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Research Article

Erythrocytes increase endogenous sphingosine 1-phosphate levels as an adaptive response to SARS-CoV-2 infection

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Low plasma levels of the signaling lipid metabolite sphingosine 1-phosphate (S1P) are associated with disrupted endothelial cell (EC) barriers, lymphopenia and reduced responsivity to hypoxia. Total S1P levels were also reduced in 23 critically ill patients with coronavirus disease 2019 (COVID-19), and the two main S1P carriers, serum albumin (SA) and high-density lipoprotein (HDL) were dramatically low. Surprisingly, we observed a carrier-changing shift from SA to HDL, which probably prevented an even further drop in S1P levels. Furthermore, intracellular S1P levels in red blood cells (RBCs) were significantly increased in COVID-19 patients compared with healthy controls due to up-regulation of S1P producing sphingosine kinase 1 and down-regulation of S1P degrading lyase expression. Cell culture experiments supported increased sphingosine kinase activity and unchanged S1P release from RBC stores of COVID-19 patients. These observations suggest adaptive mechanisms for maintenance of the vasculature and immunity as well as prevention of tissue hypoxia in COVID-19 patients.

Introduction

The clinical course of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) induced coronavirus disease 2019 (COVID-19) is highly variable. COVID-19 symptoms range from asymptomatic up to hypoxemic lung failure and acute respiratory distress syndrome (ARDS) [1]. The underlying reasons for different clinical courses and disease severity are not yet completely understood, but current data suggest that epidemiological factors such as age [2], gender [3,4] or pre-existing conditions [5] together with virus-associated factors such as viral load [6] or SARS-CoV-2 mutants [7] contribute to the outcome of patients. Besides vaccination, therapeutic strategies targeting the immune response [8], the cytokine release [9] and endothelial cell (EC) barrier [10] are under development in larger trials such as the REMAP-CAP trial [11]. However, a majority of trials is based on vague assumptions regarding the pathophysiological mechanisms of COVID-19, and development of causative treatment strategies is *hitherto* hampered [12,13].

Considering the main clinical findings in critically ill patients with COVID-19, which are hypoxemia (low paO_2) and lung edema, one may ask why the clinical courses are lethal in some individuals while others are asymptomatic. Decompensation of adaptive mechanisms to hypoxemia might contribute to this phenomenon. Sufficient oxygen supply depends on cardiac output, paO_2 and the saturation of hemoglobin

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(SpO_2) together with the concentration of hemoglobin (Hb). While paO_2 is affected by pulmonary and cardiac output by cardiovascular function it may explain why patients with pre-existing conditions are at higher risk for severe clinical courses [5].

Red blood cells (RBCs) are the main cellular transporters of O_2. Binding and release of O_2 are dynamically regulated by metabolic adaptions of RBCs and potentially decompensate as well. While high O_2 saturation favors the pentose phosphate pathway to produce reducing agents like nicotinamide adenine dinucleotide phosphate (NADPH), low O_2 saturation supports glycolysis to generate adenosine triphosphate (ATP) and 2,3-bisphosphoglycerate (2,3-BPG) [14]. The latter causes a right shift of the oxygen–Hb dissociation curve, promoting alleviated O_2 release and consequently appropriate tissue oxygenation.

We hypothesize that sphingosine 1-phosphate (S1P), a lipid signaling molecule stored and released by RBCs, may play a crucial role in the adaptation to SARS-CoV-2-induced hypoxemia, lung failure and inadequate host response for three reasons: (i) increased S1P level in RBC supports the generation of 2,3-BPG and promotes oxygen release in tissue [15]. (ii) Critically reduced serum S1P levels in blood promote edema formation in the lungs of several species, and S1P levels are reduced in human septic shock, which is accompanied by EC barrier disruption [16,17]. (iii) S1P is essential for lymphocyte migration to the side of infection, and the SARS-CoV-2-associated lymphopenia might be a consequence of a disturbed S1P homeostasis [18].

Therefore, we studied total S1P levels in plasma, serum and RBCs as well as carrier-associated S1P in a cohort of COVID-19 patients.

Methods

Study population

The first 23 COVID-19 patients treated in the intensive care unit (ICU) of the Department of Anesthesiology at Göttingen University Medical Centre (UMG) from March 2020 to May 2020 were included in the present study. The local ethics board at the UMG approved inclusion of all ICU patients under the protocol reference 25/4/19Ü. Informed consent has been obtained from patients or their legal representatives before inclusion into the study.

Clinical evaluations and assays

Sequential organ failure assessment (SOFA) scores were calculated on admission according to the published sepsis guidelines [19] and all clinical parameters were recorded from our electronical health recording system (ICCA, Phillips®). Within the first 24 h after ICU admission, plasma and serum samples were taken to measure S1P and S1P bound to its specific carrier protein: high-density lipoprotein (HDL)-S1P, low-density lipoprotein (LDL) S1P, very low-density lipoprotein (VLDL) S1P and serum albumin (SA) S1P. The Institute of Clinical Chemistry and Laboratory Medicine at UMG measured the concentration of HDL, LDL and albumin together with all other inflammatory markers. Leukocyte-free RBCs were harvested from separate samples by density gradient centrifugation, washed and stored in plasma-free conditions at 4°C.

