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Plasma monomeric ApoA1 and high-density lipoprotein bound ApoA1 are markedly decreased and associated with low levels of lipophilic antioxidants in sickle cell disease: A potential new pathway for therapy

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Abstract

Patients with sickle cell disease (SCD) exhibit high levels of reactive oxygen species and low plasma levels of lipophilic antioxidants, which may contribute to end-organ damage and disease sequelae. Apolipoprotein A1, the major apolipoprotein of high-density lipoprotein (HDL), is mainly secreted by the intestine and liver in the form of monomeric ApoA1 (mApoA1) present in plasma. Cholesterol and α-tocopherol are delivered to ApoA1 via the ATP-binding cassette transporter, subfamily A, member 1 (ABCA1). We measured cholesterol, mApoA1, ApoA1, and lipophilic antioxidants in the plasma of 17 patients with SCD and 40 healthy volunteers. Mean HDL cholesterol (-C) levels in SCD patients and healthy subjects were 59.3 and 48.1 mg/dL, respectively, and plasma lutein, zeaxanthin, and α-tocopherol were 64.0%, 68.7%, and 9.1% lower, respectively. To compare SCD to healthy subjects with similar HDL-C, we also performed subgroup analyses of healthy subjects with HDL-C above or below the mean. In SCD, the mApoA1 level was 30.4 μg/mL; 80% lower than 141 μg/mL measured in healthy volunteers with similar HDL-C (56.7 mg/dL). The mApoA1 level was also 38.4% greater in the higher versus lower HDL-C subgroups ($p = .002$). In the higher HDL-C subgroup, lutein and zeaxanthin transported by HDL were 48.9% ($p = .01$) and 41.9% ($p = .02$) higher, respectively, whereas α-tocopherol was 31.7% higher ($p = .003$), compared to the lower HDL-C subgroup. Plasma mApoA1 may be a marker of the capacity of HDL to capture and deliver liposoluble antioxidants, and treatments which raise HDL may benefit patients with high oxidative stress as exemplified by SCD.

KEYWORDS
ApoA1, HDL, lutein, zeaxanthin, α-tocopherol

Novelty Statements

What is the new aspect of your work?

There is no published information on pre-beta1 HDL (monomeric ApoA1) in sickle cell disease patients since it has never been measured in these patients.

What is the central finding of your work?

SCD patients have abnormally low mApoA1 (in spite of normal plasma HDL cholesterol levels) associated with low plasma levels of lipophilic antioxidants.

What is (or could be) the specific clinical relevance of your work?

Several behavioral (exercise, diet) and drug interventions which raise HDL and antioxidants may be beneficial to SCD patients. Lipophilic antioxidant in HDL may be a better biomarker of functionality and protective potential of HDL than HDL-cholesterol.

1 | INTRODUCTION

Sickle cell disease (SCD) results from a single mutation in the human β-globin gene leading to the production of an abnormal hemoglobin S (HbS). HbS may polymerize under low oxygen partial pressure, leading to a mechanical distortion of red blood cells (RBCs), a phenomenon called sickling. Sickled RBCs are rigid, prone to hemolysis and may initiate vaso-occlusion, thus, SCD is characterized by chronic hemolytic anemia and impaired blood rheology.[1] As a consequence of hemolysis, the accumulation of free hemoglobin and heme in plasma promotes inflammation and oxidative stress, resulting in the development of chronic vasculopathy.[2,3] Hypocholesterolemia, affecting both low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C), and increased triglycerides levels have been repeatedly reported in SCD[4] and found to correlate with inflammation,[5] hemolysis,[6] and various complications.[7,8] In addition to low plasma cholesterol levels, SCD patients have very low levels of lipophilic antioxidants; Natta et al.[9] suggested that the complete antioxidant system may be compromised in SCD and unable to face the high level of reactive oxygen species (ROS).

The hypocholesterolemia observed in SCD may occur via several mechanisms.[8,10] It also appears that the major HDL apolipoprotein (ApoA1)[11–14] and lecithin-cholesterol acyltransferase (LCAT) mass and activity are decreased in SCD patients[12] compared to healthy controls, which may result in lower HDL-C. Low plasma ApoA1 correlates with inflammation in SCD[14,15] and the use of an ApoA1 mimetic improves vascular function and enhances vasodilation in an SCD mouse model.[16] More recently, polymorphism in the cholesteryl ester transfer protein (CETP) was found to affect HDL-C levels in SCD[17] and these authors also noticed that the groups with altered levels of ApoA1 and HDL-C exhibited lower hemoglobin levels.

