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RESEARCH ARTICLE

A novel composition of endogenous metabolic modulators improves red blood cell properties in sickle cell disease

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Abstract

The most common forms of sickle cell disease (SCD) are sickle cell anemia (SCA; HbSS) and HbSC disease. In both, especially the more dense, dehydrated and adherent red blood cells (RBCs) with reduced deformability are prone to hemolysis and sickling, and thereby vaso-occlusion. Based on plasma amino acid profiling in SCD, a composition of 10 amino acids and derivatives (RCitNacQCarLKHVS; Axcella Therapeutics, USA), referred to as endogenous metabolic modulators (EMMs), was designed to target RBC metabolism. The effects of ex vivo treatment with the EMM composition on different RBC properties were studied in SCD (n = 9 SCA, n = 5 HbSC disease). Dose-dependent improvements were observed in RBC hydration assessed by hemocytometry (MCV, MCHC, dense RBCs) and osmotic gradient ektacytometry (Ohyper). Median (interquartile range [IQR]) increase in Ohyper compared to vehicle was 4.9% (4.0%–5.5%), 7.5% (6.9%–9.4%), and 12.8% (11.5%–14.0%) with increasing 20×, 40×, and 80X concentrations, respectively (all $p < 0.0001$). RBC deformability (EImax using oxygen gradient ektacytometry) increased by 8.1% (2.2%–12.1%; $p = 0.0012$), 9.6% (2.9%–15.1%; $p = 0.0013$), and 13.3% (5.7%–25.5%; $p = 0.0007$), respectively. Besides, RBC adhesion to subendothelial laminin decreased by 43% (6%–68%; $p = 0.4324$), 58% (48%–72%; $p = 0.0185$), and 71% (49%–82%; $p = 0.0016$), respectively. Together, these results provide a rationale for further studies with the EMM composition targeting multiple RBC properties in SCD.

KEYWORDS
amino acids, anemia, metabolism, red blood cell, sickle cell disease

Richard van Wijk. and Minke A. E. Rab contributed equally to this study.

1 | INTRODUCTION

Sickle cell disease (SCD) is a hereditary red blood cell (RBC) disorder with different types based on mutations in the *HBB* gene encoding the β-globin chains of hemoglobin (Hb) [1, 2]. Sickle cell anemia (SCA; HbSS) is the most common and severe type with homozygous inheritance of c.20A > T producing hemoglobin S (HbS, $\beta^{Glu6Val}$). HbS polymerizes upon deoxygenation, which generally leads to sickled, poorly deformable, dehydrated, and dense RBCs with increased adhesion. In turn, this results in hemolytic anemia, painful vaso-occlusive episodes (VOEs), and progressive organ damage [3]. Although HbS polymerization is central to SCD pathophysiology, additional processes may contribute including endothelial dysfunction, oxidative stress, inflammation, and hypercoagulability [4]. Altogether, SCD is a complex and multifactorial disease with phenotypic variability. In particular, HbSC disease, the second most common type of SCD with compound heterozygosity for HbS and hemoglobin C (HbC, $\beta^{Glu6Lys}$), is traditionally considered phenotypically "milder" than SCA. However, it is not completely understood why retinopathy and sensorineural hearing loss are more frequent [5–7]. Presence of HbC enhances HbS polymerization as it triggers potassium (K^+) and water efflux. This results in more dehydrated RBCs, thereby increasing intracellular HbC crystallization and the HbS concentration [8]. Overall, both SCA and HbSC disease lead to extensive morbidity and early mortality [3, 5, 8].

Increased knowledge on the pathophysiology of SCD has provided new therapeutic targets. SCD RBCs show alterations in various metabolites compared to healthy RBCs [9]. In addition, RBC metabolism in patients with HbSC disease differs from patients with SCA. For example, all glycolytic intermediates (except pyruvate) are significantly increased in SCA but not in HbSC disease [9]. The metabolic profile of patients with SCD could also differ over time [9, 10]. Several plasma and RBC metabolites alter on the occurrence of VOEs compared to the steady-state condition [10]. Further, the age of patients with SCA is positively correlated with higher levels of RBC creatinine and citrulline. However, in general, SCD RBCs from both patients with SCA and HbSC disease have an increased metabolic demand [11, 12]. Plasma profiling in SCD revealed significant decreases in several amino acids, which were investigated as single amino acids as well as in different compositions [13]. Ultimately, a composition containing eight different amino acids and two derivatives (arginine, citrulline, N-acetylcysteine, glutamine, carnitine, leucine, lysine, histidine, valine, and serine [RCitNacQCarLKHVS]) showed a decrease in markers of adhesion, inflammation, and chemotaxis. Furthermore, this composition showed the strongest improvement in RBC deformability [13]. Hence, this composition was selected to target multiple RBC properties, inflammation, and vascular health [13]. The components are referred to as endogenous metabolic modulators (EMMs) since they have a role in regulating signaling and metabolic pathways. In this study, the RCitNacQCarLKHVS composition was used to treat SCD blood ex vivo and to study effects on RBC properties such as hydration, deformability, and adhesion in SCA and HbSC disease.

2 | METHODS

2.1 | Patient and control samples

After obtaining written informed consent, blood from adult patients with SCA and HbSC disease was collected in steady-state conditions, irrespective of treatment (chronic RBC exchange transfusion therapy, hydroxyurea, or none), in EDTA tubes. When on chronic RBC exchange transfusion, blood was collected just before replacement with donor RBCs. The study was approved by the Medical Research Ethics Committee Utrecht and NedMec, the Netherlands (protocol numbers: 17/450 and 21/793) and conducted in accordance with the principles of the Declaration of Helsinki. Healthy control blood was obtained through our institutional's minidonor service, an ethics review board–approved blood donation facility at the University Medical Center Utrecht, the Netherlands (protocol number: 18/774).

2.2 | Ex vivo treatment with RCitNacQCarLKHVS

The EMM composition RCitNacQCarLKHVS was produced and provided by Axcella Therapeutics, Cambridge, MA, USA. The stock concentration was 400× the baseline plasma amino acid concentrations of patients with SCD [13], diluted in Dulbecco's phosphate-buffered saline (PBS). Whole blood samples were treated ex vivo with the EMM composition after standardizing the hematocrit to 20% using autologous plasma. The samples were tumbled (VWR Tube Rotator) for 4 h at 37°C before measurements were performed within 72 h after blood collection. Final concentration of the EMM composition was 20×, 40×, or 80×, and samples were compared to vehicle (Dulbecco's PBS, Sigma-Aldrich).