Lipoprotein precipitation

Lipoproteins were sequentially precipitated via an increasing $Na_3P(W_3O_{10})_4$ concentration as previously described with minor modifications [20,21]. For chylomicron and VLDL precipitation, 10 µl of 1% $Na_3P(W_3O_{10})_4$ and 10 µl of a 2 M $MgCl_2$ solution were added to 200 µl plasma. After brief mixing and 15-min incubation at room temperature (RT), the samples were centrifuged at RT for 10 min at 6000 relative centrifugal force (rcf). Supernatants were transferred to new tubes for further precipitation. For LDL precipitation, 10 µl of 4% $Na_3P(W_3O_{10})_4$ solution were added to the first supernatant. After 15-min incubation at RT, the samples were centrifuged at RT for 10 min at 6000 rcf. Again, the supernatants were transferred to fresh tubes. For HDL precipitation, 10 µl of 40% $Na_3P(W_3O_{10})_4$ and 10 µl of a 2 M $MgCl_2$ solution were added to the second supernatant. After 2-h incubation at RT, the samples were centrifuged at RT for 30 min at 20000 rcf. The supernatant was the lipoprotein-free SA containing fraction. If not used immediately, all fractions were stored at −80°C.

Extraction and quantification of S1P

S1P measurements were performed according to an established protocol using liquid chromatography coupled to triple-quadrupole mass spectrometry (LC-MS/MS) [22]. After addition of the appropriate internal standard (100 pmol C17-S1P and 300 pmol C17-sphingosine (Sph) per sample for regular analyses, 300 pmol deuteriated D7-Sph for sphingosine kinase activity and S1P release assays, Avanti Polar Lipids, Alabaster, AL, U.S.A.), samples were transferred to glass centrifuge tubes and adjusted to a final volume of 1 ml with H_2O. After addition of 0.3 ml of 6 N HCl,

Table 1 Primers used for quantitative PCR

Gene	Forward primer	Reverse primer
HPRT1	agc cta aga tga gag ttc	cac aga act aga aca ttg ata
GAPDH	gaa ggt gaa ggt cgg agt	gaa gat ggt gat ggg att tc
S1PR1	agc act ata tcc tct tct g	tga cca agg agt aga ttc
S1PR2	cat cgt cat cct ctg ttg	agt gga act tgc tgt ttc
S1PR3	cca agc aga agt aaa tca ag	cat gga gac gat cag ttg
S1PR4	gct tct gtg tga ttc tgg	cca tga tcg aac ttc aat g
S1PR5	gga aca atg atg gag att	ggc att gtc ctt gat aac
SPHK1	ctc tgg tgg tca tgt ctg ga	cag gtg tct tgg aac cca ct
SPHK2	gcc tac ttc tgc atc tac acc tac	atg agg ttg aag gac agc cc
SGPL1	ctg gca aga ggg gag agc	cca ttg aac agg gaa caa gct
SPNS2	tta ctg gct cca gcg	tga tcat gcc cag gac ag
ALDOA	agc cca tgc ttg cac tca gaa gt	agg gcc cag ggc ttc agc agg
PGK1	atg tcg ctt tct aac aag ctg a	gcg gag gtt ctc cag ca

1 ml methanol and 2 ml chloroform, samples were vigorously vortexed for 10 min. Aqueous and chloroform phases were separated by centrifugation for 3 min at 1900 rcf, and the lower chloroform phase was transferred into a new glass centrifuge tube. After a second round of lipid extraction with additional 2 ml chloroform per ml aqueous sample, the two chloroform phases were combined and vacuum-dried at 60°C for 50 min using a vacuum concentrator. The extracted lipids were dissolved in 100 µl methanol/chloroform (4:1, v/v) and stored at −20°C. Detection was performed with the QTrap triple-quadrupole mass spectrometer (Sciex, Darmstadt, Germany) interfaced with the 1100 series chromatograph (Agilent, Frankfurt, Germany) and the Hitachi Elite LaChrom column oven and autosampler (VWR, Darmstadt, Germany). Positive electrospray ionization (ESI) LC/MS/MS analysis was used for detection of S1P and C17-S1P. Multiple reaction monitoring (MRM) transitions were as follows: S1P m/z 380/264, C17-S1P m/z 366/250, Sph m/z 300/282, C17-Sph m/z 286/268, D7-Sph 307/289. Liquid chromatographic resolution of all analytes was achieved using a 2 × 60 mm MultoHigh C18 reversed phase column with 3-µm particle size (CS-Chromatographie Service, Langerwehe, Germany). The column was equilibrated with 10% methanol and 90% of 1% formic acid in H_2O for 5 min, followed by sample injection and 15-min elution with 100% methanol with a flow rate of 300 µl/min. Standard curves were generated by adding increasing concentrations of S1P to 100 pmol of the internal standard C17-S1P. Linearity of the standard curves and correlation coefficients were obtained by linear regression analyses ($r^2 > 0.99$). Data analyses were performed using Analyst 1.6 (Sciex).

Quantitative polymerase chain reaction

Total RNA isolation was performed using the PAXgene Blood RNA Kit (PreAnalytiX, Hombrechtikon, Switzerland) from 2.5 ml whole blood according to the manufacturer's instructions. One microliter of isolated RNA sample (resolubilized in 80 µl elution buffer) was used for the determination of the RNA concentration with the NanoDrop (Peqlab, Erlangen, Germany). RNA was stored at –80°C until usage. The RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Braunschweig, Germany) was used for cDNA synthesis with 1 µg template RNA by incubation for 60 min at 42°C and termination by heating at 70°C for 5 min. cDNA was stored at −80°C.