In humans, the bulk of the circulating cholesterol is transported in LDL and very low-density lipoprotein (VLDL) particles, whereas in the majority of mammals HDL is often the primary cholesterol carrier.[18] Interestingly, the same authors demonstrated the role of the ATP binding cassette transporter, sub-family A, member 1 (ABCA1) in cholesterol efflux to ApoA1[19] and in the delivery of vitamin E (α-tocopherol) to ApoA1,[20] thus linking the mechanism of cholesterol efflux to the uptake of the important and most abundant lipophilic antioxidant, α-tocopherol. Later, it was confirmed that vitamin E and cholesterol utilize the ABCA1 pathway,[21] with secretion to ApoA1 being enantioselective, favoring certain forms of vitamin E (α-tocopherol vs. β and γ-tocopherol).[22]

ApoA1 is mainly secreted by the intestine and liver as non-lipidated protein. In this manuscript, residues 137–144 (LQEKLSPL) of ApoA1, is identified by Mab55201 and referred to as monomeric ApoA1 (mApoA1). Lagerstedt et al.[23] proposed that during ApoA1 association with lipid, this region moves from β-strand to α-helix, eliminating a stabilizing β-sheet from the center of the protein and recognition by Mab55201. Castro and Fielding[24] reported that cell-derived cholesterol is first detected at the particle by the monoclonal antibody, Mab55201, and then within minutes, is transferred to larger HDL particles such as pre-β2 and larger HDL alpha particles. mApoA1 is therefore required for the first step in reverse cholesterol transport.[25]

The antioxidant activity of HDL[26] and the pivotal role of HDL in the redistribution of tocopherol towards LDL and VLDL[27] and exchange with RBC[28] have been established, nevertheless, the role of HDL in the uptake and transport of lipophilic antioxidants has rarely been investigated.[29] Because HDL is involved in the exchange of cholesterol and lipophilic substances with RBCs,[30] we investigated the relationship between the plasma level of mApoA1 and the concentration of selected lipophilic antioxidants in plasma of patients suffering from SCD. We hypothesized that plasma mApoA1 may be the active HDL sub-particle which correlates with plasma ApoA1 and with the plasma levels of lutein, zeaxanthin, and α-tocopherol. We also analyzed the distribution of lipophilic antioxidants in the non-HDL and HDL fractions in SCD patients and from healthy volunteers with comparable plasma HDL-C values.

2 | RESULTS

2.1 | Characteristics of patients

Participants included 17 patients with SCD and 40 healthy volunteers. Characteristics of the study subjects are described in Table 1. Some of the results from the healthy volunteer cohort, with different scientific objectives, were reported previously.[31,32]

2.2 | Plasma mApoA1, ApoA1, and cholesterol levels in sickle cell disease patients, healthy volunteers, and healthy volunteers with lower and higher HDL-C

ApoA1, mApoA1, total cholesterol (Total-C), HDL-C, and non-HDL-C concentrations in all healthy volunteers, subgroups of healthy volunteers with HDL-C ≤48 and >48 mg/dL, and patients with SCD are shown in Table 2 and Figure 1. The SCD patients had relatively low mean plasma Total-C (115 mg/dL), mainly due to reduced non-HDL-C levels (65.9 mg/dL), while mean HDL-C was in the upper range of normal values (59.3 mg/dL), which may have been due to the fact the SCD patients were receiving standard of care treatments such as hydroxyurea. Mean cholesterol levels in the healthy population were within normal ranges; Total-C, HDL-C, and non-HDL-C levels were 183.0, 48.1, and 127.7 mg/dL, respectively.

Mean plasma mApoA1 and ApoA1 concentrations in the SCD subjects were 30.4 μg/mL and 79.4 mg/dL, respectively. Thus, mApoA1 represented 3.8% of plasma ApoA1 in SCD subjects. In the cohort of healthy volunteers, mApoA1 and ApoA1 values were 121 μg/mL and 181 mg/dL, respectively. Thus, mApoA1 represented 6.7% of plasma ApoA1 indicating that healthy subjects had more ApoA1 and a higher percentage circulating as non-lipidated mApoA1.

TABLE 1 Characteristics of healthy volunteers and patients with sickle cell disease.

Characteristic	Healthy ($N = 40$)	SCD ($N = 17$)
Age, years, mean ± SD	21.5 ± 1.5	25 ± 12
Sex, male/female	40/0	6/11
Body mass index, kg/m^2, mean ± SD	22.4 ± 0.5	22.5 ± 3.5
Diagnosis	NA	
HbSS, *n*	—	14
HbS/β^0, *n*	—	3
Treatment	NA	
Hydroxyurea, *n*	—	14
Morphine, *n*	—	1
None, *n*	—	2

Abbreviations: HbSS, homozygosity for the sickle cell mutation; HbS/β^0, sickle cell beta-thalassemia; NA, not applicable; SCD, sickle cell disease; SD, standard deviation.

To select a group of healthy volunteers with HDL-C levels comparable to the SCD cohort, we separated the healthy cohort into two subgroups with HDL-C values above and below the mean of 48 mg/dL. In the lower HDL-C subgroup, the HDL-C mean ± SEM was 41.0 ± 1.4 mg/dL and in the higher HDL-C subgroup it was 56.7 ± 1.6 mg/dL (38.5% higher; $p < .001$). The mean HDL-C concentration of the higher HDL-C group was not significantly different from the mean HDL-C of the SCD cohort (56.7 vs. 59.3 mg/dL; ns). As expected, compared to the lower HDL-C subgroup, ApoA1 was 31.4% greater in healthy subjects with higher HDL-C (208 ± 8.79 mg/dL vs. 159 mg/dL; $p < .001$) and mApoA1 was 38.4% greater (141 ± 5.9 μg/mL vs. 102 ± 9.6 μg/mL; $p = .002$). Furthermore, the SCD patients displayed substantially lower mean ± SEM ApoA1 (79.4 ± 2.9 mg/dL) and mApoA1 (30.4 ± 5.8 μg/mL) concentrations than both the higher HDL-C subgroup (208 ± 8.79 μg/mL, $p < .0001$ and 141 ± 5.9 μg/mL, $p = .0001$, respectively) and the lower HDL-C subgroup (102 ± 9.6 μg/mL, $p < .0001$ and 159 ± 4.6 μg/mL, $p < .0001$, respectively).