2.3 | RBC characteristics and functional tests

Several RBC characteristics and functional tests were analyzed. Briefly, routine hematological parameters, the percentage of total hypochromic RBCs (mean corpuscular Hb concentration [MCHC] < 28 g/dL), and total dense RBCs (MCHC > 41 g/dL) were measured. Furthermore, high-performance liquid chromatography was performed on whole blood. Osmotic gradient ektacytometry, deformability curves, and oxygen gradient ektacytometry were performed to assess RBC deformability, expressed as the elongation index (EI), under various conditions. Targeted metabolomics was performed to quantify adenosine triphosphate (ATP) and 2,3-diphosphoglycerate (2,3-DPG) levels. In addition, p50, reflecting oxygen affinity was measured. Blood viscosity was measured and the hematocrit-to-viscosity ratio (HVR) was calculated as follows: hematocrit (%) / blood viscosity (mPa·s = cP) at a specific shear rate. Finally, RBC adhesion to laminin was assessed in in vitro flow experiments. Further methodology was provided in the Supporting Information).

TABLE 1 Baseline characteristics of healthy controls and patients with SCD.

	Healthy controls ($n = 3$)	SCA non-RCE ($n = 6$)	SCA RCE ($n = 3$)	HbSC disease ($n = 5$)
Patient characteristics				
Age, years, median (range)	62 (30–62)	29 (18–44)	31 (26–34)	32 (19–37)
Sex, female, *n* (%)	3/3 (100)	3/6 (50)	3/3 (100)	5/5 (100)
α-Thalassemia, *n* (%)				
Homozygous		0/6 (0)	0/3 (0)	1/5 (20)
Heterozygous		1/6 (17)	1/3 (33)	3/5 (60)
No		4/6 (67)	1/3 (33)	1/5 (20)
Unknown	3/3 (100)	1/6 (17)	1/3 (33)	0/5 (0)
Hydroxyurea, *n* (%)	n/a	3/6 (50)	2/3 (66)	0/5 (0)
RBC characteristics				
HbF, %	0.7 (0.3–0.8)	3.0 (2.3–5.2)	6.3 (2.1–15.4)	1.4 (0.7–1.9)
HbS, %	n/a	83.5 (79.1–86.0)	44.0 (43.2–46.4)	44.1 (43.4–45.6)
HbC, %	n/a	n/a	n/a	44.2 (44.1–45.7)
Hb, g/dL	12.8 (12.3–13.9)	8.7 (8.0–9.9)	10.1 (8.5–11.0)	10.8 (9.0—11.6)
Reticulocytes, %	1.1 (1.1–1.5)	8.9 (8.2–10.8)	9.2 (5.8–20.8)	2.5 (2.1–4.1)
MCV, fL	91 (86–94)	79 (73–95)	99 (91–104)	71 (59–78)
MCHC, g/dL	33.2 (33.0–33.4)	33.2 (32.5–34.3)	34.0 (33.5–34.0)	34.6 (33.8–35.5)
Hypochromic RBCs, %	0.4 (0.1–0.6)	6.7 (4.6–9.0)	1.6 (0.8–2.2)	0.8 (0.3–1.7)
Dense RBCs, %	0.7 (0.3–1.8)	4.5 (3.0–11.6)	5.4 (4.3–5.7)	14.3 (12.7–23.4)

Note: Numbers represent median (IQR), unless stated otherwise.
Abbreviations: dense RBCs, RBCs with a MCHC > 41 g/dL; Hb, hemoglobin; HbC, hemoglobin C; HbF, fetal hemoglobin; HbS, sickle hemoglobin; hypochromic RBCs, RBCs with a MCHC < 28 g/dL; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; n/a, not applicable; *n*, number; RCE, chronic red blood cell (RBC) exchange transfusion; SCA, sickle cell anemia; SCD, sickle cell disease.

2.4 | Statistical analysis

Statistical analysis was performed using Graphpad Prism 9.3.0. Unless otherwise stated, categorical data were summarized by counts and percentages and continuous data by descriptive statistics (median [interquartile range; IQR]). Normality tests were conducted using the Shapiro–Wilk test. An ordinary one-way ANOVA test (post hoc Tukey's multiple comparisons test with a single pooled variance) or Kruskal–Wallis test (post hoc Dunn's multiple comparisons test) was used when appropriate to compare baseline characteristics. Repeated measures ANOVA (with the Geisser–Greenhouse correction and post hoc Dunnett's multiple comparisons test) or Friedman test (post hoc Dunn's multiple comparisons test) was used when appropriate to compare treated samples with vehicle. A *p*-value ≤ 0.05 was defined as statistically significant.

3 | RESULTS

3.1 | Baseline characteristics

Seventeen adult individuals were included: three healthy controls, nine patients with SCA, of whom three were treated with chronic RBC exchange transfusion, and five patients with HbSC disease without chronic RBC exchange transfusion (Table 1). The majority were female (82%). Hydroxyurea therapy was limited to patients with SCA ($n = 5$, of whom $n = 2$ were also treated with chronic RBC exchange transfusion), which was accordingly reflected by a higher percentage of HbF compared to patients with HbSC disease ($p = 0.0186$). Routine laboratory parameters confirmed previously reported differences in RBC characteristics between patients with SCA and HbSC disease [14]. Anemia, the higher percentage of reticulocytes and the higher percentage of hypochromic RBCs were most pronounced in patients with SCA ($p = 0.1923$, $p = 0.0388$, and $p = 0.0695$, respectively) whereas patients with HbSC disease showed more microcytosis, a higher MCHC, and a higher percentage of dense RBCs ($p = 0.0256$, $p = 0.0377$, and $p = 0.0934$, respectively).

3.2 | Ex vivo treatment of SCD blood with EMMs alters RBC properties

Ex vivo treatment of whole blood from patients with SCD showed a significant dose-dependent increase with the EMM composition in mean corpuscular volume (MCV) (Figure 1A). Median (IQR) MCV of vehicle, 20×, 40×, and 80× concentration, were 78.7 (70.4–94.7) fL, 79.7 (71.0–96.6) fL ($p = 0.0004$), 81.5 (71.9–98.3) fL ($p < 0.0001$), and 82.1 (73.4–101.4) ($p < 0.0001$). This increase in MCV was seen in treated samples of all groups (Figure 1B), accompanied by a significant decrease in MCHC (Figure 1C,D). Median (IQR) MCHC of vehicle, 20×, 40×, and 80× concentration, were 34.0 (33.3–35.3) g/dL, 33.1 (32.2–34.6) g/dL ($p = 0.0474$), 33.0 (32.2–34.4) g/dL ($p = 0.0019$), and 32.2

