reduced PK/HK ratio compared to healthy controls. This reduced ratio can result from either decreased PK activity or increased HK activity. Increased HK activity could, theoretically, enhance glycolytic flux, leading to elevated ATP production. However, patients with SCD also have elevated 2,3-DPG levels, indicating impaired glycolysis downstream in the Embden-Meyerhof pathway, similar to observations in patients with PKD. Therefore, we believe that the PK/HK ratio reflects decreased PK activity rather than abnormally elevated HK activity in patients with SCD. Clinical trials demonstrated that PK activation therapy ameliorates pathophysiological effects in SCD. Phase 1/2 studies of mitapivat and etavopivat in patients with SCD have demonstrated increased ATP production and reduced intracellular 2,3-DPG levels, leading to higher hemoglobin levels [15–17]. Moreover, improvements in markers of RBC sickling and oxygen affinity were observed, and treatment with PK activator therapy resulted in a reduction of reticulocytes and hemolytic parameters, such as lactate dehydrogenase and bilirubin [15–17]. Reduced hemolysis and, hence, increased RBC survival alters the distribution of RBC subpopulations. We, therefore, performed an RBC density separation in a subset of patients to investigate whether the PK and sickling properties are RBC age/density dependent. Importantly, RBC density is not identical to RBC age, but reticulocytes are less dense with increased MCV and decreased MCHC. During cell aging, RBCs shed parts of their membrane resulting in smaller and denser cells [27, 32]. RBC fractions with the highest density had the lowest enzymatic PK and HK activity, lowest RBC deformability, and sickled earlier upon deoxygenation. Understanding the PK properties in the RBC fractions can help in evaluating the treatment responses, as the distribution of these fractions differs between patients and may also be affected by (PK activator) therapy. Measuring the PK and HK activity and PK thermostability exclusively in Fraction 1 was not feasible. This is because this fraction contained an excessive number of leukocytes and platelets, which interfere with enzyme activity, and an insufficient volume to perform RBC isolation and enzyme activity measurements. Overall, the results of the density separation experiments in a subset of SCD patients show that each RBC fraction possesses unique characteristics, with a decrease in PK and HK activity but no difference in PK thermostability according to density. The varying proportions of these RBC fractions among patients could explain the heterogeneity in RBC characteristics in SCD. To accurately assess potential PK impairment, PK activity in SCD patients could be compared to that of individuals with a similar reticulocyte count. Future studies should aim to investigate a population with high reticulocytes but without RBC defects to provide clearer insights into PK.

Four PK isoforms are encoded by two genes: *PKLR* (PKL in the liver, PKR in RBCs) and *PKM* (PKM1 in musle and brain, PKM2 in embryonic cells and various adult tissues, including erythroblasts) [4, 33]. The PK thermostability and PK/HK ratio assays were initially developed as a diagnostic tool for PKD [34]. The PK thermostability test measures the ability of

the PK enzyme to maintain its structure and function when exposed to heat [22]. In PKD the *PKLR* gene is mutated, often resulting in decreased PK activity and thermostability of the variant PK. However, why the PK enzyme in patients with SCD is less active and less resistant to heat exposure despite the absence of a mutation in the *PKLR* gene is yet unknown. A decreased relative PK activity is not limited to PKD and SCD but has also been reported in other hereditary hemolytic anemias, including hereditary spherocytosis, beta thalassemia, and hereditary xerocytosis (also known as dehydrated stomatocytosis), as well as in patients with myelodysplastic syndrome [35–37]. In hereditary spherocytosis, it was hypothesized that membrane instability causes the loss of glycolytic enzyme complexes from the RBC membrane, including PK, thereby reducing the local availability of PK [36]. In SCD and thalassemia, oxidative stress could affect the structure and/or function of the PK enzyme [38]. This hypothesis is supported by data showing inhibition of PKM2 by reactive oxygen species [39]. While most research on PKM2 had focused on its role in cancer cells, the mechanism observed in PKM2 may also be relevant to PKR. Currently, it is unclear whether the relatively decreased PK activity observed in the above conditions is primarily caused by oxidative stress, the loss of glycolytic enzyme complexes due to membrane instability, and/or another underlying factor. However, impaired PK activity is a rationale for PK activator therapy in all these red cell disorders and is currently under investigation [40–42].

