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HDL proteome and apolipoproteins concentrations in severe ICU COVID-19 patients

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Abstract

Background SARS-CoV-2 infection affects both lipid metabolism and lung function. The severity of the disease has been associated with reduced levels of both high-density lipoprotein (HDL) and low-density lipoprotein cholesterol. Despite the crucial role that these nanoparticles play in SARS-CoV-2 infection, few studies have examined their structure during COVID-19 beyond HDL quantity.

The study aimed to assess apolipoprotein levels in COVID-19 patients who either survived or died following ICU admission. In addition, ICU survivors and non-survivors were compared for HDL particle size and proteome.

Methods Between February and April 2020, our study enrolled 37 COVID-19 patients upon their intensive care unit admission. Among them, 18 survived the disease, while 19 succumbed to it. We used mass spectrometry to assess plasma levels of 14 apolipoproteins and LCAT. Additionally, we analyzed HDL subpopulation distribution by utilizing native polyacrylamide gel electrophoresis. HDL particles were isolated from both surviving and non-surviving patients using ultracentrifugation, followed by characterization of their proteomes with NanoLC-MS/MS.

Results Plasma apolipoproteins, including Apo A-II, Apo Cs (I, II, III), Apo H, Apo J, Apo M, and LCAT, were decreased in patients who did not survive COVID-19. However, no alterations were noted in the distribution of HDL subpopulations in relation to mortality. HDL composition was further altered based on mortality, displaying a decline in Apo H and paraoxonase 3.

Conclusion In conclusion, we have shown an alteration in plasma apolipoproteins and HDL composition between surviving COVID-19 patients and non-survivors. Some markers, such as Apo H, are more predictive than baseline lipid concentrations such as HDL-C. These markers appear to provide a more accurate indication of mortality during COVID-19 compared with baseline lipid concentrations such as HDL-C.

Keywords COVID-19, Mortality, HDL particle, Apolipoproteome, Apolipoprotein, Proteomic, Intensive care unit, HDL subpopulation, HDL proteome

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Introduction

Coronavirus disease 2019 (COVID-19) pandemic, which started in Wuhan, China in December 2019, is caused by a Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) infection and has caused more than 6 million deaths worldwide [1, 2]. COVID-19 presents a broad range of symptoms and gravity, varying from ambulatory mild disease (including asymptomatic forms) to severe cases, that may result in systemic failure and death [3, 4]. Severe forms of COVID-19 are associated with a hyperinflammatory response marked by a cytokine storm resulting in excessive production of pro-inflammatory cytokines like monocyte chemotactic protein-3 (MCP-3), interleukin -1 (IL-1), and interferon α/β (IFNα/β). These three cytokines are closely associated with the severe form of COVID-19, although the cytokine profile of COVID-19 patients varies [5–7].

Lipoproteins are macromolecular complexes composed of apolipoproteins and lipids. Apolipoproteins regulate the lipoprotein structure, lipid transport, enzyme coactivation, and provide anti-inflammatory and antioxidant effects [8]. The apolipoproteins are classified into subfamilies, including Apo A, B, C, D, E, L, F, H, M, and (a). These proteins are distributed across various lipoproteins, including low-density lipoproteins (LDL) and high-density lipoproteins (HDL) [9, 10].

During a SARS-CoV-2 infection, the concentrations of total cholesterol, LDL-cholesterol (LDL-C), and HDL-cholesterol (HDL-C) are reduced and are linked to the severity of the disease [11–14]. Interestingly, numerous studies have also shown that lipid and lipoprotein metabolism are disrupted during the acute phase of SARS-CoV-2 infection, along with other illnesses [15].

Some apolipoproteins, including Apo A-I and Apo B100, can be assayed routinely like HDL-C, LDL-C, TC. Moreover, Apo A-I, A-II, and B are present in low levels in COVID-19 patients and are linked to an increased likelihood of developing severe forms of the disease [16]. In a previous study, our team found lower plasmatic concentrations of nine apolipoproteins in COVID-19 patients compared to controls [13].