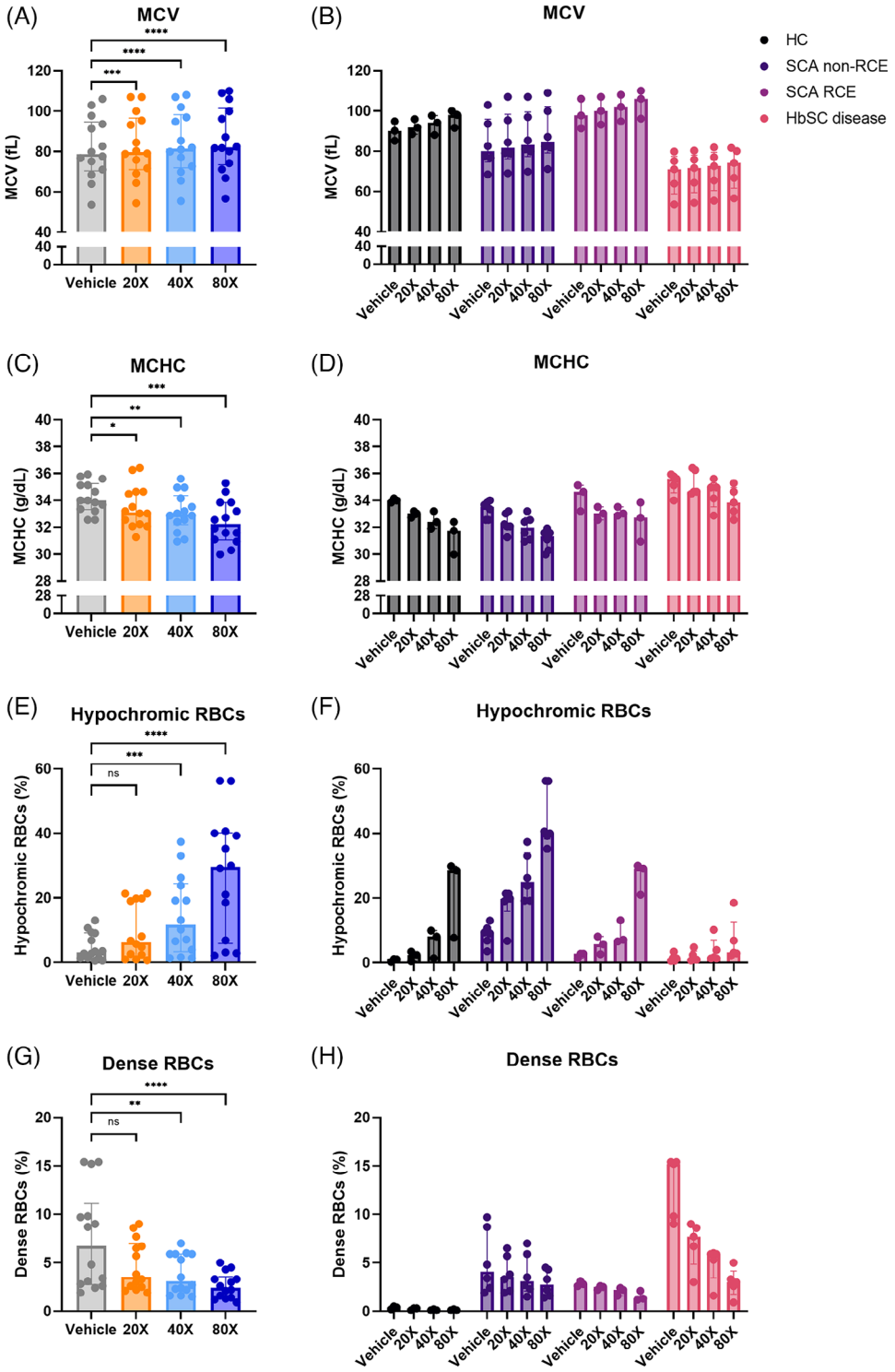

FIGURE 1 **Effects of ex vivo treatment with the endogenous metabolic modulator (EMM) composition on red blood cell (RBC) characteristics.** The composition with 10 different amino acids and derivatives, RCitNacQCarLKHVS, referred to as EMMs, showed a dose-dependent increase (with 20× [orange], 40× [light blue], 80× [dark blue] concentrations compared to vehicle [gray]) in (A) mean corpuscular volume (MCV) and (E) the percentage of hypochromic RBCs, and a decrease in (C) mean corpuscular Hb concentration (MCHC) and (G) the percentage of dense RBCs in the samples of patients with sickle cell disease (SCD, $n = 14$). Although baseline values differed, trends were similar in the different types of SCD (B, D, F, H): sickle cell anemia without chronic RBC exchange transfusion (SCA non-RCE) ($n = 6$; dark purple), sickle cell anemia with chronic RBC exchange transfusion (SCA RCE) ($n = 3$; light purple), and HbSC disease ($n = 5$; pink). Healthy controls (HCs) were included for reference values ($n = 3$; black). Dots (individual values) and medians are presented. Error bars represent the interquartile range (IQR). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$; ns, not significant. MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; hypochromic RBCs, red blood cells with an Hb concentration < 28 g/dL; dense RBCs, red blood cells with an Hb concentration > 41 g/dL.