PowerUp SYBR Green Master Mix (Thermo Fisher Scientific) was used to determine S1P receptor isoforms 1–5 (S1PR1, S1PR2, S1PR3, S1PR4, S1PR5), sphingosine kinase isoforms SPHK1 and SPHK2, S1P-lyase (SGPL1), Spinster homolog 2 (SPNS2), fructose-bisphosphate aldolase (ALDOA), phosphoglycerate kinase 1 (PGK1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression in human blood cells according to the manufacturer's protocol. Each PCR was performed in 10 µl reaction mixture containing 5 µl of 2× PowerUp Green Master Mix, 0.1 µM forward and reverse primers and 100 ng template cDNA. Real-time analysis was performed using the MasterCycler Realplex system (Eppendorf, Hamburg, Germany). Thermocycling program was 40 cycles of a denaturation step for 15 s at 95°C followed by annealing and extension step for 1 min at 60°C with an initial activation step for 2 min at 50°C and 2 min at 95°C. Specific exon-spanning primers (Table 1) were used to determine expression of transcripts of interest (S1PR1–5, SPHK1-2, SGPL1, SPNS2, ALDOA, PGK1 and GAPDH) in human blood cells with hypoxanthine phosphoribosyltransferase (*HPRT1*) as reference gene. Expression levels were evaluated using the change-in-threshold (C_t) values normalized by the C_t values of the housekeeping gene, *HPRT1*. Relative changes in gene expression were determined by the $2(-\delta\delta C(T))$ method with the relative expression software tool (REST) based on a mathematical model and the pairwise fixed reallocation randomization test [23].

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Sphingosine kinase activity assay

Ten microliters of pelleted RBCs (1 × 10E7 cells) were dissolved with 89 μl PBS and supplemented with 1 μl of 10 mM C17-Sph. Samples were incubated overnight at 37°C under constant agitation. The reaction was stopped by adding 1 ml methanol. After addition of 10 μl of 30 μM D7-Sph as internal standard, samples were stored overnight at −80°C. Proteins were subsequently precipitated by centrifugation at 14000 rcf for 5 min, and the supernatant was analyzed for the conversion of C17-Sph into C17-S1P by LC-MS/MS as described above.

S1P release assay

Ten microliters of pelleted RBCs (1 × 10E7 cells) were dissolved with 89 μl PBS and supplemented with 1 μl of 10 mM C17-Sph. Samples were incubated overnight at 37°C under constant agitation. After centrifugation at 2600 rcf for 5 min, RBCs were washed twice with PBS and incubated overnight in PBS supplemented with 50 mg/ml SA at 37°C under constant agitation. Samples were subsequently centrifuged at 2600 rcf for 5 min, and the supernatant was transferred into new tubes. Proteins were precipitated with 1 ml methanol followed by incubation at −80°C overnight. Following centrifugation at 14000 rcf for 5 min, the supernatant was analyzed for the presence of C17-S1P by LC-MS/MS as described above.

Statistical analysis

The primary variables were S1P in plasma, serum and RBC, and S1P bound to HDL (HDL-S1P), serum albumin (SA-S1P), LDL (LDL-S1P) and VLDL (VLDL-S1P). Outliers were identified according to the ROUT method with the desired maximum false discovery rate Q set to 1% [24]. Differences between two groups were tested for significance using the unpaired Student's *t* test with nonparametric correction using the Mann–Whitney U-test. A P-value <0.05 was considered to be significant. Statistical analyses were performed using GraphPad Prism 7.0a, April 2016 (La Jolla, CA, U.S.A.).

Results

S1P levels were significantly increased in HDL particles of COVID-19 patients

In order to investigate modulations of S1P signaling due to SARS-CoV-2 infection, blood samples of 23 COVID-19 patients were collected at the time of ICU admission. Samples from 17 healthy volunteers were used as controls. The clinical severity monitored by the SOFA and the simplified acute physiology score (SAPS II) were 9 (5–11, SOFA) and 41 (34–49, SAPS II), respectively, with a predicted mortality rate of 26.6% (15.3–43.8%, Table 2). While neutrophil counts were significantly increased in the blood of COVID-19 patients, those of lymphocytes, monocytes and eosinophils were decreased (Table 2). Significant reductions in Hb, hematocrit, RBC counts, mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), SA, total cholesterol, LDL and HDL were also observed (Table 2). Quantification of S1P with LC-MS/MS in plasma and serum of COVID-19 patients revealed a statistically significant decrease with a median concentration of 585 nM (plasma) and 1024 nM (serum) compared with 897 nM (plasma) and 1302 nM (serum) in healthy volunteers, with slightly higher values in serum compared with plasma (Figure 1). In line with total plasma and serum S1P levels, both main carriers of S1P in plasma, SA and HDL, were also significantly reduced by 65% (HDL) and 56% (SA) in COVID-19 patients compared with controls and considerably below standard values (Table 2). We therefore performed sequential lipoprotein precipitations and analyzed the respective fractions containing VLDL and chylomicrons (A), LDL (B), HDL (C) and lipoprotein-free SA (D) for their S1P content by LC-MS/MS. Strikingly, the lipoprotein-free SA fraction (D) revealed the most dramatic decrease of S1P content by 82% (Figure 2A). S1P concentrations in the remaining fractions were also significantly reduced by 40% (VLDL and chylomicrons), 48% (LDL) and 42% (HDL). Looking at the S1P concentrations per molecule, the S1P content per HDL particle increased by 60% in COVID-19 patients compared with healthy controls, while the remaining SA was occupied with 60% less S1P than normal (Figure 2B), demonstrating a remarkable shift of S1P from SA to HDL in COVID-19 patients.

