Global metabolomics and lipidomics approaches to probe virus-host interactions

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Chapter 5

Altered plasma lipidome is associated with disease severity in COVID-19 patients

Based on

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Biomolecules, under revision.

Abstract

The severity of COVID-19 is linked to an imbalanced immune response. Dysregulated metabolism of small molecules and bioactive lipids has also been associated with disease severity. To promote understanding of the disease biochemistry and provide targets for intervention, we applied a range of LC-MS platforms to analyse over 100 plasma samples from patients with varying COVID-19 severity and with detailed clinical information on inflammatory responses (>30 immune markers). This is the third publication in a series, and it reports the results of comprehensive lipidome profiling using targeted LC-MS/MS. We identified 1076 lipid features across 25 subclasses, including glycerophospholipids, sterols, glycerolipids, and sphingolipids, among which 531 lipid features were dramatically changed in the plasma of intensive care unit (ICU) patients compared to patients in the ward. Patients in the ICU showed 1.3-57 fold increases of ceramides, (lyso-) glycerophospholipids, diglycerides, triglycerides, and plasmagen phosphoethanolamines and 1.3-2 fold lower levels of a cyclic lysophosphatidic acid, sphingosine-1-phosphates, sphingomyelins, arachidonic acid-containing phospholipids, lactosylceramide, and cholesterol esters compared to patients in the ward. Specifically, phosphotidylinositols (PIs) have shown a strong fatty acid-saturation dependent behavior, with SFA- and MUFA-derived PI decreasing and PUFA-derived PI increasing. We also found ~4000 significant Spearman correlations between lipids and multiple clinical markers of immune response with $|R| \geqslant 0.35$ and FDR corrected $Q<0.05$. Except for lysophosphatidic acid, lysophospholipids were positively associated with the CD4 fraction of T cells, and the cytokines IL-8 and IL-18. In contrast, sphingosine-1-phosphates were negatively correlated with innate immune markers such as CRP and IL-6. Further indication of metabolic changes in moderate COVID-19 disease were demonstrated in recovering ward patients compared to early hospitalization, where 99 lipid species were altered (6 increased by 30-62%; 93 decreased by 1.3-2.8 fold). Overall, these findings support and expand on early reports that dysregulated lipid metabolism is involved in COVID-19.

Keywords: SARS-CoV-2; COVID-19; lipidomics; lipids; cytokines; inflammation

1. Introduction

The coronavirus (COVID-19) pandemic has presented a significant global challenge due to the rapidly growing number of new variants, which highlights the urgent need for a characterization of SARS-CoV-2 pathogenicity and host immune response [1]. Patients with COVID-19 experience a spectrum of clinical symptoms of different severity, ranging from asymptomatic to critical pneumonia, acute respiratory distress syndrome (ARDS), and death [2]. Furthermore, patients exhibit high inter-individual variability in response to SARS-CoV-2 infection, which makes it difficult to identify those with risks of adverse outcomes. The involvement of lipids in the COVID-19 disease is far reaching. Corona viruses first react with the host cell membrane for entry and infection, which has brought novel insights into the involvement of cellular lipids [3–7]. Lipids are the main building blocks of cell membranes and play a crucial role in the viral replication process, affecting the host lipid metabolism [4,8]. Membrane lipids also release precursors of eicosanoid and docosanoid polyunsaturated fatty acids (PUFA) to regulate the immune responses during an infection [9]. Several lipidomic studies have described an altered lipidome profile in COVID-19 patients [10–13]. In an early pandemic study, patients with varying severity of COVID-19 (compared to healthy subjects) showed decreased serum levels of sphingolipids, glycerophospholipids and choline, while phosphocholine was increased [10]. Another study reported decreased plasma diacylglycerols (DG) and increased levels of sphingomyelins (SM) and monosialodihexosyl gangliosides (GM3s) in COVID-19 patients compared to healthy controls [11]. Suggesting overall alteration in lipid balance in COVID-19, additional studies reported decreased serum total cholesterol, HDL and LDL alongside increased triglycerides (TG) [12,13]. Another study showed that a comprehensive lipid mapping unveils host dependency factors that remain consistent among various SARS-CoV-2 variants [14].

Previously we reported COVID-19 plasma perturbations in amines which reflected inflammation and oxidative stress [15], and also presented altered signalling lipid metabolism which was suggested to reflect excessive immune response and disrupted shift to resolution of inflammation [16]. To further study lipidome changes in COVID-19 patients, we analysed 103 plasma samples from 44 patients at varying disease severity and conducted a comprehensive profiling of over 1000 lipids by targeted LC-MS/MS. The measured lipids

underwent differential analysis based on disease severity (i.e., hospitalization status) and were also correlated with over 30 immune response markers obtained for the same cohort [17]. The results of this study contribute to the gathering evidence of lipid alteration in COVID-19, and provide further insight into the cellular mechanisms involved in the disease progression.

2. Results

2.1 Altered lipid profiles in COVID-19 patients in ward and ICU

During the first wave of COVID-19 and before the improvements made to the wards of hospitals during the later waves, the more severely affected patients were transferred to the ICU for more intense care, mechanical ventilation, and generally improved monitoring. The location of the patient is thus a proxy for severity of COVID-19. To determine the potential relationships between plasma lipids and disease severity, we profiled 25 plasma samples from 7 patients in the ICU and 78 plasma samples from 37 patients in the ward, using targeted lipidomics approaches. A description of the COVID-19 patient cohort with samples collected at varying hospitalisation days is summarised in **Table 1** and further detailed in **Table S1**.

Table 1. Demographics of the COVID-19 patient in the lipidomics study. Values are n (%) or median (full range). Information about comorbidities and medication (4 weeks pre-admission) is missing for 25% of patients (n=12), smoking status is missing for 9 patients, remaining hospitalisation days for 2 patients. All data per patient is available in **Table S1**.