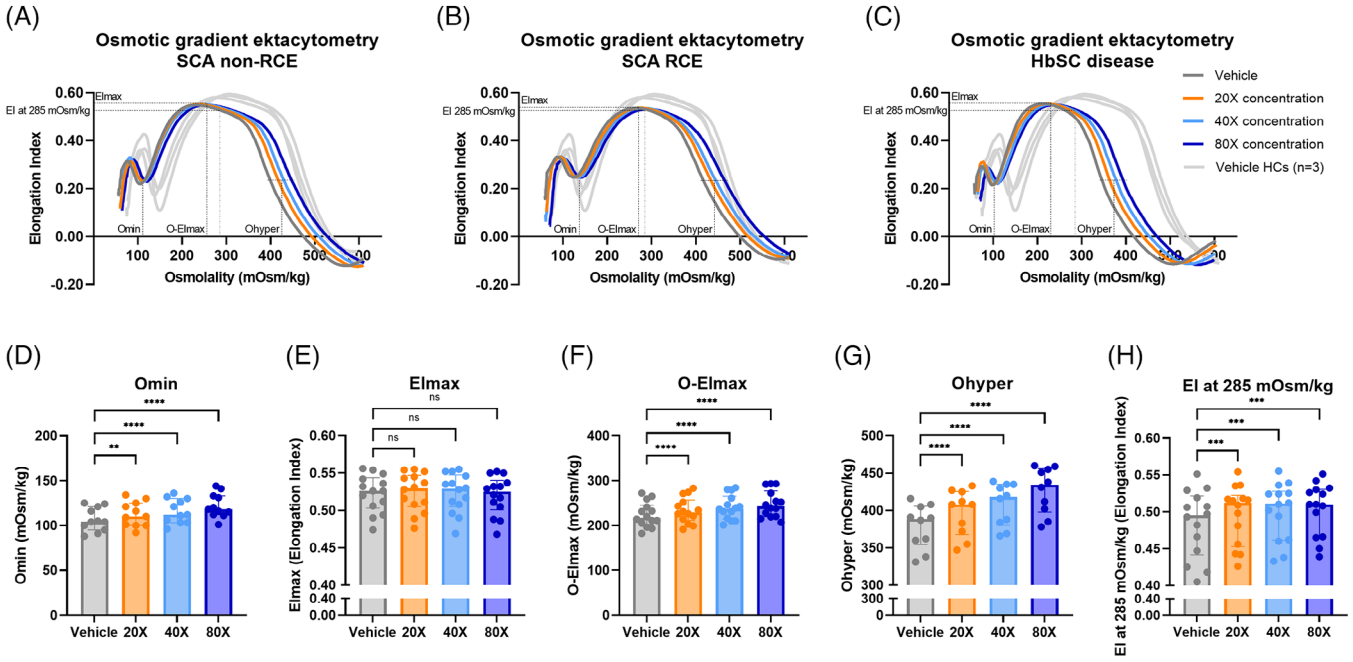

FIGURE 2 **Ex vivo treatment with the endogenous metabolic modulator (EMM) composition shifts the curves obtained by osmotic gradient ektacytometry toward normal in patients with sickle cell disease (SCD).** Representative osmotic gradient ektacytometry curves are shown for (A) a patient with sickle cell anemia (SCA) non-RCE, (B) a patient with SCA RCE, (C) a patient with HbSC disease upon ex vivo treatment with the different concentrations of the EMM composition (20× [orange], 40× [light blue], 80× [dark blue] concentrations compared to vehicle [gray]). All parameters improved toward normal ($n = 3$ HCs with vehicle [light gray]) in the samples of patients with SCD ($n = 14$): (D) Omin (osmolality at minimum RBC deformability [EImin], reflecting membrane surface area to volume ratio; NB missing data of $n = 2$ SCA non-RCE and $n = 1$ HbSC disease), (E) EImax (Osmoscan) (reflecting maximum RBC deformability), (F) O-EImax (the osmolality of EImax), (G) Ohyper (the osmolality corresponding to 50% of EImax, reflecting RBC hydration), and (H) EI at 285 mOsm/kg as a physiological osmolality. Dots (individual values) and medians are presented. Error bars represent the IQR. $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$; ns, not significant.

(31.1–33.8) g/dL ($p = 0.0008$). The percentage of hypochromic RBCs significantly increased in a dose-dependent manner, while the percentage of dense RBCs significantly decreased (Figure 1E and G). This suggests an improvement in hydration status of RBCs from patients with both SCA and HbSC disease as well as healthy RBCs (Figure 1F and H), also when individual results were normalized to vehicle (100%) (Supporting Information Figure S1). In addition, RBC characteristics in blood from patients with SCA treated with hydroxyurea responded in the same way as patients with SCA not treated with hydroxyurea (Supporting Information Figure S1). Together, these results indicate that ex vivo treatment with the EMM composition improved the amount of dense, dehydrated RBCs in SCD irrespective of genotype and concomitant therapy.

3.3 | Ex vivo treatment of SCD blood with EMMs improves ektacytometry-derived parameters

We found a dose-dependent right shift of the osmotic gradient ektacytometry curves toward normal (i.e., compared to vehicle condition of healthy controls) (Figure 2A–C). Whereas the maximum EI (EImax [Osmoscan]) did not change, the osmolality at the minimum EI (Omin; representing the membrane surface area-to-volume ratio of RBCs), the osmolality at EImax (O-EImax), the osmolality at 50% of EImax (Ohyper; reflecting RBC hydration status), and the EI at a physiological osmolality of 285 mOsm/kg all significantly increased by increasing concentration of the composition RCitNacQCarLKHVS (Figure 2D–H and Supporting Information Figure S2). This reflects a decreased RBC membrane surface area-to-volume ratio, increased RBC hydration status, and increased RBC deformability at a physiological osmolality. For example, median (IQR) increase in Ohyper compared to vehicle was 4.9% (4.0%–5.5%), 7.5% (6.9%–9.4%), and 12.8% (11.5%–14.0%) with increasing 20×, 40×, and 80× concentrations, respectively (all $p < 0.0001$). In addition, deformability curves and oxygen gradient ektacytometry showed a significant dose-dependent increase in the EI at 3 Pa and EImax (Oxygenscan) at 30 Pa, respectively, reflecting improved RBC deformability at normoxia (Figure 3A–E and Supporting Information Figure S3). Median (IQR) increase in EImax (Oxygenscan) with the 20×, 40×, and 80× concentration was 8.1% (2.2%–12.1%; $p = 0.0012$), 9.6% (2.9%–15.1%; $p = 0.0013$), and 13.3% (5.7%–25.5%; $p = 0.0007$), respectively. DeltaEI (the difference between EImax [Oxygenscan] and minimum EI [EImin]) and Area (the area under the curve between a partial pressure of oxygen [pO_2] of 10 and 100 mmHg) also increased (Figures 3G and I). However, PoS, reflecting the pO_2 where there is a 5% decrease in EImax and when RBCs start to sickle, and EImin, representing minimal deformability resulting from changes in shape and orientation of SCD RBCs upon deoxygenation, did not significantly change (Figure 3F and H). Besides, recovery (the EI upon