S1P was significantly increased in RBCs of COVID-19 patients

While the demonstrated shift of S1P from SA to HDL was probably preventing an even more dramatic decrease in total S1P in plasma and serum of COVID-19 patients, the main source of the additional S1P bound to HDL remained unclear. We therefore analyzed the S1P content of RBCs, which are one of the main sources for S1P in plasma. Notably, the S1P content in RBCs of COVID-19 patients increased by 40% with a median of 4333 pmol (3471–4727 pmol) per

Table 2 Characteristics of the study cohort

Parameter	Normal range	COVID-19		
SARS-CoV-2, GE/reaction	N/A	468 (28–22011)		
Age, years	N/A	69 (66–75)		
Died, *n* (%)	N/A	7 (30%)		
ICU days	N/A	16 (7–24)		
ECMO, *n* (%)	N/A	3 (13%)		
SAPS II	N/A	41 (34–49)		
Predicted mortality, %	N/A	26.6 (15.3–43.8)		
SOFA	N/A	9 (5–11)		
FiO_2	N/A	0.50 (0.37–0.75)		
paO_2, mmHg	65–105	73 (66–88)		
$paCO_2$, mmHg	36–42	44 (36–62)		
paO_2/FiO_2	N/A	133 (86–217)		
SpO_2	94–98	96 (95–98)		
pH	7.34–7.45	7.42 (7.34–7.46)		
Temperature, °C	N/A	38.0 (37.0–38.5)		
D-Dimers, mg/l	<0.5	1.34 (0.72–3.52)		
C-reactive protein, mg/dl	≤5	118.7 (29.7–186.6)		
Ferritine, μg/l	22-275	732 (163–1337)		
Procalcitonin, μg/l	<0.07	0.50 (0.15–1.30)		
Interleukin-6, pg/ml	<7	75.4 (45.1–514.1)		
Lactate, mmol/l	≤1.8	1.1 (0.6–1.6)		
Parameter	**Normal range**	**COVID-19**	**Healthy**	***P*-value**
n		23	17	
Hemoglobin, g/dl	13.5–17.5	10.5 (8.8–12.5)	14.8 (14.3–15.6)	<0.0001
Hematocrit, %	39–51	33 (27–37)	44 (42–46)	<0.0001
Red blood cells, $10^6/\mu l$	4.4–5.9	3.79 (3.17–4.26)	4.9 (4.6–5.3)	<0.0001
MCV, fl	81–95	87 (77–93)	89 (87.5–91.5)	0.2383
MCH, pg	26–32	28 (24–31)	31 (29–32)	<0.05
MCHC, g/dl	32–36	32.6 (31.5–33.3)	34.0 (33.8–34.3)	<0.0001
Platelets, $10^3/\mu l$	150–350	195 (131–327)	285 (223–304)	0.1890
Leukocytes, $10^3/\mu l$	4.0–11.0	11.1 (5.6–13.6)	5.7 (5.0–7.6)	<0.05
Lymphocytse, %	20–45	9.5 (5.9–25.1)	32 (28–35)	<0.001
Monocytes, %	3–13	7.1 (4.2–8.2)	9.0 (7.5–10.5)	<0.05
Eosinophils, %	≤8	1.0 (0.35–1.5)	3.0 (2.0–3.0)	<0.001
Basophils, %	≤2	0.40 (0.15–1.0)	0.05 (0.03–0.08)	N/D
Neutrophies, %	40–76	69 (56–82)	54 (51–62)	<0.05
Serum albumin, g/dl	3.4–5.5	2.1 (1.7–2.3)	4.4 (4.1–4.7)	<0.0001
Cholesterol, mg/dl	≤200	120 (96–143)	183 (160–225)	<0.0001
Triglycerides, mg/dl	≤150	135 (102–243)	120 (88–149)	0.1290
LDL, mg/dl	≤115	71 (50–85)	131 (119–161)	<0.0001
HDL, mg/dl	>40	18 (11–32)	51 (43–65)	<0.0001

Abbreviation: GE, genome equivalent.
Values represent the median with interquartile range (25^{th}–75^{th} percentile), groups were compared using the Mann–Whitney U test.

10E10 RBCs compared with healthy controls with 3068 pmol (2301–3546 pmol) (Figure 3). To uncover the reason for the observed increased S1P levels, we first checked for defects in the S1P exportation in RBCs from COVID-19 patients. *Ex-vivo* incubations with C17-S1P loaded RBCs demonstrated no difference of S1P exportation between RBCs isolated from COVID-19 patients and healthy controls (Figure 4A). We then continued to look at the activity of sphingosine kinases, which are the main producers of S1P. Incubation of RBCs from COVID-19 patients with Sph resulted in almost 50% higher phosphorylation rates and C17-S1P production than RBCs isolated from healthy volunteers (Figure 4B). This significantly increased sphingosine kinase activity could explain the observed difference in S1P content of RBCs isolated from COVID-19 patients compared with healthy controls.