	Patients (n=44)	**Samples (n=103)**
Age, years	73 [49-87]	71 [49-87]
Male (%)	30 (68%)	65 (63%)
BMI	27 [19-42]	27 [19-42]
Diabetes mellitus (DM)	9 (20%)	
Chronic kidney disease (CKD	3 (7%)	
Cardiovascular disease (CVD)	7 (16%)	
Chronic obstructive pulmonary disease (COPD)	8 (18%)	
past smoker	17 (39%)	

pre-admission beta-blockers, ACE inhibitors	14 (32%)	
pre-admission corticosteroids	8 (18%)	
pre-admission chloroquine	27 (61%)	
Days with symptoms till hospitalisation	8 [1-19]	
Total hospitalisation days	7 [2-62]	
Admitted to ward	37 (84%)	78 (76%)
Admitted to ICU	7 (16%)	25 (24%)
Organ failure	7 (16%)	
Deceased	9 (20%)	
Unfavourable outcome (ICU or death)	12 (27%)	36 (35%)
Invasive breathing support (intubated)	6 (14%)	
post-admission chloroquine	35 (80%)	
post-admission corticosteroids	2 (5%)	
post-admission antibiotics	38 (86%)	
CRP, mg/L (normal <10)		104.5 [3-577]
IL6, pg/mL (normal <8)		19.3 [1-397]
Ferritin, ng/mL (normal 10-400)		1035 [84-4807]
Leukocytes, 10^9/L (normal 4.5-11)		8 [4-20.5]
Lymphocytes, 10^9/L (normal 0.8-5.0)		0.95 [0.26-3.15]
Neutrophils, 10^9/L (normal 1.7-6.5)		6.36 [2.3-17.5]

In order to visualize the distribution of this cohort, all 1076 lipid features that passed the quality control process were first analysed using a principal component analysis (PCA) which shows some separation between the ward and ICU patients (**Figure 1**). Next, a linear regression model for univariate analysis was employed, adjusting for age, sex, BMI, and count of samples per patient, to identify the most important biomarker candidates distinguishing the patients at ward from ICU. A total of 531 lipids (including the ratio of sphinganine-1-phosphate (Spha1P) 18:0/ sphingosine-1-phosphate (S1P) 18:1) across 19 lipid classes with fold change $\geqslant$ 1.3 or $\leqslant$ 0.7 and FDR Q value < 0.05 were considered significant (**Table S3**), which indicates a widely changed lipidome in severe COVID-19 patients.

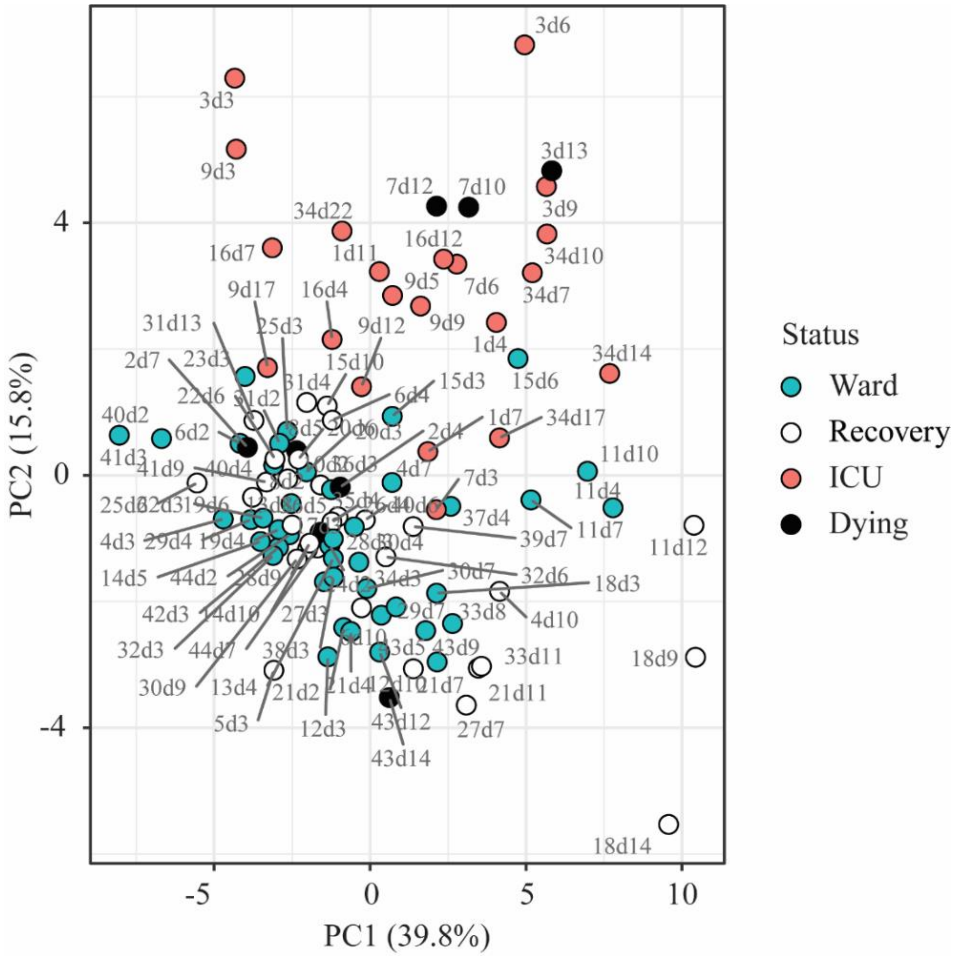

Figure 1. PCA scores plot of samples from patients admitted to ward (blue markers) or ICU (red markers), based on all lipid features data (cube-root transformed and Pareto-scaled). Data points of samples taken within a day of release from hospital ("recovery") are depicted by open circles, and samples taken within 4 days of death are in black. Each data point is tagged with the patient ID and sample day (corresponding with Table S2). Ward patients #11 and #15 were situated in close proximity to the ICU patients. Ward patient #11 was suffering from three disease complications-CKD, CVD, and diabetes, while Ward patient #15 was on different drug regimens (including antibiotics, chloroquine, and corticosteroids) during their hospital stay.