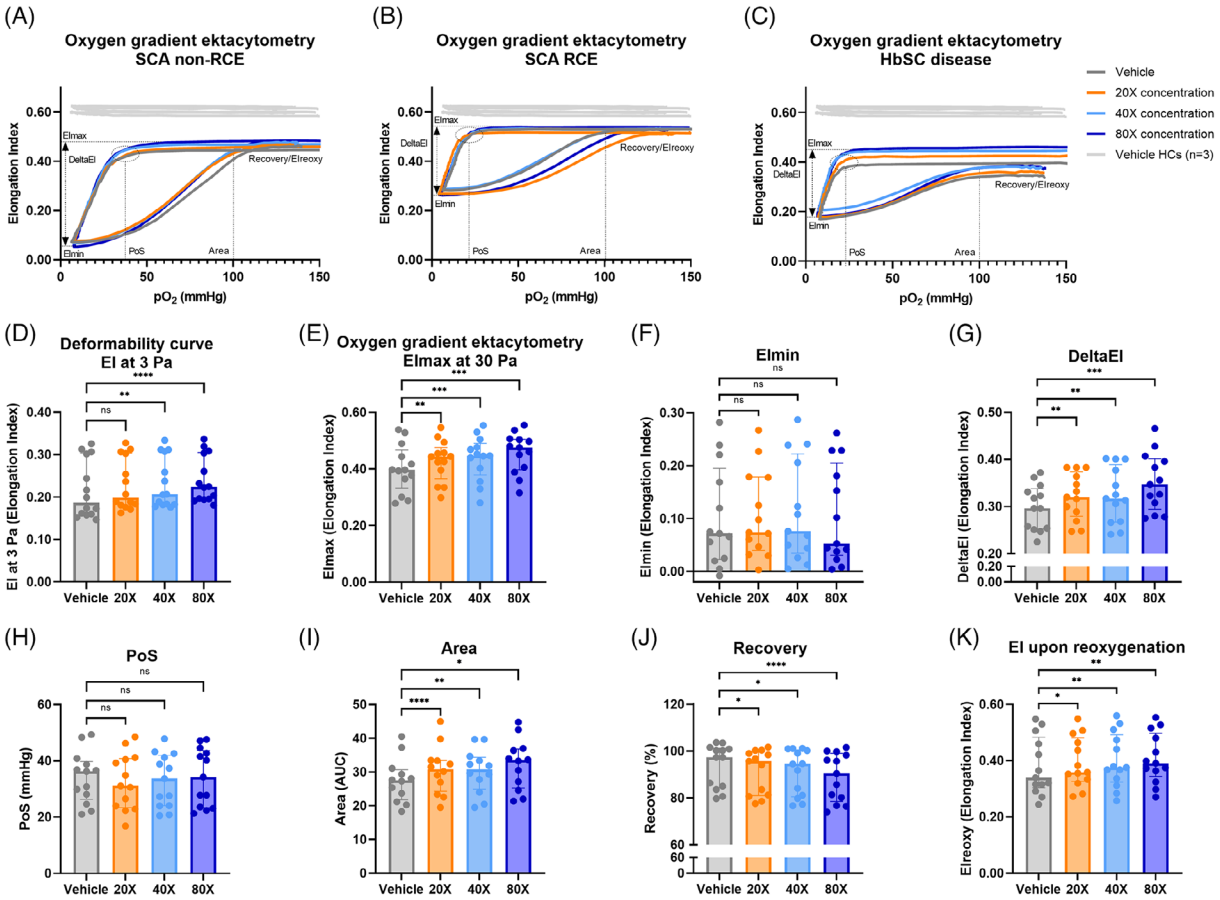

FIGURE 3 **Several parameters of deformability curves and oxygen gradient ektacytometry changed upon ex vivo treatment with the different concentrations.** Representative oxygen gradient ektacytometry curves are shown for (A) a patient with SCA non-RCE, (B) a patient with SCA RCE, (C) a patient with HbSC disease upon ex vivo treatment with the different concentrations of the EMM composition (20× [orange], 40× [light blue], 80× [dark blue] concentrations compared to vehicle [gray]). Several parameters significantly changed in treated samples of the patients with SCD when compared to vehicle ($n = 13$; NB missing data of $n = 1$ SCA non-RCE): (D) EI at 3 Pa reflecting one of the physiological shear stresses of the deformability curve, (E) EImax (Oxygenscan) (reflecting maximum RBC deformability before deoxygenation), (G) DeltaEI (difference between EImax and EImin), (I) Area (the area under the curve between 10 and 100 mmHg; NB missing data of $n = 1$ SCA RCE), and (J) recovery (the EI upon reoxygenation [EIreoxy; K] expressed as the percentage of EImax). No significant changes were demonstrated in (F) EImin (reflecting the minimum deformability upon deoxygenation) and (H) point of sickling (PoS, the partial pressure of oxygen at which a 5% decrease of EImax is observed). Dots (individual values) and medians are presented. Error bars represent the IQR. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$; ns, not significant.

reoxygenation [EIreoxy] expressed as the percentage of EImax [Oxygenscan]; reflecting the unsickling process) significantly decreased although the absolute EIreoxy improved (Figure 3J,K). This implicates that the overall RBC deformability upon reoxygenation is better with ex vivo treatment compared to vehicle. Generally, recovery was lower in HbSC disease compared to other types of SCD. Notably, there were no clear differences in trends between ektacytometry-derived parameters in SCA with or without concomitant hydroxyurea (Supporting Information Figures S2 and S3).

3.4 | 2,3-DPG levels in SCD blood decrease in a dose-dependent manner upon ex vivo treatment with EMMs

ATP and 2,3-DPG levels were measured in a subset of samples due to technical issues ($n = 3$ patients with SCA without chronic RBC exchange transfusion, $n = 3$ patients with HbSC disease, and $n = 2$ healthy controls as reference). Overall, we found no significant changes in ATP levels, but a dose-dependent decrease was observed in 2,3-DPG levels in the treated SCD samples when compared to vehicle (Figure 4A,B). ATP/2,3-DPG ratio significantly improved with the 20× and 40× concentration when compared to vehicle, but not with the 80× concentration (Figure 4C). The p50 values showed no significant differences in the SCD samples (Figure 4D).

3.5 | HVR is stable with the 20× and 40× EMM composition

We found a dose-dependent increase in viscosity at all measured shear rates in the patient samples (Figure 5A; data only shown at a shear rate of 300 s^{-1}). Since viscosity is partly dependent on hematocrit, and