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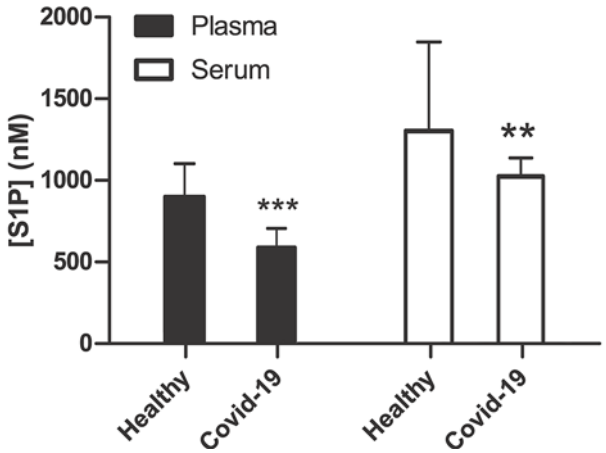

Figure 1. S1P levels in plasma and serum

Shown are medians with interquartile ranges of S1P levels in plasma and serum from 17 healthy volunteers and 23 severe Covid-19 patients. Statistical significance was tested with unpaired Student's *t* test with nonparametric correction using the Mann–Whitney U-test; $^{**}P<0.01$, $^{***}P<0.001$.

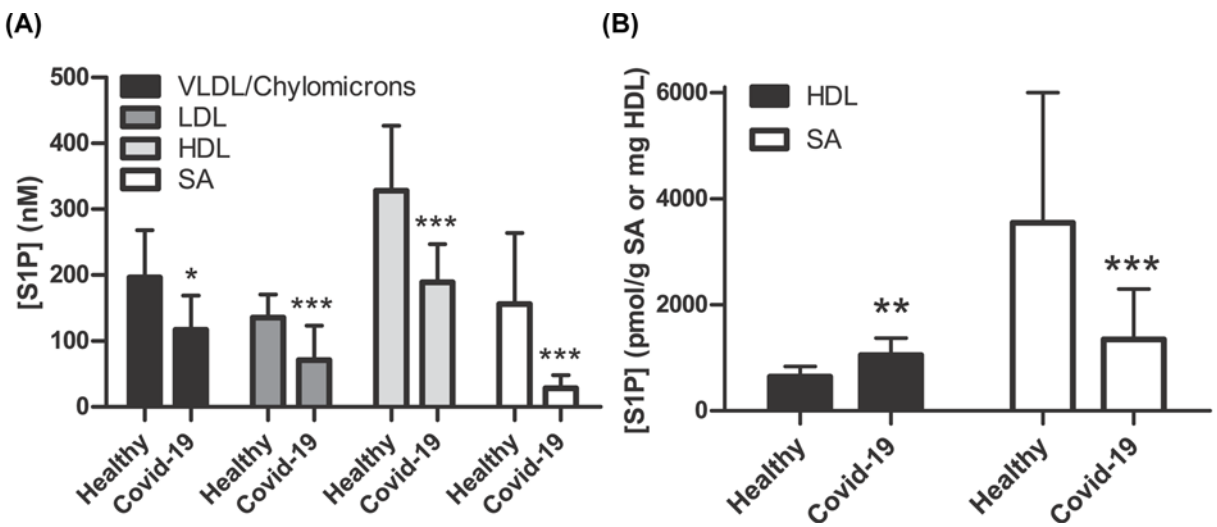

Figure 2. Distribution of S1P in different lipoprotein and lipoprotein-free compartments

(**A**) Total plasma S1P concentrations separated according to the association with VLDL and chylomicrons, LDL, HDL and SA. (**B**) Plasma S1P concentrations associated with either 1 mg HDL or 1 g SA. (A,B) Shown are medians with interquartile ranges of S1P concentrations from 17 healthy volunteers and 23 severe Covid-19 patients. Statistical significance was tested with unpaired Student's *t* test with nonparametric correction using the Mann–Whitney U-test, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

S1P-related metabolic differences in COVID-19 patients

To confirm the results obtained from LC-MS/MS measurements and functional studies, we performed quantitative polymerase chain reaction (qPCR) experiments and analyzed the expression of S1P receptors (S1PR) and different enzymes and transporters of the sphingolipid and glycolysis pathways in blood cells. These analyses confirmed a metabolic switch in COVID-19 patients towards increased production and release of S1P with significantly increased expression of SPHK1 (Figure 5A) and unaltered expression of the S1P exporter Spinster homolog 2 (SPNS2, Figure 5B). In contrast, SPHK2 (Figure 5C) and the S1P lyase (SGPL1, Figure 5D), both enzymes involved in the degradation of blood-borne S1P [25], were significantly down-regulated. S1PR1 (Figure 5E) and S1PR5 (Figure 5F) were also down-regulated, indicating an activated immune status. In contrast, receptor isoforms 2–4 (S1PR2-4, Figure 5G–I) were not altered. Importantly, the significant increase in glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Figure 5J), fructose-bisphosphate aldolase (ALDOA, Figure 5K) and phosphoglycerate kinase 1 (PGK1, Figure 5L) suggested