All significantly changed species in the lipid classes TG, ceramides (Cer), DG, lysophoshatidylcholines (LPC), lysophosphatidylethanolamines (LPE), lysophosphatidylglycerols (LPG), lysophosphatidylserines (LPS), lysophosphatidylinositols (LPI), phosphatidylcholines (PC), alkyl phosphatidylcholines

PC(O-), phosphatidylglycerols (PG), phosphatidylethanolamines (PE), lysophosphatidic acids (LPA) increased, while all significantly changed species in the lipid classes cholesteryl esters (CE), lactosylceramides (LacCer), S1P and SM decreased in the ICU patients when compared to ward patients as shown in FC plot (**Figure 2a**) and specific examples (**Figure 2b-e**). The whole classes of PS, alkyl phosphatidylethanolamines (PE (O-)) increased in ICU patients, except for two AA- precursor containing species, PS 18:1/20:4 and PE O-16:0/20:4, which were decreased in the ICU patients.

PIs have shown a strong fatty acid-saturation dependent behavior with SFA-& MUFA-derived PI decreasing and PUFA-derived PI increasing in ICU patients. Among all those differences, TGs and PE (O-)/ alkenyl phosphatidylethanolamines (PE (P-)) showed the biggest increased levels with FCs up to 57-fold in ICU patients (**Figure 2a; Table S3**).

2.2 Paired analysis in ward patients

To quantify the metabolic changes towards recovery of COVID-19 patients, paired analysis was performed on samples taken from 16 patients in the ward, hence reflecting moderate disease only. The patients were selected based on sample availability at the start of hospitalisation (days 1-4 since admission) and towards the end of hospitalisation (within 1 day before release from the hospital), with a minimum interval of three days between the two time points. The analysis was restricted to ward patients, since there were not enough relevant samples available from ICU patients. Compared with the widely changed lipidome in patients in the ICU vs. ward, here only 99 lipids were significantly altered (FC $\geqslant$ 1.3 or $\leqslant$ 0.7 and an FDR Q value < 0.05) towards recovery in the ward (**Table S4**). Most of the significant changes were found in phospholipids and lysophospholipids, that increased towards recovery (see examples in **Figure 3**). Of note, other lipid classes, especially TG and SM remained mostly unchanged along the hospitalization in the ward, suggesting higher relevance to severe disease.

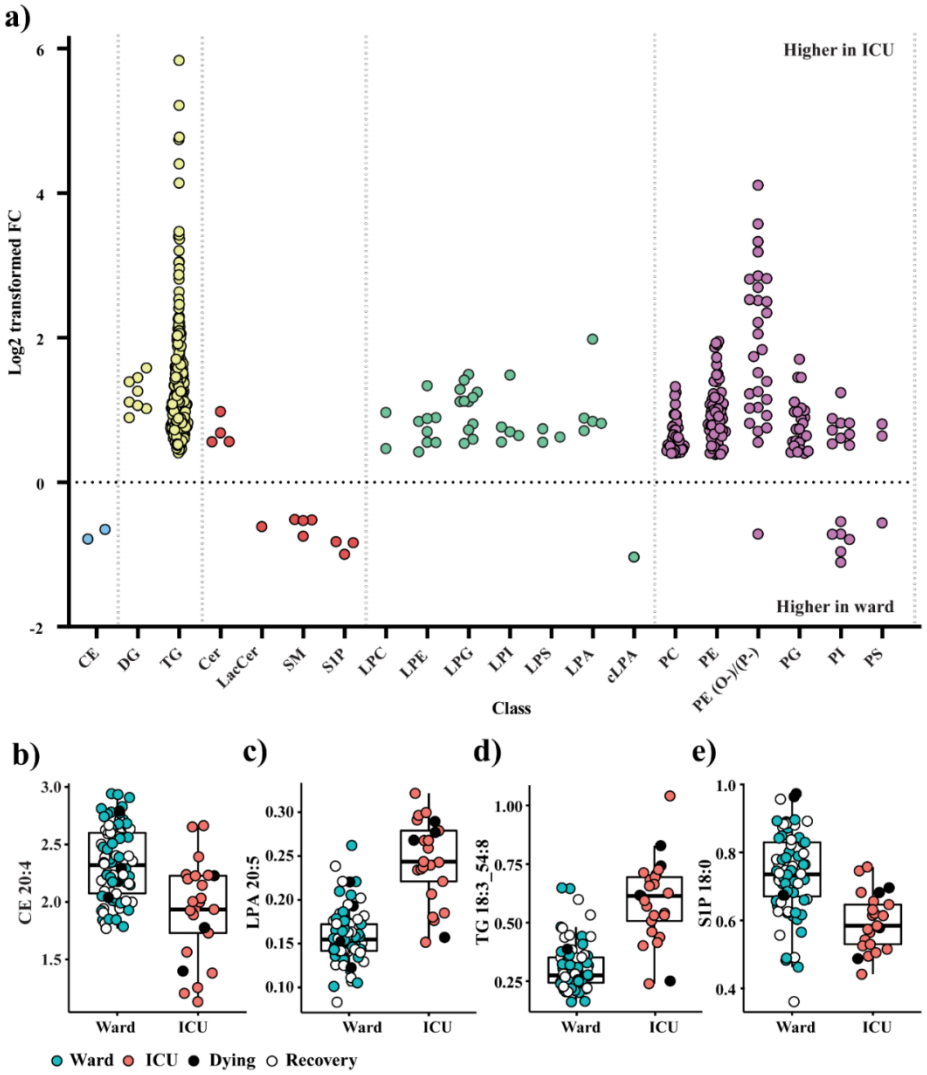

Figure 2. (a) Distribution of log2 transformed fold changes of 531 significantly changed lipid features with FC $\geqslant$ 1.3 or $\leqslant$ 0.7) and an FDR Q value < 0.05 between ICU and ward. Different colours represent different lipid (sub)categories: light blue: CE; yellow: Glycerolipids; red: (Glyco)sphingolipids; green: (Lyso)phospholipids; purple: phospholipids; (b-e) Box and whisker and scatter plots of lipids (Q<0.005) differentiating between hospitalisation status: ICU (red) vs. ward (blue; open markers for recovering patients within 24 h of release). Black markers represent patients who died within 4 days. Prior to plotting, lipid peak area ratios with internal standards were cuberoot-transformed. Lipids: (b) CE 20:4; (b) LPA 20:5; (d) TG 18:3_54:8; (e) S1P 18:0; The detailed results are in **Table S3**.