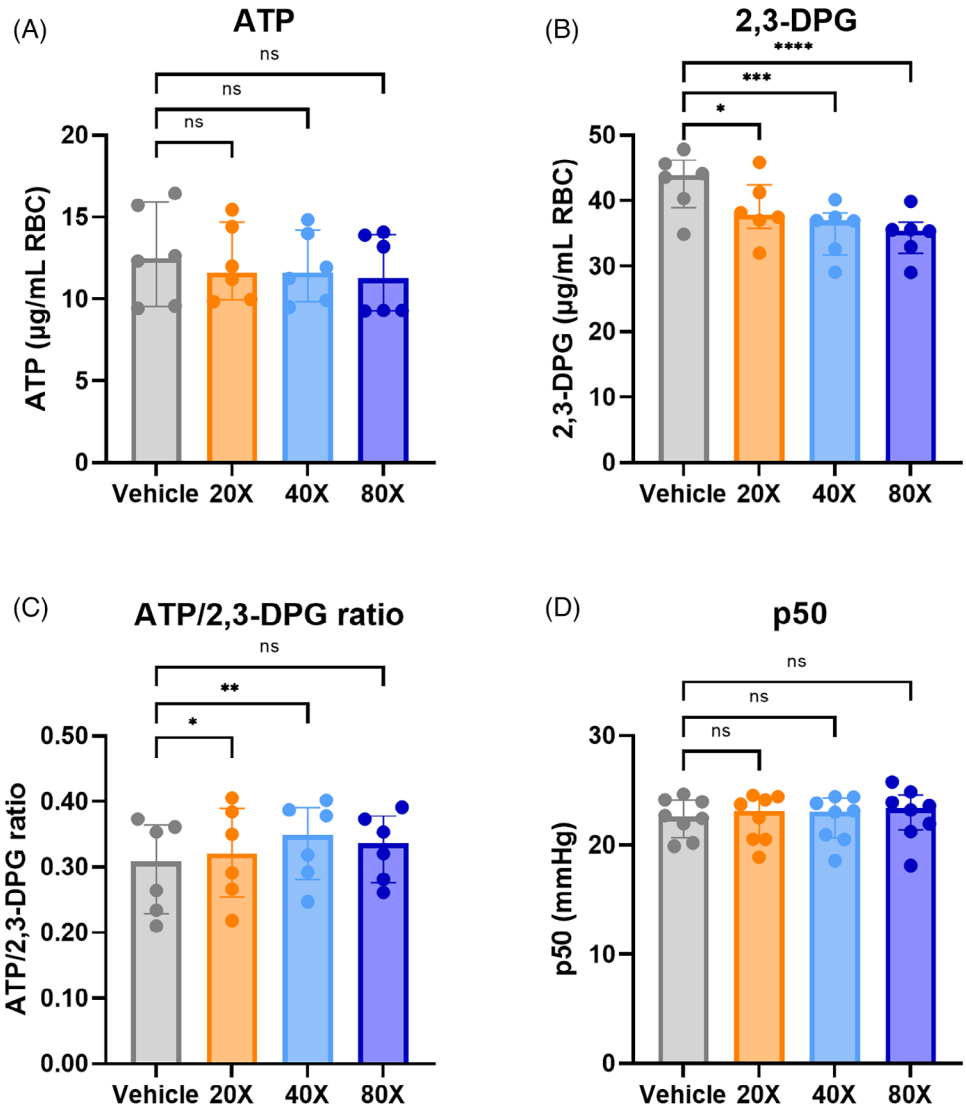

FIGURE 4 **Improvements in 2,3-DPG level and the ATP/2,3-DPG ratio are not reflected in changes in Hb-oxygen affinity.** ATP levels did not significantly change with the different concentrations of the EMM composition (20× [orange], 40× [light blue], 80× [dark blue] concentrations compared to vehicle [gray]) (A), but a dose-dependent decrease was observed for 2,3-DPG levels in the treated samples of patients with SCD when compared to vehicle ($n = 6$ in total; $n = 3$ patients with SCA without chronic RBC exchange transfusion, and $n = 3$ patients with HbSC disease) (B). The ATP/2,3-DPG ratio significantly improved with the 20× and 40× concentration ($n = 6$) (C). However, p50 values, which reflect the oxygen tension when hemoglobin is 50% saturated with oxygen, showed no significant differences in the SCD samples ($n = 8$ in total: $n = 3$ patients with SCA without chronic RBC exchange transfusion, $n = 3$ patients with chronic RBC exchange transfusion, $n = 2$ patients with HbSC disease) (D). Dots (individual values) and medians are presented. Error bars represent the IQR. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$; ns, not significant. ATP, adenosine triphosphate; 2,3-DPG, 2,3-diphosphoglycerate.

hematocrit was higher in samples treated with higher concentrations of the EMM composition (Figure 5B), HVR was calculated as a measure of RBC oxygen transport efficiency [15, 16]. With the 20× and 40× concentration, HVR did not significantly change in the patient samples at all measured shear rates. However, with the 80× concentration, a decrease was seen in HVR (Figure 5C; data only shown at a shear rate of 300 s^{-1}). This result of ex vivo treatment with the 80× concentration was not seen in healthy control samples and appeared to be more pronounced in samples of patients with SCA compared to HbSC disease (Figure 5D).

3.6 | RBC adhesion to laminin improves after ex vivo treatment with EMMs in both SCA and HbSC disease

Overall, although baseline adhesion was variable within all groups, the percentage of adherent RBCs to laminin decreased upon ex vivo treatment with RCitNacQCarLKHVS in the patient samples (Figure 6A–C, Supporting Information Figure S4). As expected, healthy controls showed the lowest absolute number of adherent RBCs, with limited to no (additional) effect of treatment. In contrast, samples of patients