In the complete cohort of healthy volunteers, a significant correlation was observed between plasma ApoA1 and mApoA1 ($r = 0.59$; $p = .0008$) (Supplementary Figure 1). This correlation was weaker, but still significant, between mApoA1 and HDL-C ($r = 0.47$; $p = .01$) (Supplementary Figure 2).

2.3 | Plasma lipophilic antioxidants in sickle cell disease patients, healthy volunteers, and healthy volunteers with lower and higher plasma HDL-C

Concentrations of lipophilic antioxidants in plasma, HDL and non-HDL fractions in all healthy volunteers, in subgroups of healthy volunteers with higher and lower HDL-C, and in SCD patients are shown in Table 3. As expected from their relative lower lipophilicity (Supplementary Table 1) the di-hydroxy xanthophylls, lutein and zeaxanthin, were preferentially found in the HDL fraction whereas the more lipophilic β-carotene and α-tocopherol tended to partition into the non-HDL (LDL/VLDL) fraction (Figure 2A). There were no significant differences in the amounts of β-cryptoxanthin and β-carotene carried by HDL compared to non-HDL particles suggesting that the differences observed for lutein, zeaxanthin, and α-tocopherol were relatively specific to these lipophilic antioxidants. In healthy volunteers and SCD patients, α-tocopherol was the most abundant antioxidant with 63% transported in non-HDL particles and 37% in HDL particles. Furthermore, the subgroup of healthy volunteers with higher HDL-C levels, compared to the lower HDL-C subjects, had significantly more lutein (48.9% higher; $p = .01$), zeaxanthin (41.9% higher; $p = .02$), and α-tocopherol (31.7% higher; $p = .003$) (Table 3, Figure 2B), suggesting a relative enrichment of the HDL fraction with the measured antioxidants. The pattern of lipophilicity-driven distribution of lipophilic antioxidants in HDL versus non-HDL was also observed in SCD patients despite their lower non-HDL-C levels. The smaller non-HDL fraction in patients with SCD likely led to a greater recovery of lipophilic antioxidants in the HDL fraction when

TABLE 2 mApoA1, ApoA1, Total-C, HDL-C, and non-HDL-C in all healthy volunteers, subgroups of healthy volunteers with lower or higher HDL-C (≤ and >48 mg/dL), and patients with sickle cell disease.

	Healthy volunteers[a]			
Participants	All ($N = 40$) Mean ± SEM	Low HDL-C ($N = 22$)	High HDL-C ($N = 18$)	SCD patients ($N = 17$)
mApoA1, μg/mL	120.7 ± 6.7	101.8 ± 9.6	140.9 ± 5.9	30.4 ± 5.8
ApoA1, mg/dL	181.0 ± 6.1	158.6 ± 4.6	208.4 ± 8.7	79.4 ± 2.9
Total-C, mg/dL	183.0 ± 5.6	179.3 ± 8.5	187.4 ± 7.2	115.0 ± 7.9
HDL-C, mg/dL	48.1 ± 1.6	41.0 ± 1.4	56.7 ± 1.6	59.3 ± 6.2
Non-HDL-C, mg/dL	127.7 ± 4.3	125.0 ± 6.7	130.8 ± 5.1	65.9 ± 3.5

Abbreviations: ApoA1, apolipoprotein A1; HDL-C, high-density lipoprotein cholesterol; mApoA1, monomeric apolipoprotein A1; non-HDL-C, non-high-density lipoprotein cholesterol; SCD, sickle cell disease; SEM, standard error of the mean; Total-C, total cholesterol.
[a]Numbers of participants are as labeled except mApoA1 for all healthy volunteers ($n = 29$), healthy volunteers with lower HDL-C ($n = 15$), and healthy volunteers with higher HDL-C ($n = 14$), and ApoA1 for SCD patients ($n = 16$).

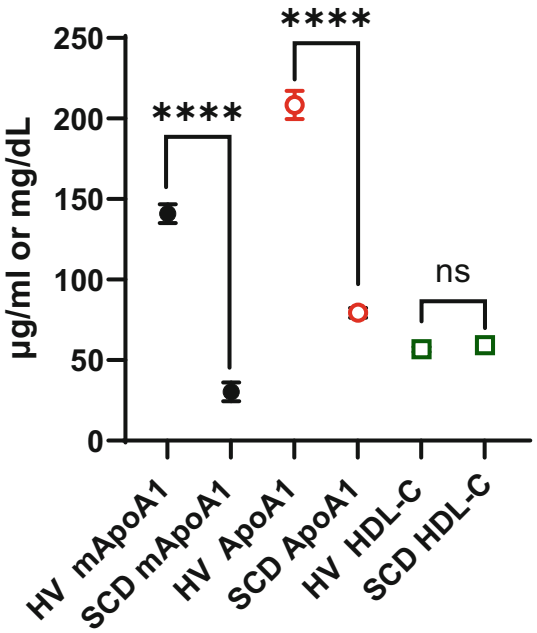

FIGURE 1 mApoA1 (μg/ml) filled circles, ApoA1 (mg/dL) open circles and HDL-C (mg/dL) green squares in SCD patients compared to healthy volunteers (HV) with similar HDL-C levels. ****$p < .0001$.

compared to healthy volunteers (Figure 3). For example, the mean ± SEM ratio of lutein in the HDL versus non-HDL fraction was 1.78 ± 0.13 in all healthy volunteers and 2.93 ± 0.41 in the SCD cohort ($p = .002$).