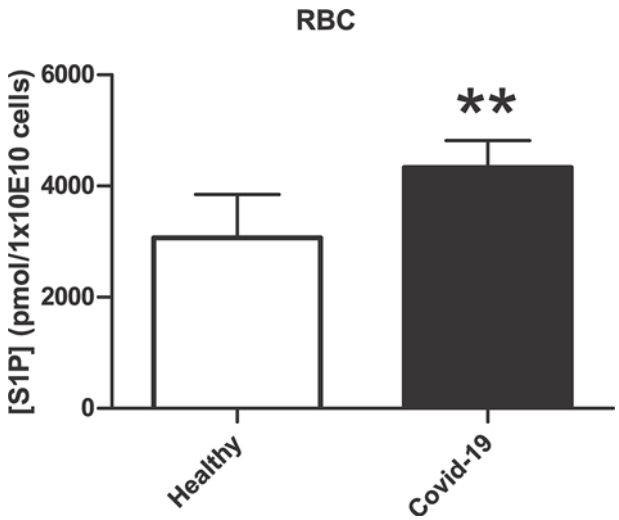

Figure 3. S1P levels in RBC

Shown are medians with interquartile ranges of S1P levels in RBCs from 17 healthy volunteers and 23 severe Covid-19 patients. Statistical significance was tested with unpaired Student's *t* test with nonparametric correction using the Mann–Whitney U-test, $^{**}P<0.01$.

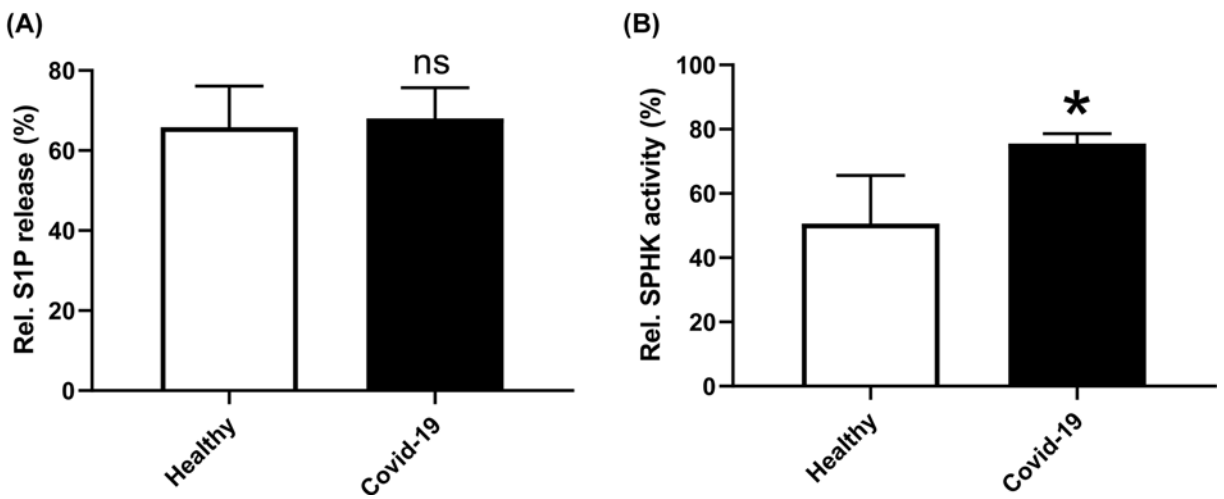

Figure 4. Phosphorylation and release of S1P by RBC

(**A**) Release of C17-S1P by RBCs into PBS supplemented with 50 mg/ml SA. (**B**) Phosphorylation of C17-Sph by sphingosine kinases (SPHK) in RBC. (A,B) Shown are means ± SD of freshly isolated RBCs from five healthy volunteers and four severe Covid-19 patients. Statistical significance was tested with unpaired Student's *t* test with nonparametric correction using the Mann–Whitney U-test; $^{*}P<0.05$; ns, not significant.

a metabolic switch towards increased glycolysis, which was reported as an adaptive mechanism in RBCs with elevated S1P levels.

Discussion

We report that S1P levels were significantly decreased in plasma and serum of patients with severe COVID-19 while S1P levels in RBCs isolated from those patients were increased.

Other studies also demonstrated decreased S1P levels in serum [26,27], but unchanged S1P levels in RBCs of COVID-19 patients [28]. The patient cohort in our study was exclusively derived from the ICU. Accordingly, all patients were characterized by a high SOFA score. The patient cohorts of other studies were less well defined, particularly