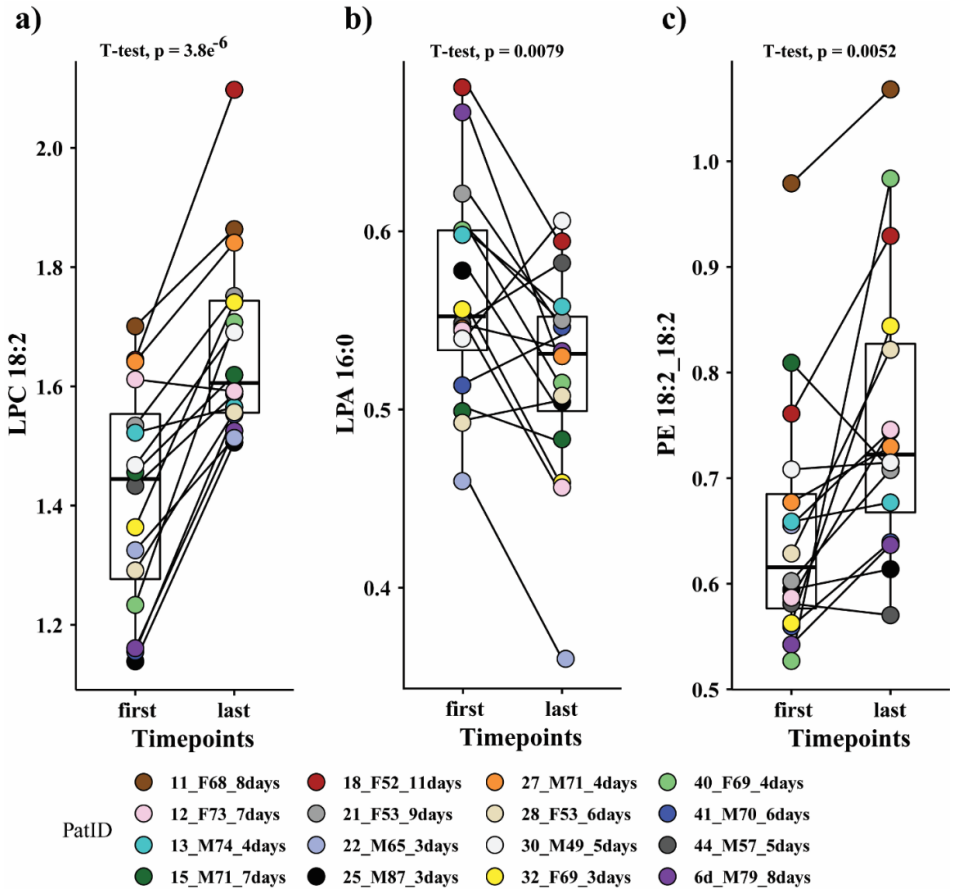

Figure 3. Box and whisker and scatter plots of paired changes of lipids levels in COVID-19 ward patients. A line connects each patient's paired samples, with the first time point being not more than 4 days from admission, and last time point during the 24 h before release from hospital. Lipid peak area ratios with internal standards were cuberoot-transformed. Lipids: (a) LPC 18:2; (b) LPA 16:0; (c) PE 18:2_18:2; The legend shows the individual patient by marker colour, with indication of patient number, sex, age, and number of days between time points. Patient information is provided in **Table S2**. FDR-corrected paired t-tests, gender differences and fold changes are provided in **Table S5**.

2.3 Correlation between lipids and immune response markers

The concentration of 37 immune response markers, including different leukocytes, chemokines, cytokines, and others, were also measured (**Table S2**). We performed Spearman correlation analysis on lipids and cytokine data from the whole dataset, and a heatmap summarizing the correlation results of each immune marker with each lipid class is presented in **Figure 4**. In total, 3995 significant correlations were observed with the

threshold of $|R| \geqslant 0.35$ and $Q<0.05$, among which 37 with strong association with $|R| \geqslant 0.6$ and $Q<0.05$, (**Table S5**). We observed that SM and S1P were negatively correlated with clinical indices of systemic inflammation including pro-inflammatory markers IL-6, CRP, TNFα, neutrophils, CCL2, GM-CSF, CXCL10, IFNG and macrophage-activation markers (soluble (s) CD206 and CD163), and positively correlated with the CD8 fraction of T cells (**Figure 4; Table S5**). Ceramides were positively correlated with IL-6, IL-8, IL-18, CD206, CD163, CD8, TNFα, ferritin, and T cell count. Lysophospholipids except for LPA were positively correlated with IL-18, IL-8, and IL-7 and negatively correlated with CXCL10 (IP10). Selected plots with strong correlations ($|R| \geqslant 0.60$ and $Q<0.05$) are shown in **Figure 5**. The correlations of S1P with immune markers were found to be mostly driven by the dramatic and consistent differences in lipid levels between patients in the ICU and the ward (**Figure 5b**), while the LPLs and TG were independent of the hospitalization status (**Figure 5c-f**). The results of the correlation analysis are further utilised in the biochemical discussion section.