Compared to the complete cohort of healthy volunteers, plasma lutein, zeaxanthin, and α-tocopherol levels were 63.8%, 68.7%, and 9.39% lower in patients with SCD (Table 3). Furthermore, despite their similar HDL-C levels, the healthy volunteers in the higher HDL-C subgroup had higher levels of lutein, zeaxanthin, β-cryptoxanthin, β-carotene, and α-tocopherol compared to patients with SCD. Except for α-tocopherol, SCD patients also had lower mean concentrations of lutein, zeaxanthin, β-cryptoxanthin, and β-carotene than healthy volunteers within the lower HDL-C subgroup of healthy volunteers.

In healthy volunteers, a significant correlation was observed between the plasma level of mApoA1 and the HDL fraction levels of lutein ($r = 0.52$; $p < .005$) (Supplementary Figure 3), zeaxanthin ($r = 0.42$; $p = .026$) (Supplementary Figure 4), and α-tocopherol ($r = 0.54$; $p = .003$) (Supplementary Figure 5).

3 | METHODS

3.1 | Participants and ethical approval

1. *Healthy volunteer participants* were recruited via invitations from the National Preterm Birth Register managed by the University Clinical Centre in Ljubljana, Slovenia using medical record screening and telephone/email-based individual interviews. Healthy volunteer and SCD participants were matched for age, height, and body mass index (BMI). This study was performed according to the Declaration of Helsinki. The experimental protocol for the original trial in which the samples were collected that were utilized in this investigation was registered at ClinicalTrials.gov (NCT04739904), and ethical approvals were obtained from both the University of Ljubljana, Faculty of Sport Ethics Committee (8/2020-316) and the Aosta Hospital Ethical Committee (06/05/2021.0038781.I). Some of the results from the project, with different scientific objectives, were reported previously.[31,32]
2. *Sickle cell disease patients* 17 subjects (14 HbSS patients [homozygous for HbS] and 3 HbS/β0 patients [sickle cell beta-thalassemia]) under hydroxyurea treatment ($n = 14$) or not ($n = 3$) were included in the study. SCD patients were recruited from the Edouard Herriot Hospital, Lyon, France. Thirteen were in clinical steady-state at the time of the study, that is, without a vaso-occlusive crisis or other acute medical complication within the last 2 months and without blood transfusion for at least 3 months before inclusion.

TABLE 3 Xanthophylls and α-tocopherol in total plasma and HDL and non-HDL fractions in all healthy volunteers, subgroups of healthy volunteers with HDL-C ≤ and >48 mg/dL, and patients with sickle cell disease.

	Healthy volunteers[a]			
	All ($n = 38$)	Low HDL-C ($n = 21$)	High HDL-C ($n = 17$)	SCD patients ($n = 17$)
Antioxidants	Mean ± SEM			
Lutein, μg/mL				
Plasma	149.3 ± 11.2	133.9 ± 16.6	166.4 ± 14.3	54.1 ± 6.2
HDL	67.4 ± 5.4	55.3 ± 5.5	82.4 ± 8.8	46.4 ± 6.9
Non-HDL	39.7 ± 2.8	38.1 ± 4.0	41.7 ± 3.8	18.8 ± 2.8
Zeaxanthin, μg/mL				
Plasma	30.9 ± 2.9	28.6 ± 3.8	33.6 ± 4.5	9.7 ± 1.8
HDL	17.4 ± 1.4	14.7 ± 1.3	20.8 ± 2.4	8.3 ± 1.7
Non-HDL	9.8 ± 0.7	9.7 ± 0.9	10.1 ± 1.0	3.2 ± 0.6
β-cryptoxanthin, μg/mL				
Plasma	117.6 ± 11.0	108.6 ± 17.0	128.1 ± 13.1	18.6 ± 2.6
HDL	29.8 ± 2.9	27.1 ± 4.0	33.0 ± 4.0	11.4 ± 2.7
Non-HDL	32.1 ± 2.8	33.7 ± 4.6	30.2 ± 2.8	9.6 ± 1.8
β-carotene, μg/mL				
Plasma	227.2 ± 13.9	234.8 ± 19.9	218.7 ± 19.9	47.2 ± 9.2
HDL	12.2 ± 1.1	11.1 ± 1.3	13.5 ± 1.8	14.8 ± 3.3
Non-HDL	24.9 ± 1.9	26.1 ± 2.8	23.4 ± 2.6	32.3 ± 6.0
α-tocopherol, μg/mL				
Plasma	7048 ± 275	6560 ± 307	7627 ± 448	6386 ± 675
HDL	2810 ± 138	2461 ± 154	3242 ± 201	3703 ± 251
Non-HDL	4756 ± 196	4987 ± 267	4469 ± 280	3323 ± 310

Abbreviations: HDL, high-density lipoprotein; HDL-C, high-density lipoprotein cholesterol; non-HDL, non-high-density lipoprotein; SCD, sickle cell disease; SEM, standard error of the mean.