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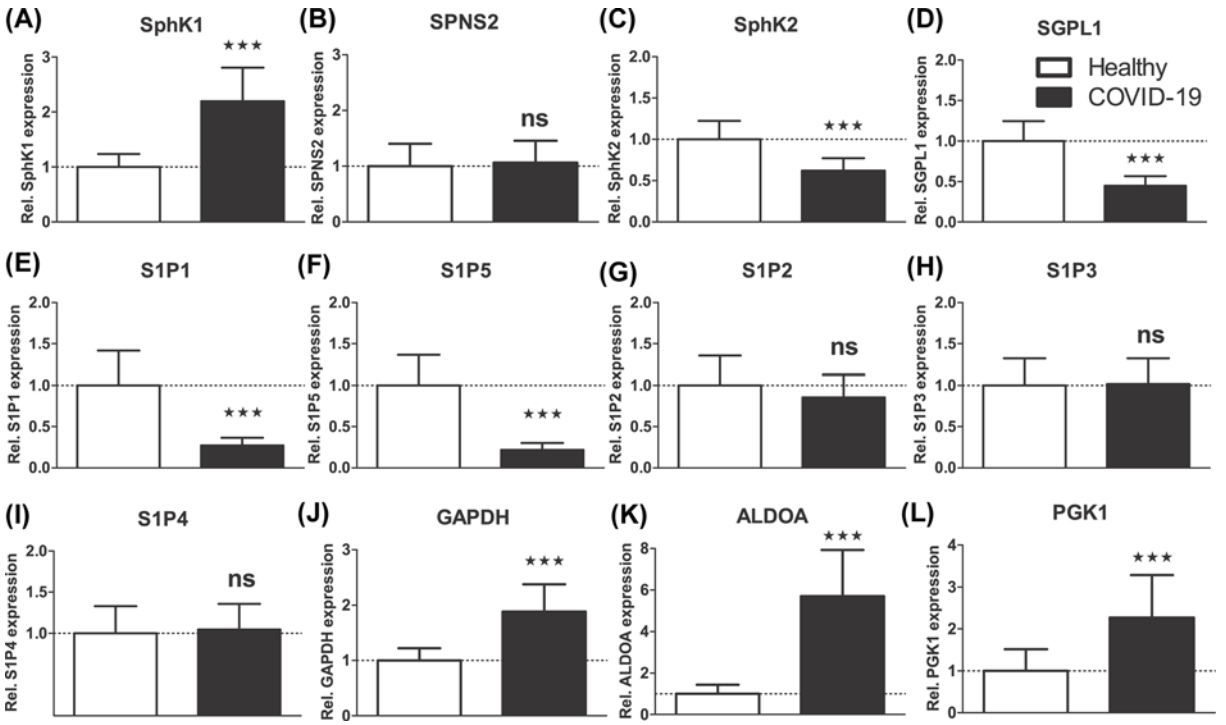

Figure 5. Blood cell expression of S1P-generating and metabolizing enzymes, S1P receptors and enzymes of the glycolytic pathway

mRNA expression of (**A**) the extracellular S1P producing enzyme sphingosine kinase 1 (SPHK1) and (**B**) the S1P exporter SPNS2, (**C**) SPHK2 and (**D**) the lyase SGPL1, both involved in blood-borne S1P degradation (25), the S1P receptors (**E**) S1PR1, (**F**) S1PR5, (**G**) S1PR2, (**H**) S1PR3, and (**I**) S1PR4, and the glycolytic enzymes GAPDH (**J**), ALDOA (**K**) and PGK1 (**L**), all normalized to HPRT1. (A–L) Shown are means ± SEM for 17 healthy volunteers and 18 severe Covid-19 patients. Statistical analysis was performed using the pairwise fixed reallocation randomization test (23), ***$P<0.001$; ns, not significant.

regarding disease severity [26–28]. Not considering different disease outcomes may result in incomplete phenotypical observations due to increased variations and consequently insignificant results. This may explain the unchanged S1P levels in RBCs of COVID-19 patients reported in a different study that only looked at positive SARS-CoV-2 tests without discrimination of disease severity [28].

The differential use of S1P carriers in COVID-19 patients detected in the present study was also observed in patients with surgical trauma and sepsis [17]. The switch of S1P carriers from SA to HDL with concomitantly dropping SA levels could function as an adaptive response mechanism to maintain normal homeostasis. In contrast with SA-S1P, HDL-S1P impairs lymphopoiesis and neuroinflammation [29] and dampens vascular inflammation [30]. Less efficient activation of S1PR1 was suggested as a potential mechanism that also contributed to the observed protective effect of HDL-S1P in atherosclerosis [30]. Importantly, HDL-S1P was shown to induce sustained endothelial barrier stabilization, which is also compromised in severe COVID-19 patients [31]. Thus, the preferential use of HDL as the predominant S1P carrier seems to be a compensatory response to adjust for decreased SA and SA-S1P levels in order to maintain EC barrier function and to prevent an excessive inflammatory response.

Although total plasma and serum S1P levels in severe COVID-19 patients dropped significantly by more than 30%, reductions in SA and HDL levels were even more severe with 56% for SA and 65% for HDL. A similarly severe drop of S1P levels was probably prevented by the observed additional S1P production in RBCs and increased S1P association with HDL. A drop of SA and HDL was also observed in septic shock patients [32]. Here, total S1P levels in plasma and serum were reduced to a similar extent of both, SA-S1P and HDL-S1P [17]. Septic shock patients were also characterized with a high SOFA score similar to the herein analyzed severe COVID-19 patients. But in contrast with septic shock patients, severe COVID-19 patients had more S1P bound to HDL. The reason for this difference might be the observed elevated S1P levels in RBCs due to increased formation and decreased degradation. RBCs are the main source for S1P in serum and plasma and play an important role in the stabilization of the vascular endothelial barrier particularly after an immune challenge [20,33,34]. It was demonstrated before that HDL was more efficient in

extracting S1P from RBCs than SA, and loading of RBCs with excess amounts of S1P resulted in predominant binding and extraction of S1P by HDL, not SA [35]. Thus, it is feasible to suggest that increased S1P levels in RBCs of severe COVID-19 patients were responsible for enhanced binding and extraction of S1P by HDL, which finally might have limited the observed drop of S1P levels in plasma fairly well despite the overall reduced HDL and SA levels.