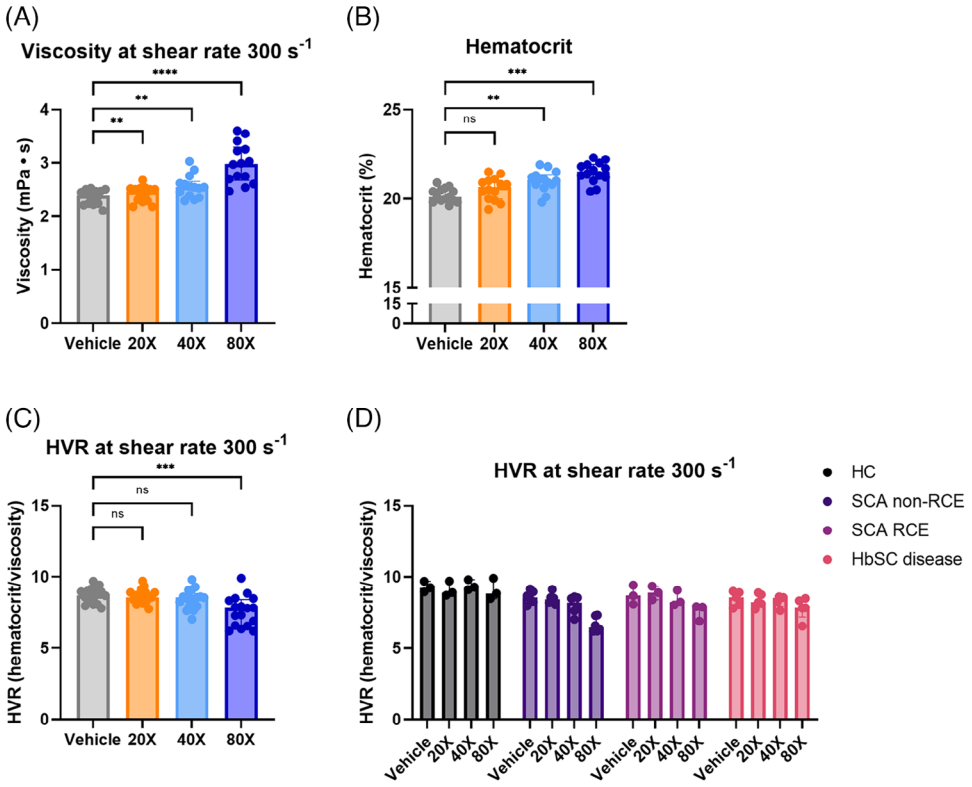

FIGURE 5 **Hematocrit-to-viscosity ratio changed only upon ex vivo treatment with the highest concentration.** Overall, viscosity of the different samples of patients with SCD ($n = 14$) treated with the EMM composition increased (A; data only shown at a shear rate of 300 s^{-1}, but the same trends were observed at all other shear rates). However, since viscosity strongly depends on the number and volume of RBCs and thereby hematocrit, the hematocrit to viscosity ratio (HVR; calculated as hematocrit (%) / blood viscosity (mPa·s = cP) at 300 s^{-1}) was used. HVR, estimating RBC oxygen transport efficiency, decreased only in the highest, 80× concentration, especially in blood from patients with SCA non-RCE (C-D; data only shown at a shear rate of 300 s^{-1}, but the same trends were observed at all other shear rates). However, HVR remained stable in the lower concentrations up to 40× concentration despite the dose-dependent increases in MCV (Figure 1A,B) and hematocrit (B). Healthy controls were included for reference values ($n = 3$; black). Dots (individual values) and medians are presented. Error bars represent the IQR. $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$; ns, not significant.

with SCA as well as HbSC disease responded compared to vehicle to treatment with 20×, 40×, and 80× concentration with a median (IQR) decrease of 43% (6%–68%; $p = 0.4324$), 58% (48%–72%; $p = 0.0185$), and 71% (49%–82%; $p = 0.0016$), respectively.

4 | DISCUSSION

In this study, ex vivo treatment of SCD blood with a composition of 10 amino acids and derivatives, RCitNacQCarLKHVS, demonstrated significant dose-dependent improvements in RBC hydration, as assessed by hemocytometry (MCV, MCHC, percentage of dense RBCs), osmotic gradient ektacytometry (Ohyper), and oxygen gradient ektacytometry (EImax). Furthermore, 2,3-DPG levels and adhesion properties of SCD RBCs decreased. Importantly, these effects were irrespective of genotype (SCA or HbSC disease) and concomitant treatment (e.g., RBC exchange transfusion therapy, which is known to lower amino acid levels) [17]. Overall, our results indicate that the multitargeted EMM composition has the potential to improve RBC properties involved in SCD pathophysiology.

Maintenance of RBC hydration is a dynamic process, which is impaired in SCD and investigated as therapeutic target [18–20]. Clinical studies with senicapoc, an inhibitor of the calcium-activated K^+ efflux (Gardos) channel, showed significant improvements in hemolysis, dense RBCs and in the high dose group also in Hb level and MCV [21, 22]. However, a phase 3 study was terminated early since there was no effect on VOEs [22]. Of note, efficacy of senicapoc depends on RBC membrane damage. In addition, efficacy on clinical complications in HbSC disease could have been missed since no patients in the phase 2 study and 5%–6% in the phase 3 study had HbSC disease [8]. In contrast to the senicapoc studies, MCHC decreased in our ex vivo study. In addition, we performed functional assays of RBC sickling, adhesion, and viscosity to not only assess RBC hydration but also other RBC properties. Thus, the multitargeted EMM composition could have more multidimensional effects than agents only targeting RBC hydration.

The selection of the EMM composition was based on plasma profiling. For example, plasma levels of L-arginine, a precursor of nitric oxide (NO), were reduced in patients with SCD [13]. This reduction is mainly caused by hemolysis and scavenging of NO by cell-free plasma Hb, increasing plasma arginase activity. Functional NO deficiency

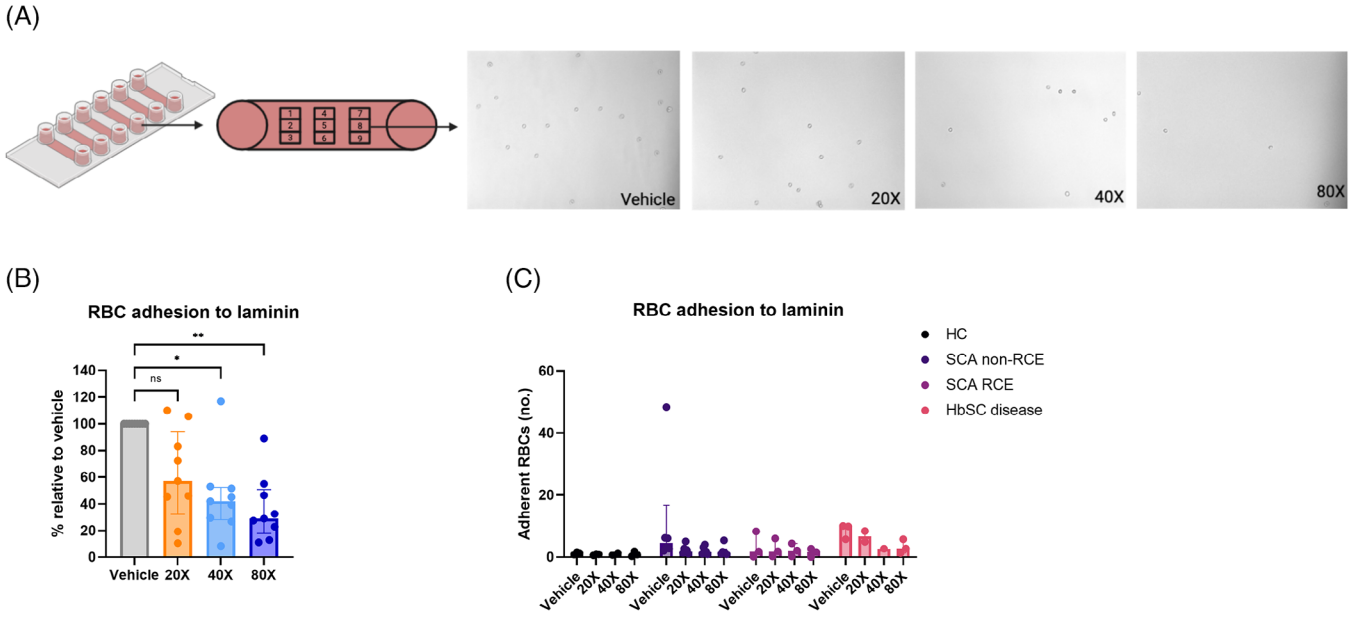

FIGURE 6 **RBC adhesion to laminin decreased upon ex vivo treatment with the EMM composition.** Schematic overview of the experimental set-up with the nine places where images were taken using a microfluidic device (A; on the left), and one series of images obtained by microscopy (20× objective) of samples from a patient with HbSC disease with vehicle and different concentrations of the EMM composition (A; on the right). Although variability in the percentage (B) and number (no.) (C) of RBCs adherent to laminin exists in samples of patients with SCD ($n = 10–12$ with $n = 6$ SCA non-RCE [dark purple], $n = 2–3$ SCA RCE [light purple] and $n = 1–3$ HbSC disease [pink] for the different concentrations due to missing data and excluding samples without RBC adhesion in panel B), a significant decrease was found in the samples treated with the 40× and 80× concentration. HCs were included for reference values ($n = 2–3$ for the different concentrations; black). Dots (individual values) and medians are presented. Error bars represent the IQR. $^{*}p < 0.05$; $^{**}p < 0.01$; ns, not significant. The schematic overview in panel A is created with BioRender.com.