[a]Numbers of participants are as labeled except in healthy volunteers, plasma lutein and plasma β-carotene ($n = 36$), plasma zeaxanthin and plasma α-tocopherol ($n = 35$), and plasma β-cryptoxanthin ($n = 37$); in the lower HDL subgroup, plasma lutein, plasma zeaxanthin, plasma β-carotene, and plasma α-tocopherol ($n = 19$), and plasma β-cryptoxanthin ($n = 20$); and in the higher HDL subgroup, plasma zeaxanthin and plasma β-tocopherol ($n = 16$).

Three patients were suffering from a vaso-occlusive crisis at the day of inclusion but had not been transfused for at least 3 months. The study was conducted in accordance with the guidelines set by the Declaration of Helsinki, approved by the Regional Ethics Committees (L14-127) and the subjects gave informed consent to participate. Additional blood parameters are provided in Supplementary Table 3.

3.2 | Monomeric ApoA1 (mApoA1) assay

Venous blood was taken from the antecubital vein and collected into EDTA tubes. mApoA1 was analyzed using a specific enzyme-linked immunosorbent assay[17] with a monoclonal antibody, Mab55201, which identifies residues 137–144 (LQEKLSPL) of the mature protein uniquely present in mApoA1 HDL in a non-alpha helix conformation, that is implicated in early cholesterol transport from cell membranes to plasma. The sandwich ELISA using Mab55201 was purchased from IMMBIOMED GmbH, Germany product N° 289194 and used according to the provider's protocol.

The assay has been validated for precision, recovery, linearity, and lower limit of detection (Hartis Pharma internal report) and was previously compared to a 2D gel separation of HDL sub-particles[33] and used in a number of clinical studies.[34,35] Interestingly this ELISA has also been used successfully to measure mApoA1 in cynomolgus monkeys, despite the replacement of Q by H in the epitope recognized by Mab55201.[36] Indeed, mApoA1 can only be detected and quantified by 2D gel electrophoresis followed by a cumbersome quantification of HDL sub-particles with a radiolabelled antibody to ApoA1.[37] Fielding et al.[38] identified a monoclonal antibody (Mab55201) that binds to a sequence of ApoA1 molecules (forming a β-sheet) which, upon dimerization and/or lipidation,[39] changes conformation to an α-helix (helix 5) that is undetectable by Mab55201. Binding of Mab55201 to its epitope prevents cholesterol efflux via ABCA1 to ApoA1[40] suggesting its potential involvement in the initial interaction of ApoA1 with ABCA1. Miyazaki et al.[41] characterized the

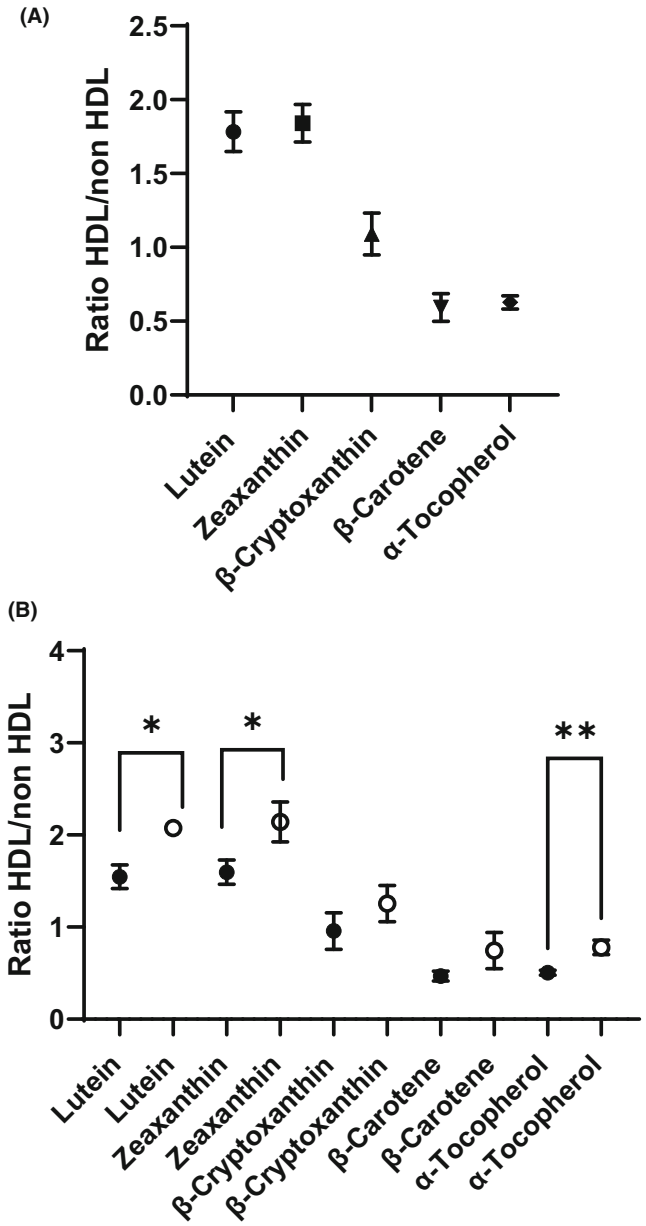

FIGURE 2 (A) Ratio of lutein, zeaxanthin, β-cryptoxanthin, β-carotene and α-tocopherol in HDL versus non-HDL fractions in all healthy volunteers. (B) Ratio of lutein, zeaxanthin, β-cryptoxanthin, β-carotene and α-tocopherol concentrations in HDL and non-HDL fractions in low HDL-C subjects filled circles and high HDL-C subjects open circles. $^{*}p < .05$; $^{**}p < .01$.

plasma component detected by Mab55201 present in human plasma and demonstrated that it is indistinguishable from lipid free ApoA1.