Increased expression and activity of sphingosine kinase 1 coincided with increased S1P levels in RBC. Earlier reports demonstrated a contributing role of sphingosine kinase 1 produced S1P in RBCs for the right-shift of the oxygen–Hb dissociation curve in people at high altitude [15]. This shift facilitates the plain release of oxygen from RBCs into tissues and prevents local tissue hypoxia. Here, we report for the first time that S1P accumulation due to both increased sphingosine kinase 1 expression and activity also occurred in severe COVID-19 patients, which suffer even more from hypoxemia than people living at high altitude because of impaired lung function. High S1P levels in RBCs could therefore be an adaptive mechanism to cope with hypoxic conditions due to a severe SARS-CoV-2 infection. People living at high altitude were protected from SARS-CoV-2 infections and revealed a lower severity of the disease compared with people living at sea level [36]. This finding suggests that pre-conditioning with low-grade hypoxemia may prevent the most severe disease states of COVID-19.

Studying the histopathology of COVID-19 autopsies, recent reports highlighted both, a systemic endothelial activation and strongly pronounced derangement and endothelialitis especially of the pulmonary endothelium [37] and propose COVID-19 as a vascular disease [38]. Our data support the concept that the observed shift induced by S1P redistribution might contribute to an improved global oxygen supply at the pulmonary side of alveolar integrity (oxygen uptake) as well as in tissues with their microcirculatory terminal vessels (oxygen release). Irrespective the fact that systemic endothelial activation and resulting dysfunction is observed in a broad range of viral and bacterial infections such as sepsis and septic shock [39], the herein described distinct adaptive mechanism in COVID-19 patients might contribute to an improved evaluation of more specific therapeutic interventions, which is driven by a better understanding of host's response against SARS-CoV-2 infection.

Despite these promising new results, our study is also confronted with certain limitations. It was carried out at a single center with a limited number of patients admitted to the ICU. Although sufficiently powered for the presented results, the size of the cohort does not allow for the analysis of certain subsets like survivors and non-survivors, ventilated and non-ventilated, or high and low plasma, serum or RBCs' S1P levels. Furthermore, we cannot exclude that our results are biased by sample size or treatment strategies. We measured S1P associated with HDL, SA and RBCs and correlated the measured values with clinical and laboratory parameters. But our observations cannot explain cause–consequences at the end. Nonetheless, our data highlight potential compensatory and adaptive S1P signaling pathways in COVID-19 patients that warrant further investigations of the role of S1P in HDL and RBC to help the body maintain homeostasis.

Clinical perspectives

- **Background:** The signaling lipid metabolite S1P is an immune modulator typically involved in infections.
- **A brief summary of results:** Blood S1P association in severe COVID-19 patients shifted from SA to HDL as carrier. Although this shift could not prevent a significant decrease in total plasma S1P levels, it may have prevented an even more severe drop of S1P levels. RBCs as a major source for S1P in plasma revealed significantly increased S1P levels compared with healthy controls due to increased sphingosine kinase activity.
- **The potential significance of the results to human health and disease:** Our data point to adaptive mechanisms in severe COVID-19 patients to support HDL-driven endothelial barrier stabilization and RBC-driven oxygen release by distinct shifts of S1P concentrations towards its carrier HDL and its producing cell RBCs.

Data Availability

All supporting data are included within the main article and available by contacting the corresponding author.

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Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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CRediT Author Contribution

Martin Sebastian Winkler: Conceptualization, Supervision, Investigation, Visualization, Writing—original draft, Project administration, Writing—review & editing. **Ralf Alexander Claus:** Methodology, Formal analysis, Writing—review & editing. **Mareike Schilder:** Methodology, Writing—review & editing. **Stefan Pöhlmann:** Resources, Writing—review & editing. **Sina M. Coldewey:** Resources, Writing—review & editing. **Julian Grundmann:** Writing—review & editing. **Torben Fricke:** Writing—review & editing. **Onnen Moerer:** Resources, Writing—review & editing. **Konrad Meissner:** Resources, Funding acquisition, Writing—review & editing. **Michael Bauer:** Resources, Funding acquisition, Writing—review & editing. **Heike Hofmann-Winkler:** Methodology, Writing—review & editing. **Markus H. Gräler:** Conceptualization, Resources, Formal analysis, Supervision, Funding acquisition, Investigation, Visualization, Methodology, Writing—original draft, Project administration, Writing—review & editing.

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Abbreviations

COVID-19, coronavirus disease 2019; EC, endothelial cell; Hb, hemoglobin; HDL, high-density lipoprotein; ICU, intensive care unit; LC-MS/MS, liquid chromatography coupled to triple-quadrupole mass spectrometry; LDL, low-density lipoprotein; RBC, red blood cell; rcf, relative centrifugal force; RT, room temperature; SA, serum albumin; SARS-CoV-2, severe acute respiratory syndrome coronavirus-2; SOFA, sequential organ failure assessment; Sph, sphingosine; S1P, sphingosine 1-phosphate; UMG, University Medical Center Göttingen; VLDL, very low-density lipoprotein; 2,3-BPG, 2,3-bisphosphoglycerate.

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