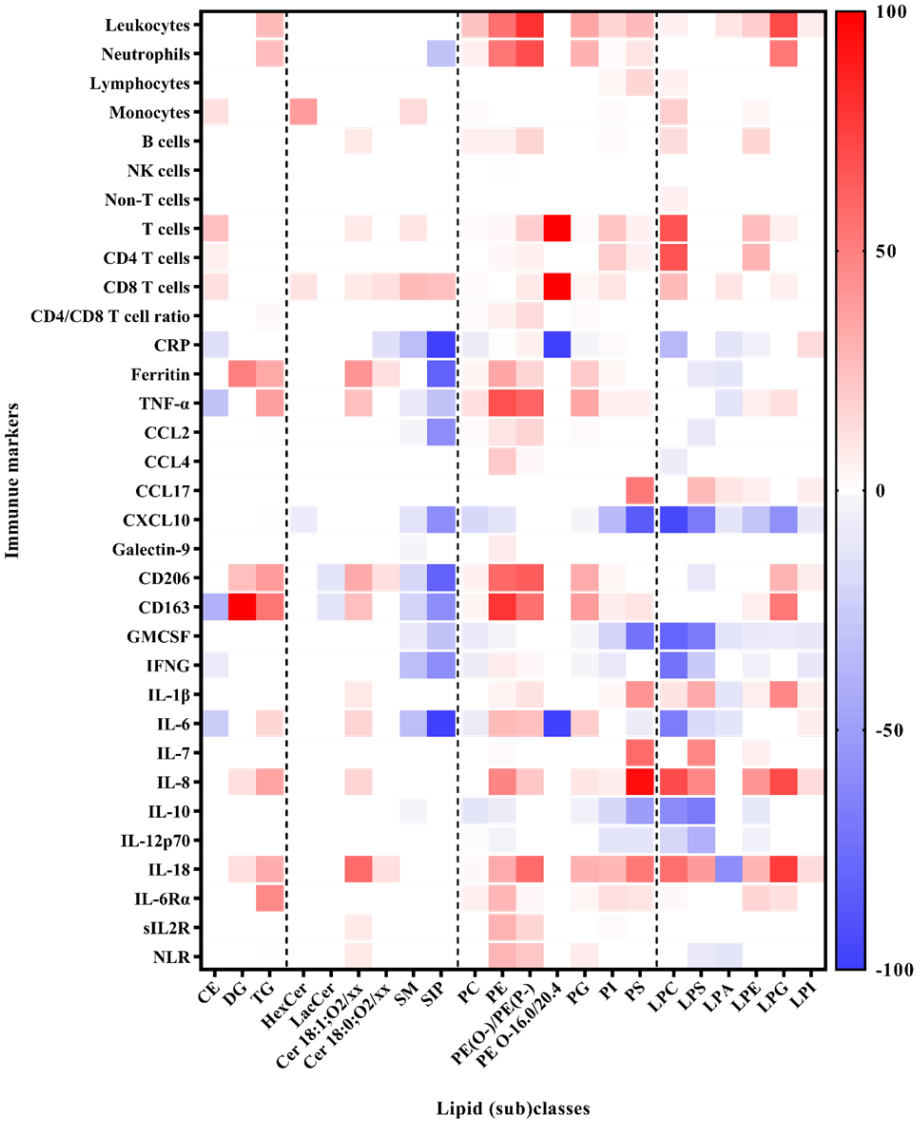

Figure 4. Heatmap of Spearman correlation results between metabolites and immune response markers (all cuberoot-transformed). Color bars represents the percentage of the lipid species with significant correlations with $|R| \geqslant 0.35$ and $Q<0.05$ per class, red for positive correlations and blue for negative correlations. Complete correlation matrices (with R, P, Q values) are provided in **Table S5**.

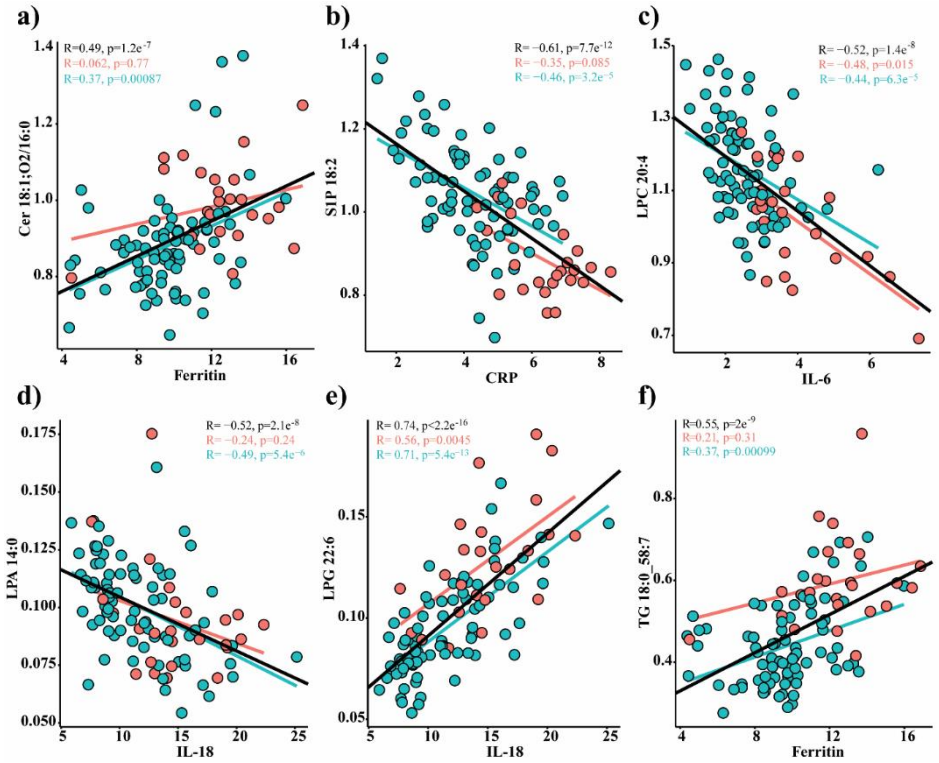

Figure 5. Selected Spearman correlation scatter plots between metabolites and immune response markers (cuberoot-transformed). Red markers are samples from ICU patients, and blue markers are from ward patients. The regression lines and Spearman R values and p values (uncorrected) are in black for all samples, red for ICU, and blue for ward. Lipids: (a) Cer 18 :1;O2/16 :0 vs. Ferritin; (b) S1P 18:2 vs. CRP; (c) LPC 20:4 vs. IL6; (d) LPA 14:0 vs. IL18; (e) LPG 22:6 vs. IL18; (f) TG 18:0_58:7 vs. Ferritin; The full correlation results are in **Table S5**.

3. Discussion

Our study reveals noteworthy changes in the lipidome associated with COVID-19 disease severity, including in sphingolipids, glycerolphospholipids and glycerolipids. Biochemical processes relevant to the changes in measured metabolites and various immune response markers and their correlations are discussed here.