In addition, the sequence recognized by Mab55201, plays a crucial role in both binding and activating LCAT, which allows the enlargement of HDL particles by accumulation of cholesteryl esters within the core of the HDL particles. Pairing of β-strands leads to the formation of the loop centered at residue 139 known as the loop belt model.[42] Mutation in the amino acids in this sequence such as Leu141 (ApoA1 Pisa)[43] and Leu144[44] (ApoA1 Zaragosa) affect LCAT activity

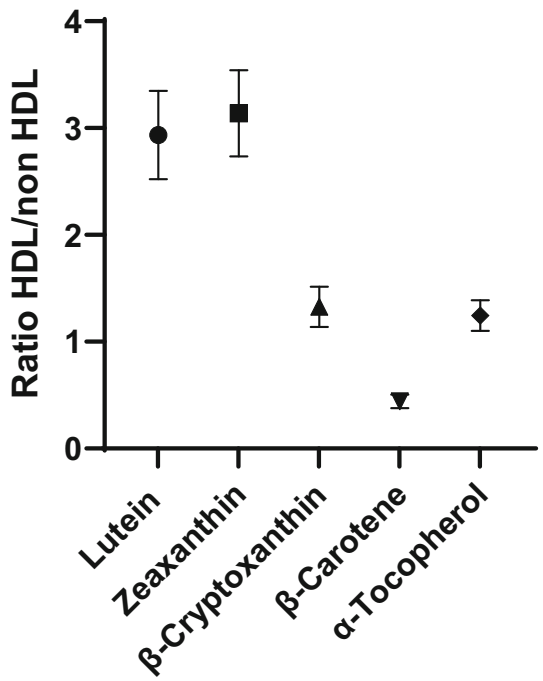

FIGURE 3 Ratio of lutein, zeaxanthin, β-cryptoxanthin, β-carotene, and α-tocopherol concentrations in HDL versus non-HDL fractions in SCD patients.

in humans. Mutation of Pro143Arg (ApoA1 Giessen)[45] and Arg151 (Arg151Cys, ApoA1 Paris)[46] also decrease LCAT activity. Thus, the epitope LQEKLSPL of mApoA1, well conserved across mammalian species, especially in primates (Supplementary Table 2), is a key biomarker of HDL function as it changes conformation and controls both cholesterol efflux and LCAT activity.

3.3 | Precipitation of ApoB lipoproteins with phosphotungstate/$MgCl_2$

To separate efficiently HDL from non-HDL particles we used a precipitation method which has been shown to be comparable to ultracentrifugation[47] to further analyze carotenoid and tocopherol. HDL was isolated by precipitation of Apo B-containing particles.[48] To 250 µL plasma, 12.5 µL phosphotungstate (12 mM) and 6.25 µL $MgCl_2$ (2 M) were added, mixed by vortex, and centrifuged at 6000*g* for 10 min at 4°C. Supernatants were harvested and pellets re-suspended in 250 µL Na_2CO_3 (0.5 M).

3.4 | ApoA1 assay and cholesterol determination

Plasma ApoA1 was measured with the Diazyme (Switzerland) Apolipoprotein A1 Assay kit according to the provider (ref DZ141A-K) with DZ141A-CAL as calibrator. Cholesterol was measured with the colorimetric assays from Elabscience (Switzerland), reference E-BC-K109-S according to the manufacturer's protocol.

3.5 | Lipophilic antioxidants

Concentrations of lutein, zeaxanthin, β-cryptoxanthin, β-carotene, and α-tocopherol in plasma and its β-precipitation fractions (HDL and non-HDL) were measured by liquid chromatography–tandem mass spectrometry (Waters UPLC-MS/MS) in ESI mode. For the ß-precipitation samples (precipitate or supernatant) and plasma samples, 100 µL aliquots were combined with methanol (900 µL), mixed by vortex, and centrifuged (16.1 relative centrifugal force [rcf], 10 min). An aliquot of supernatant 100 µL was combined with 50 µL of a solution of internal standard, MTBE/MeOH (1:1) (500 µL) and water (350 µL) and mixed by vortex prior to injection A matrix calibration curve was prepared from pooled plasma was and used for quantification by the standard addition method. For the measurement of non-HDL and HDL samples, the corresponding matrix was prepared from pooled plasma and used as the basis for the calibration standards. Analytes were analyzed on a Cortecs Phenyl (1.6 µm; 2.1 × 100 mm) by gradient elution with ammonium acetate (20 mM) and acetonitrile:methanol:MTBE (76:15:9 v:v:v) as the mobile phase. Injection volume: 10 µL, column temp: 35°C, flow rate: 0.5 mL/min, Injection volume: 10 µL, column temp: 35°C, flow rate: 0.5 mL/min. Under these conditions the retention time of zeaxanthin is 4.24 min, lutein: 4.36 min, α-tocopherol: 8.19 min, β-cryptoxanthin: 10.19 min and β-carotene: 13.21 min.