Although SCD is caused by a single nucleotide mutation, the pathophysiology is highly complex, leading to significant phenotype variability [43]. Many biomarkers have been studied in SCD, each related to different aspects of disease pathophysiology. For decades, HbF has been a well-known biomarker and key modulator of SCD severity [44]. HbF plays a protective role in SCD by reducing the HbS concentration and thus inhibiting RBC sickling. This protective mechanism can be induced with HbF induction therapies, such as hydroxyurea [45]. Patients with higher HbF levels generally experience a milder clinical disease course. However, even patients with elevated HbF levels as a result of hydroxyurea treatment may still suffer from clinical complications [45]. Therefore, other pathophysiological targets are needed that can be addressed by therapy, such as PK thermostability and the PK/HK ratio. The correlation between PK thermostability and the PK/HK ratio with both laboratory and SCD-related clinical characteristics is a rationale for PK activation therapy. Future studies should investigate whether patients with reduced PK thermostability and/or a low PK/HK ratio will particularly benefit from PK activator therapy by measuring these specific biomarkers before initiation of therapy.

In conclusion, decreased PK thermostability and PK/HK ratio are associated with compromised RBC properties and a higher prevalence of SCD-related clinical complications in patients with SCD. Enhancing PK activity and stability with PK activator therapy may therefore improve other pathophysiological features of SCD beyond RBC metabolism. As the development of disease-modifying therapies for patients with SCD is urgently needed, identification and further research into new biomarkers and promising approaches such as PK activators are essential.

Author Contributions

M.J.M.T., R.v.W., and M.A.E.R. designed the study. M.J.M.T., J.F.B., L.v.P., M.J.v.D., B.A.v.O., and J.R.A.d.W. developed laboratory methodology and performed laboratory experiments. M.J.M.T., S.v.d.V., S.E.M.S., M.N.L., M.H.C., E.N., B.J.B., A.W.R., E.J.v.B., and M.A.E.R. recruited and included patients. M.J.M.T. collected clinical data and wrote the manuscript. R.v.W. and M.A.E.R. supervised the research. All authors revised the manuscript and approved the final version.

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Ethics Statement

Study procedures were approved by the local medical ethical committees in accordance with the Declaration of Helsinki (protocol number: 17/392). Blood samples from healthy controls were obtained by the Mini Donor Service, a blood donation facility at the University Medical Center Utrecht, the Netherlands (protocol number: 18/774).

Consent

All patients provided verbal and written consent for participation in this study.

Conflicts of Interest

M.J.M.T., M.J.v.D., and J.R.A.d.W. received research funding from Agios Pharmaceuticals Inc. M.N.L. received research funding from ZonMW, LeoPharma, and INVENT-VTE; reports consultancy fees (all to institute) from BMS/Pfizer, Amgen, Inari, Viatris, Servier, and AbbVie. M.H.C. received research funding from Takeda, Pfizer, Bayer, CSL Behring, Novo Nordisk, Novartis, Nordic Pharma; reports advisory or consulting role with Roche, Bayer, and Novartis. E.N. received research funding from Novartis; reports consulting role with Novartis and Vertex. B.J.B. received research funding from Sanquin, BMS, Pfizer, and Novartis; received honoraria from Sanofi; reports advisory board participation with Celgene, CSL Behring, Pfizer, and Novo Nordisk. E.J.v.B. received research funding from Agios Pharmaceuticals Inc.; reports consulting role with Agios Pharmaceuticals Inc. and Pfizer. A.W.R. received honoraria from Pfizer, Vertex pharmaceuticals, BMS, and Servier. R.v.W. received research funding from Agios Pharmaceuticals Inc. and Pfizer; reports consulting role with Agios Pharmaceuticals Inc., Pfizer, and RR Mechatronics. M.A.E.R. received research funding from Agios Pharmaceuticals Inc. and Pfizer; reports consulting role with RR Mechatronics. The other authors declare no conflicts of interest.

Data Availability Statement

Data and protocols are available upon request (e-mail M.A.E.Rab@umcutrecht.nl). Data will be shared as is compliant with the General Data Protection Regulation and European Union privacy laws.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.