3.1 Sphingolipids metabolism

Sphingomyelin itself doesn't have a direct link to COVID-19, however Sphingolipids are

essential components of membrane lipid rafts which mediate signal transduction and immune activation processes [18,19]. Specifically, two protein targets of COVID-19, ACE2 and TMPRSS2, are embedded in lipid rafts and can actively participate in viral infection [20,21]. On the other hand, lipoproteins, including those carrying sphingomyelin, play a role in the body's immune response. COVID-19 affects multiple systems, including the cardiovascular and immune systems, and lipoproteins are involved in both. Recent studies have shown that lipoproteins, especially high-density lipoproteins (HDL), may have a protective role against severe complications from COVID-19. HDL particles are known to possess anti-inflammatory and antioxidant properties that can help modulate the immune response and potentially mitigate the severity of inflammatory reactions, such as those seen in severe COVID-19 cases. Sphingomyelin, as a component of these lipoproteins, indirectly contributes to their functions. However, the exact relationship between sphingomyelin within lipoproteins and COVID-19 isn’t yet fully understood and remains an area of ongoing research. In our study, we observed increases in ceramide levels and decreases in SM and LacCer in severe COVID cases (ICU), which are consistent with previously reported serum lipid alteration in COVID-19 [19]. Ceramides exhibited positive correlations with markers of macrophage activation (CD163 and CD206) that typically increase during the innate immune response in COVID-19. IL-18 was also correlated with ceramides, and this aligns with macrophage activation, as IL-18 can be produced by cells like macrophages. IL-18 has a role in maintaining Th1 inflammatory response to viral infection, and it induces downstream production of IFNG [24]. It was suggested to be associated with lower risk of developing severe COVID-19 [25]. Ceramides also positively correlated with IL-8, which mediates the inflammatory reaction in the respiratory system (as demonstrated in COVID-19 [26]), promoting neutrophil activation. Positive associations of ceramides with ferritin levels suggest an interplay between iron metabolism and reactive oxygen species (ROS) production during the COVID-19 disease process [27,28]. Ferritin is a surrogate marker for hyper-immune response and elevation of ferritin occurs when intracellular iron concentration and production of hepcidin increases, which can be an indicator of cellular damage [28]. As expected, SM that were higher in patients in the ward compared to ICU, showed negative correlations with an array of immune response markers that characterize hyper-inflammation (CRP, macrophage activation markers, CXCL10, TNF-α, IL-6). This further strengthens the link between SM and better

health outcome.

The gathered results may also be linked to the active role of sphingolipids in the development of enveloped viruses at the early stage [3,22,23], which leads to cell apoptosis and immunoescape by lipid raft remodeling [29–32]. The ceramide-sphingomyelin signalling system has a central role in the viral infection of human epithelial cells [33]. Sphingomyelins are derived from ceramides in cell membranes via the activity of sphingomyelin synthase. During an adaptive immune response, the membrane-embedded inert sphingomyelins will be hydrolysed by acid sphingomyelinase (aSMase) and cause rapid and transient formation of ceramides, which is a hallmark of adaptive responses and cellular repair [23]. This has been shown to be important in COVID-19 as antidepressants have been used to diminish the production of ceramides level via inhibiting the aSMase levels and preventing the SARS-Cov-2 binding in cell models [23]. Furthermore, ceramides have been reported as important biomarkers for metabolic disease, e.g cardiovascular disease [34] and diabetes [35], which are co-morbidities associated with a worse outcome in COVID-19.

3.2 Sphingoid base 1-phosphates

Sphingoid base 1-phosphates are important immune modulators [36]. They are part of the sphingolipid signalling cascade, and play a role in immune cell trafficking and endothelial function, depending also on the expression of specific cell surface receptors [37]. S1P is a reported prognostic marker for COVID-19 outcome and the lower circulating level of S1P in severe patients suggest the loss of the protective effect of S1P [38,39]. S1P 18:1 is the most researched metabolite in this group [40,41], and its extracellular S1P gradient regulates the excretion of lymphocytes such as mature dendritic cells from/to lymphoid organs (low S1P concentration) and into the blood (high S1P concentration) [36,42,43]. This occurs via binding of S1P to receptors such as S1PR1 on the surface of T cells, while the receptors expression is induced by endothelial chemokines (CXCL10 etc.) [44]. S1P was related to inflammation resolution, for example by secretion from alveolar macrophages in acute lung injury [45]. Our results support the link to adaptive immune response, showing consistent and strong declines of four sphingoid-base 1-phosphates in ICU patients, alongside a moderate increase towards recovery of ward patients. Similar observations were reported

for S1P in patients with COVID-19 [11,38,46]. Aligning with the proposed beneficial effects of S1P, we found negative correlations between the four metabolites and the acute immune response markers CRP (S1P 18:2 in **Fig.5b**), TNF-α, IL-6, ferritin and others, mostly in a homogenous manner across the four metabolites (**Table S6**). Analogues of sphingosine-1-phosphate were suggested as low-risk supportive treatment of COVID-19 patients, to reduce the inflammatory response, mitigate lung damage and even lower the viral load [8,29,47,48].

3.3 Glycerophospholipids

We observed an extensive increase in glycerophospholipid levels in ICU patients. Glycerophospholipids (PC, PE, PI, PG and PS) are major components of cell membranes and play multiple roles in response to viral infections. Apart from interacting with glycoproteins on the plasma membrane, viruses can also utilize host-derived lipid membranes in their intercellular transmission to conceal and evade the host's immune system [49,50]. Glycerophospholipids can undergo degradation, producing lysophospholipids and a free fatty acid. Lysophospholipids are immune modulators and are involved in several pathophysiological processes such as cell proliferation, migration and tumorigenesis [51]. Our study revealed a strong negative correlation between LPCs and leading markers of hyper-inflammation (GM-CSF, CXCL10, IL-6, CRP). This affinity between better health status and higher levels of LPCs is also demonstrated by increased levels towards recovery in the ward. CXCL10 is linked to various lung inflammatory conditions through both pro- and anti-fibrotic effects, as well as its role in recruiting cells that secrete or respond to IFNG [52]. Lysophosphocholines (LPCs) produced by phospholipase A2 (PLA2) can be further metabolized by lysophospholipase D/autotaxin (ATX), leading to their conversion to LPA, which is involved in the innate immune response [51]. Indeed, we observed a decrease in LPA towards recovery of patients in the ward. When LPCs release a PUFA from their sn2 position, it can serve as a precursor for oxylipins, which play a significant role in regulating the immune response during viral infection [16]. PUFAs are essential precursors for a diverse array of oxylipins that are stored in an esterified form and later released by enzymes like COX-1, 12-lipoxygenase (12-LOX), and CYPs found in platelet [53]. A significant alteration in levels of AA-containing precursors was observed in phospholipids. Our previous analysis of signaling lipids in the same cohort found that ICU