3.6 | Statistical analysis

Mean, SD, SEM, paired and unpaired *t*-tests as well as Pearson correlation coefficients were calculated using GraphPad Prism 10.2.0 software (GraphPad Software, Boston, MA, USA) Statistical significance was set at $p < .05$.

4 | DISCUSSION

SCD patients have low plasma and RBC tocopherol[49] and their RBCs are prone to hemolysis and irreversible sickling.[50] The heightened oxidative stress in SCD patients who suffer from hemolysis[51,52] and have increased plasma free hemoglobin and oxidative heme is a major contributing factor to end-organ damage and disease complications. Indeed, heme binds and can oxidize lipids in lipoproteins increasing their inflammatory and atherogenic potential.[53] In SCD, RBC membranes are highly oxidized.[54] Thalassemic patients display almost no detectable tocopherol in HDL particles.[55] Antioxidant carotenoids have been shown to decrease hemolysis[56] and, albeit in a single study with a small number of patients, vitamin E supplementation was shown to benefit SCD patients.[50] Our results quantified for the first time a significant decrease in mApoA1, which is sometimes referred to as pre-β1 HDL, in patients suffering from SCD. Furthermore, we showed that this reduction is associated with a marked decrease in plasma lipophilic antioxidants. We confirmed lower ApoA1 levels in SCD patients as reported previously by Soupene et al.[57] who also demonstrated decreased HDL functionality, as evidenced by a reduced HDL-ApoA1 exchange, during vaso-occlusive crises in SCD.

Since it has been published that males and females SCD patients do not differ in their HDL-C levels,[58] we do not expect a major gender contribution to the difference observed between our SCD patients, composed of males and females and the male healthy volunteer group.

It is generally well established that SCD patients have low total plasma cholesterol and low LDL-C; HDL-C levels are also generally low[4–6] or similar to controls,[7,59] as observed in our cohort. The differences in HDL-C and ApoA1 levels among cohorts of SCD patients may be explained by differences in standard of care in different countries and recent availability of new drug treatments.[17] Both HDL-C and ApoA1 were observed by Yalcinkaya et al. to be markedly decreased in a study of 35 pediatric SCD patients[14] and to be associated with hemolysis and inflammation, respectively. These authors concluded from their investigations that hemolysis was associated with decreased HDL-C, and inflammation was linked to decreased ApoA1 levels in SCD patients, and suggested that the HDL particle is altered during the course of the disease. The well accepted involvement of HDL in cholesterol exchange with RBCs,[60] and in the removal of excess cholesterol from tissues and RBC cell membranes, suggest that HDL may be a major player in the RBC abnormalities observed in SCD.[30]

Another important finding in our investigation was that in these young healthy volunteers the plasma concentration of mApoA1 was better correlated with total plasma ApoA1 than with HDL-C. Healthy volunteers with higher HDL-C (>48 mg/dL) displayed a higher level of ApoA1 as well as a higher concentration of mApoA1 compared to those with HDL-C ≤48 mg/dL. More importantly, mApoA1 correlated with plasma lutein, zeaxanthin, and α-tocopherol in these subjects. These results support the hypothesis that a major role of ApoA1 and HDL in healthy subjects is to transport and distribute lipophilic antioxidants to lipoproteins with lower densities and to tissues such as RBCs as illustrated in Figure 4. In addition, ApoA1 and HDL may be involved in the removal of excess RBC membrane cholesterol often observed in RBCs from SCD patients.[30]

We confirmed that in the healthy volunteer population, lipophilic antioxidants (lutein, zeaxanthin, and α-tocopherol) are associated with the HDL fraction and distributed to the non-HDL fraction according to their relative lipophilicity. This is in agreement with previous investigations where HDL was shown to transport preferentially the dihydroxy xanthophylls lutein and zeaxanthin, whereas β-cryptoxanthin and β-carotene, which are much more lipophilic, were preferentially transported by the LDL and VLDL particle subfractions.[29,61,62] The correlation between mApoA1 and the level of lipophilic antioxidants supports the hypothesis that mApoA1 is involved in the uptake of dietary or tissue stored lipophilic antioxidants. Other transporters such as CETP and/or phospholipid transfer protein may be involved in the transfer of tocopherol[63] and xanthophylls[64] from HDL to larger lipoproteins particles.

Exercise and treatments, such as the CETP-inhibitor dalcetrapib, which increase HDL levels and increase the levels of plasma and