contributes to endothelial dysfunction in SCD [23, 24]. Early-phase studies provided evidence for pain reduction in patients experiencing VOEs by supplementation of L-arginine or L-citrulline, its precursor [25–29]. In 2017, L-glutamine was approved by the US Food and Drug Administration for patients with SCD aged ≥5 years. L-glutamine increases the synthesis of RBC antioxidants (i.e., the reduced form of nicotinamide adenine dinucleotides), which resulted in fewer VOEs in a phase 3 study and in real-world data [30, 31]. However, L-glutamine monotherapy did not show improvements on markers of hemolysis and some RBC-specific parameters such as the percentage of dense RBCs, although the studies did not include patients with HbSC disease, and ambiguous effects on RBC adhesion [13, 30–32]. Fewer VOEs were also observed in children with SCD after treatment with L-carnitine, which plays a critical role in energy metabolism, particularly in the long-chain fatty acid metabolism [33]. Antioxidants such as N-acetylcysteine have been proposed as therapeutic potential in SCD, but so far no strong evidence for clinical benefit was found [34–36]. In our study, we do not know which effect on RBC parameters is caused by which specific component of the EMM composition, although especially the multifaceted effects are promising.

VOEs in patients with SCD are multifactorial and may result from increased RBC adhesion to the endothelium [3]. The EMM composition is clinically promising as an oral supplement based on the dose-dependent decrease in RBC adhesion to laminin in the patient samples. Laminin is the ligand of lutheran/basal cell adhesion molecule (Lu/BCAM), which is overexpressed on SCD RBCs and activated upon oxidative stress and/or high intracellular calcium, thereby inducing hemolysis [37–39]. Indeed, hydroxyurea has antiadhesive effects and is shown to reduce VOEs, although crizanlizumab, a humanized monoclonal antibody against P-selectin, only showed a reduction in annualized VOE rate in a phase 2, but not in preliminary results of a phase 3 study [40–45]. Of note, crizanlizumab did not change other RBC parameters as evaluated with the EMM composition in our study [36, 40].

Limitations of our study include the unknown plasma profiles of the study participants and the small heterogeneous sample size. However, similar effects were found in all patient groups, irrespective of genotype and treatment. Data were stratified on hydroxyurea and nonhydroxyurea use (Supporting Information Figures S1–S4), but subanalysis of these small groups was not performed. Further, despite improvements on ektacytometry-derived parameters of RBC hydration and RBC deformability [46], no significant effects could be measured on some other such as the PoS. This may be due to the limited 4-h ex vivo treatment with EMMs, interassay variability or interindividual differences in our study [47–49]. It is known that the PoS is higher in nontransfused patients with SCA than in transfused patients with SCA or patients with HbSC disease [48, 50]. While in nontransfused patients with SCA a higher PoS is associated with more SCA-related clinical complications, including VOEs, acute chest syndrome and cerebral infarction, this association was not found in a small cohort of patients with HbSC disease [51, 52]. In HbSC disease, PoS could therefore not be the best parameter to monitor treatment

efficacy. Moreover, ex vivo treatment with EMMs might have more general effects on RBC properties, but no direct effects on RBC sickling, supported by the observed changes in RBC parameters of healthy controls. It is also possible that treatment with the EMM composition has the most beneficial effects on a subpopulation of (the most fragile) RBCs, which may escape detection by ektacytometry. Indeed, in some SCD patients the parameter recovery increases above 100%, likely reflecting lysis of fragile, less deformable RBCs during oxygen gradient ektacytometry [48, 49]. The dose-dependent decrease we observed in recovery could support the fact that the EMM composition prevented lysis of these fragile RBCs during oxygen gradient ektacytometry, although EImax and EIreoxy increased. Such a hypothesis is supported by increases in recovery measured upon storage of whole blood, and thereby probably lysis of the most fragile RBCs, for up to 8 days at 4°C [48]. Notably, in HbSC disease, recovery is generally lower when compared to SCA indicating distinct processes of (un)sickling likely due to crystallization of HbC, but not fully elucidated [8].

In conclusion, our study shows beneficial dose-dependent effects of ex vivo treatment with RCitNacQCarLKHVS on multiple RBC properties, which are compromised or altered in SCD pathophysiology. Although our ex vivo study shows the treatment rationale of restoring EMMs to target different RBC-related pathways involved in SCD pathophysiology, it is unknown which concentration is reached and optimal in vivo. Future (animal and dose-finding) studies should be conducted to investigate the pharmacokinetics/pharmacodynamics (e.g., drug half-life), efficacy, and safety and how the ex vivo improvements induced by EMMs translate into clinical benefits for both patients with SCA and HbSC disease.

AUTHOR CONTRIBUTIONS

M.v.D., M.T., B.v.O., M.K., R.W., R.v.W., and M.R. designed the study and developed methodology. M.v.D. and M.T. recruited patients and collected clinical data. M.v.D., M.T., B.v.O., J.d.W., and J.B. performed laboratory experiments and acquired data. T.J.J.R. and J.J.M.J. performed and analyzed 2,3-DPG- and ATP-level measurements. M.v.D., M.T., B.v.O., R.v.W., and M.R. analyzed and interpreted data. M.v.D. wrote the manuscript. All authors including W.v.S and E.v.B. revised the paper critically. R.v.W. and M.R. directed the research. All authors revised the paper and approved the final version.

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CONFLICT OF INTEREST STATEMENT

R.v.W. and M.R. received research funding from Axcella Therapeutics and Pfizer. E.v.B. and R.v.W. are consultants for Pfizer. E.v.B., R.v.W., and M.R. receive research funding and are consultants for Agios Pharmaceuticals Inc. M.J.K. and R.W. are employees of Axcella Therapeutics. The remaining authors declare no competing financial interests.

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DATA AVAILABILITY STATEMENT

For original data and protocols, please contact the corresponding author.

ETHICS STATEMENT

The study was approved by the Medical Research Ethics Committee Utrecht and NedMec, the Netherlands (protocol numbers: 17/450 and 21/793) and conducted in accordance with the principles of the Declaration of Helsinki.

PATIENT CONSENT STATEMENT

Written informed consent was obtained prior to collecting blood.

CLINICAL TRIAL REGISTRATION

The authors have confirmed that clinical trial registration is not needed for this submission.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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