patients had significantly lower levels of AA compared to ward patients [16], which may be linked to metabolic requirements or an altered level of PLA2 activity [46,54]. One study in COVID-19 patients showed significantly decreased plasma phospholipids alongside increased lysophospholipids, which may indicate enhanced activity of (PLA2) [2]. In contrast, we observed extensive increases in glycerophospholipids, including PC, PE, PI, PG, PS, together with the corresponding lysophospholipids. Disagreement between studies can reflect various cohort differences and treatment (80% received chloroquine that can increase phospholipid levels [55]; however, less than 5% received corticosteroids that inhibit PLA2). Another factor is the analytical methods and the choice of blood product for lipidomics analysis. Plasma is preferred over serum, as it prevents a skewed profiling of oxylipins, sphingoid-based compounds, and lysophospholipids, among other lipids altered by coagulation [56].

3.4 Glycerolipids and other neutral lipids

Glycerolipids, including TG and DG, showed higher abundance in the plasma of ICU patients compared to those in the ward. In agreement with the link to worse disease state, TGs also correlated with markers of innate immune response (TNF-α, neutrophils), markers of macrophage activation, and ferritin. Typically, triglycerides serve as energy reservoirs for free fatty acids, and the liver is heavily involved in triglyceride metabolism to ensure a steady supply of energy and proper distribution of lipids throughout the body [57,58]. Individuals with pre-existing conditions such as diabetes and heart disease, which are characterized by elevated triglyceride levels and chronic inflammation, are predisposed to an increased risk of developing severe COVID-19 [59].

Studies have shown hypertriglyceridemia in COVID-19 patients, which highlights the biochemical significance of TGs, potentially indicating elevated adipose tissue lipolysis [60] and liver function abnormalities, as indicated elsewhere [61,62]. In addition, this is also supported by the lower total CE recorded in ICU patients in our study, which is often observed in liver damage [63]. Another study also found increased TG and decreased CE in patients with severe symptoms or elderly patients, which is consistent with the hepatic impairment associated with COVID-19 [7]. Beyond the hypermetabolic state and under-nutrition, such alterations in plasma lipids between ward and ICU can be attributed to

various metabolic pathways associated with the viral infection and the host immune response [64]. Triglyceride-rich lipoproteins have been associated with innate immunity [65] and all lipoprotein classes can sequester and prevent the excessive inflammation [64].

Altogether, the evidence presented in this study suggests that viral infection and following hospital treatment have a profound impact on the systemic lipid metabolism in COVID-19 patients. The pathophysiological effects of the disease seem long lasting; therefore, it is important to monitor the health state after discharge. The blood lipid profile can provide a sensitive array of marker linked to inflammation and disease severity.

4. Materials and Methods

4.1 Cohort

The cohort consisted of 44 adults admitted to the regional Amphia hospital in Breda, the Netherlands, on 24 March 2020-14 April 2020. **Table 1** summarises key characteristics of the 44 patients and 103 collected blood samples. **Table S1** provides background information and hospitalisation details per patient, such as comorbidities, treatment, and outcome. All patients reported COVID-19-related complaints and tested positive for the SARS-CoV-2 by a PCR.

4.2 Samples

EDTA blood samples were collected in intervals of 3-4 days throughout the study, as detailed in **Table S1** per patient. A small aliquot of the collected blood was immediately taken for flow cytometric immune profiling. Plasma was isolated from the remaining blood, aliquoted, and stored at -20 ◦C until serological analysis, or until transportation to the analytical chemistry laboratory, where kept at -80 ◦C until sub-aliquoting and LC-MS analysis.

4.3 Haematological and Serological Analysis

Flowcytometric leukocyte analysis and serological analysis of cytokines and soluble cell surface molecules have been reported previously by Schrijver et al. [17]. All assays were

performed according to manufacturer's protocol. The measured parameters, values, and units are detailed in **Table S2**.

4.4 Plasma Lipids Analysis

The complete details of sample preparation and analytical method are provided in **Supplementary Document S1**. Plasma samples were prepared by liquid-liquid extraction using butanol:MTBE (1:1, v/v), and analysed by two different UHPLC-MS/MS methods (high pH and HILIC-MS/MS). The chromatography was conducted on a Shimadzu Nexera X2 UHPLC (Shimadzu Corporation, Kyoto, Japan). For the high pH method, a Kinetex EVO C18 column was utilised (2.1 × 50 mm, 1.7 µm; Phenomenex Inc., Torrance, CA, USA). The HILIC-MS/MS method used a Luna amino column (100 mm × 2 mm, 3 µm, Phenomenex). Mass Spectrometry was conducted using a Shimadzu 8050 system in the high pH method [66], and a Sciex QTRAP 6500 MS (Sciex, Framingham, MA, USA) in the HILIC-MS/MS method [67]. ESI-MS was performed with polarity switching and multiple-reaction-monitoring (MRM). The acquired LC-MS data were processed using the vendor software (Sciex MultiQuant v3.0.2; Shimadzu Labsolutions v3.3), integrating the assigned MRM peaks and further correcting according to the peak areas of matched internal standards. In-house quality-control software (mzQuality) was utilised to assess and correct the analytical performance based on study QC replicates, blank samples, and internal standards. A total of 1076 lipid features with RSDQC <30% measured by the two platforms passed quality control and were utilised in the statistical analysis.