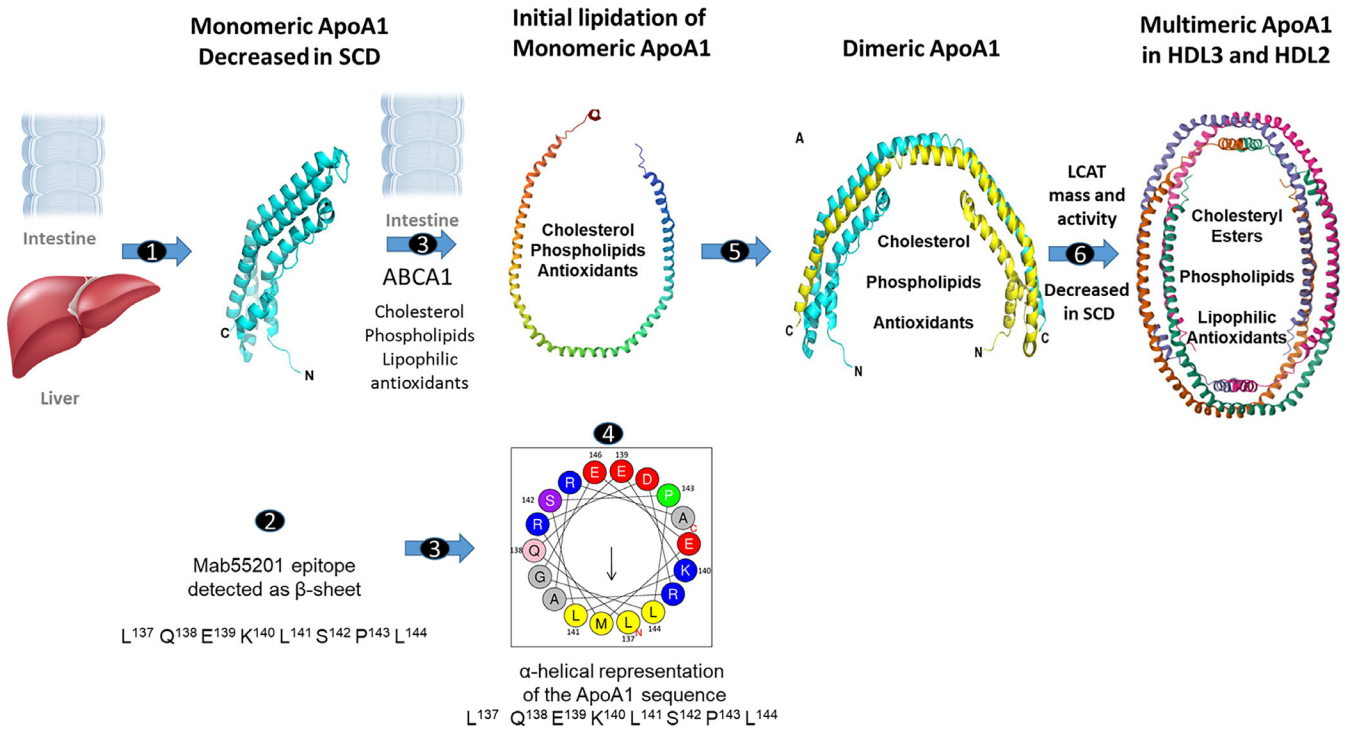

FIGURE 4 1-ApoA1 secreted mainly by the intestine and liver as monomeric ApoA1 (mApoA1). 2-Amino acid sequence L^{137} Q^{138} E^{139} K^{140} L^{141} S^{142} P^{143} L^{144} recognized by mAb55201 as beta-sheet. 3-Upon lipidation via ABCA1, mApoA1, acquires cholesterol, phospholipids and lipophilic antioxidants. 4-Lipidation leads to the formation of Helix 5 including amino acid sequence LQ E K L S P L not recognized by mAb55201 and with the hydrophobic amino acids L^{137} L^{141} L^{144} (yellow) on one side of the alpha-helix. 5-Dimerization of ApoA1 induces the formation of the LCAT binding site. 6-LCAT activity allows further lipidation, addition of ApoA1 molecules and enlargement of HDL particles. In Sickle Cell Disease mApoA1, LCAT mass and activity are dramatically diminished.

tissue lipophilic antioxidants, might have a place in the armamentarium against SCD[65] and may benefit these patients by decreasing end-organ damage from ROS and RBCs from hemolysis. In addition, lutein, zeaxanthin, and α-tocopherol protect the retina and brain from ROS; patients suffering from macular degeneration and Alzheimer's Disease may also benefit from better delivery of these antioxidants to the retina[66,67] or the brain.[68,69]

We propose the simultaneous measurement of mApoA1 and lipophilic antioxidants in HDL and non-HDL lipoproteins as a better estimate of the HDL functionality compared to HDL-C levels in healthy subjects as well as SCD patients. Similarly, these parameters may determine the potential benefits of exercise, dietary supplementation and/or treatments aiming at restoring an optimal concentration of these antioxidants in plasma and tissues.

AUTHOR CONTRIBUTIONS

Eric J. Niesor: Conception and design of the work. Coordination of analysis performed on samples form the Healthy volunteer and SCD cohorts. Drafting first manuscript. **Serge Rezzi**: Coordination of analysis of samples and responsible for accuracy or integrity of the analytical work. **Andrew Hodgson** and **Stephane Canarelli**: Responsible for analytical chemistry, coordination and execution, reporting of analytical chemistry and blood chemistry data. **Gregoire Millet** and **Tadej Debevec**: Coordination of healthy volunteer cohort samples. **Claire Bordat** and **Elie Nader**: Coordination and analysis of blood samples from the SCD cohort. **Philippe Connes**: Coordination and execution blood sample analysis of the SCD cohort. Manuscript writing.

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CONFLICT OF INTEREST STATEMENT

As CEO of Hartis-Pharma SA and as former Roche AG employee is co-author of several applied patents covering the use of HDL raising and mimicking interventions for the treatment of SCD and other pathologies.

DATA AVAILABILITY STATEMENT

Data will be available on request.

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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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