4.5 Statistical Analysis

All statistical analyses were performed in R, and graphs were plotted using the packages ggpubr and stats. Overall, all lipid features presented zero missingness; therefore, no imputations were performed. Cytokine and immune marker data (n = 37) were analysed as provided (**Table S2** [17]). All variables were cuberoot-transformed prior to statistical analyses. We could not identify clear outliers; therefore, no samples were removed from the dataset. Differential analysis between ICU and ward patients incorporating all samples was performed using linear regression correcting for age, sex, and BMI, grouped by patient, and weighted by the inverse number of observations per patient. Paired analyses between two

time points of the same patient were performed using a paired t-test assuming unequal variances. This approach enabled patient-corrected analysis of metabolic changes, and a more meaningful metabolite fold-change than when calculated in non-paired analysis. Metabolite fold change values were calculated based on the untransformed data, per patient in the paired t-test analysis, or by dividing the medians of experimental classes, in non-paired analysis. Spearman correlation analyses between metabolites and immune markers were conducted for all samples together and per hospitalisation status (ICU or ward), plotted as three regression lines to provide complementary information. The p-values obtained in all tests were adjusted for multiple testing using the Benjamini–Hochberg method implemented in the p.adjust R function (v.4.0.3), and termed Q-values. Significance levels were defined as $|R| \geqslant 0.35$ and $Q<0.05$. The corrections were for either the number of variables in univariate tests (n =1079) or for the number of unique correlations in the Spearman correlation tests (n = 40888).

5. Conclusions

In conclusion, our study highlights that the plasma lipidome profiles of COVID-19 patients differ at different stages of the disease. Our findings also demonstrate the interplay between pro-inflammatory cytokines and host metabolism in COVID-19. Despite the relatively small sample size, this work provides insights to further assist in drug development and treatment of COVID-19 and expands the essential resources promoting further research on the viral diseases' pathogenesis. Future investigation should explore the long-term effects of COVID-19 on lipid metabolism, the utility of metabolic changes as prognostic indicator of disease severity or outcome, and the efficacy of metabolic-targeted therapies for treating COVID-19.

Author contributions

Conceptualisation, R.C.H.V., W.A.D., A.W.L., V.H.J.v.d.V., and T.H.; Data curation, Z.Z., N.K., A.K., L.L., A.J.v.G., A.A.M.E., and L.P.; Formal analysis, Z.Z., N.K., A.K., M.S., L.L., A.J.v.G., and A.A.M.E.; Funding acquisition, R.C.H.V. and T.H.; Investigation, A.J.v.G. and T.H.; Methodology, Z.Z.; A.K., L.L., and L.P.; Project administration, A.C.H. and T.H.; Resources, A.C.H.; Software, A.K.; Supervision, A.C.H., V.H.J.v.d.V., and T.H.;

Visualisation, Z.Z. and A.K.; Writing-original draft, Z.Z. and NK; Writing-review and editing, Z.Z., N.K., A.K., M.S., L.L., A.C.H, A.J.v.G., R.C.H.V., W.A.D., A.W.L., and V.H.J.v.d.V. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: All data utilised in the statistical analyses are available as part of supplementary files.

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Conflicts of Interest

The authors declare no conflict of interest.

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SUPPLEMENTARY MATERIAL

Sample preparation procedure

For the lipid LC-MS analysis, all 103 samples were aliquoted to 150 µL for High pH method and 50 µL for HILIC MS/MS method. Study quality control (QC) samples were prepared by a pool of all study samples.

Sample preparation procedure for High pH method

EDTA plasma samples (150 µL) were thawed on ice and immediately treated with 5 µl antioxidants (0.2 mg/ml BHT/EDTA). A 10 µL mixture of isotope-labeled analogues (ISTDs) of the LPA and S1P (Table chemicals) was added to all samples followed by the addition of 150 µL citric acid/phosphate buffer (pH 4.5). Lipid mediators were extracted from the plasma by adding 1 mL of a butanol:MTBE (1:1;v:v) mixture. Samples were shortly vortexed before they were left to settle for 20 min. Sample preparation was continued by a 2 min bullet blender step and 10 min centrifugation at 13000 rpm (4˚C). The upper organic phase (850 µL) was transferred into clean Eppendorf tubes and dried in a vacuum centrifuge. Reconstitution of the samples occurred with 50 µL of an ice-cold methanol:acetonitrile mixture (70:30) with 100 µM CUDA standard after which the samples were transferred to HPLC vials for injection.

Sample preparation procedure for HILIC MS/MS based method

A total of 25 µL of plasma (human K2 EDTA plasma/COVID-19 patient plasma) was extracted according to the methyl tert-butyl ether (MTBE) method. A volume of 34 µL of the one-IS mix (concentration of this mix is defined in previous publication [1]. was added to 25 µL of plasma and vortexed. To this mixture, 231 µL of methanol (MeOH) and 770 µL of MTBE were added. The sample was incubated at room temperature on an orbital shaker for one hour followed by the addition of 192.5 µL of water thus making final ratio MTBE:MeOH:Water (10:3:2.5, v/v/v). The mixture was again incubated at room temperature for 10 minutes and then centrifuged at 15800 rcf for 10 minutes. A volume of 520 µL of upper layer was collected and dried in a vacuum concentrator followed by reconstitution in 200 µL of acetonitrile:methanol (3:7). This mixture was vortexed and

centrifuged for 10 minutes. The supernatant was collected and injected in the LC-MS for analysis.

The COVID-19 study batch design includes solvent blanks, procedure blanks (with IS), clinical study samples and quality control (QC) samples. These QC samples were a pool of all the study plasma samples and were analyzed at regular intervals in the study batch to determine the performance of the method.

1 Zhang Z, Singh M, Kindt A, Wegrzyn AB, Pearson MackenzieJ, Ali A, et al. Development of a targeted hydrophilic interaction liquid chromatography-tandem mass spectrometry based lipidomics platform applied to a coronavirus disease severity study. *Journal of Chromatography A* 2023; 1708 :464342.

Table S1-S5: In supplemental excel.

Table S1. Description of the cohort patients

Table S2. Immune markers measurement results

Table S3. Significance values for metabolites differentiating between ICU and ward

Table S4. Paired t-tests comparing a first and last time point of selected ward patients

Table S5. Spearman correlation coefficients and FDR Q values between metabolites and immune markers