Beyond quantitative changes, few studies have focused on the structure and function of HDL during COVID-19. Nonetheless, HDL possesses pleiotropic properties, including antioxidant and anti-inflammatory effects, in addition to its primary role in reverse cholesterol transport [11, 15, 17–19]. In a recent study of our team comparing COVID-19 patients versus controls, with an enrichment of acute phase proteins and a depletion of apolipoproteins, HDL particles proteome was profoundly altered in COVID-19 patients [20]. Moreover, in our in vitro analyses, we underlined that HDL from COVID-19 patients was much less endothelioprotective [20]. During SARS-CoV-2 infection, alterations in HDL functionality have been identified as a potential marker of disease severity. In particular, this study has demonstrated a significant impairment in HDL cholesterol efflux capacity, paraoxonase-1 (PON-1) arylesterase activity, and antioxidant properties. Furthermore, low HDL cholesterol efflux capacity has been associated with an increased risk of mortality in patients with SARS-CoV-2 infection, regardless of HDL-C concentrations [21].

Whereas previous studies mostly compared COVID-19 patients with controls, the aim of the present work was to compare apolipoprotein concentrations of deceased patients with those of patients who survived an episode of COVID-19 leading to severe form of the disease. The second objective of this work was to characterize HDL particles (size and proteome) at admission to ICU between deceased patients and survivors.

Materials and methods

Study population

We conducted an analysis with data from the prospective French COVID cohort (NCT04262921) [22]. The French COVID cohort includes hospitalized patients with a PCR-confirmed COVID-19 and who gave written consent. The study was sponsored by INSERM, conducted in accordance with the Declaration of Helsinki, and Ethics approval was given by the French Ethics Committee CPP Ile-de-France VI (ID RCB: 2020-A00256-33).

We selected adult patients from the French COVID cohort study, from February 1, 2020 until July 31, 2020 (corresponding to the first wave of pandemic in France). Pregnant women were excluded. None of the patients received corticosteroids. In this study, patients selected from the French COVID cohort had to meet the following criteria:—admitted in ICU;—at least 1 ml of plasmas for lipoprotein isolations and analyze;—not be on long-term statin therapy.

Quantification of lipoprotein subpopulation by tube gel electrophoresis (Quantimetrix Lipoprint)

Plasma relative proportions of HDL subpopulations were determined by polyacrylamide gel electrophoresis (PAGE) using Quantimetrix Lipoprint HDL (Quantimetrix Corporation, Redondo Beach, CA, USA). Lipoprint was performed and analyzed according to the manufacturer's protocol. Briefly, 25 µL EDTA plasma was mixed with 300 µL loading gel, and this sample gel was applied to the Lipoprint HDL gel tube before photopolymerization for 35 min at RT. Gel tube electrophoresis was performed at 3 mA/gel tube until albumin reached 1.5 cm at the end of the gel tube (± 50 min). After resting in the dark for 30 min, the tubes were scanned and analyzed using Lipoware HDL software. After tube analysis,

three main subpopulations were determined from the 10 subpopulations measured: large HDL (HDL-1 to HDL-3), intermediate HDL (HDL-4 to HDL-7) and large HDL (HDL-8 and HDL-10).

Lipoprotein isolation from plasma

All subsequent steps were performed under a Class II microbial safety bench. Lipoproteins were isolated from EDTA plasma by three-step ultracentrifugation. All ultracentrifugation steps were performed at 252,000 g for 20 h at 10 °C using ultracentrifuge tubes placed in a 50.4 Ti rotor in a Beckman Coulter Optima L-80 XP ultracentrifuge. For the first ultracentrifugation step, plasma was weighed to a density of 1.019 using a potassium bromide (KBr) solution with a density of 1.35. The weighted plasma was covered with a 1.019 density KBr solution in ultracentrifugation tubes. After ultracentrifugation, the upper fraction corresponding to VLDL was collected. For the second ultracentrifugation step, plasma was collected and weighted to a density of 1.063 using a KBr solution with a density of 1.35. The weighted plasma was covered with a 1.063 density KBr solution in ultracentrifuge tubes. After ultracentrifugation, the upper fraction corresponding to LDL was collected. For the final ultracentrifugation step, plasma was weighted to a density of 1.21 using a KBr solution with a density of 1.21. The weighted plasma was covered with a 1.21 density KBr solution in ultracentrifugation tubes. After ultracentrifugation, the upper fraction corresponding to HDL was collected. After isolation, lipoproteins were washed five times with saline EN buffer (0.15 M NaCl, 1 mM EDTA, 0.025% NaN3, pH 7.3) using a centrifugal filtration tube (Amicon Ultra, 3 kDa cut-off, UFC500396). Tubes were centrifuged at 14,000 g for 15 min at 4 °C, and the volume was concentrated to 100 µL. Lipoprotein proteins were then assayed by the bicinchoninic acid method (BCA, Sigma), and HDL purity was verified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie blue staining.

Fast protein Liquid Chromatography (FPLC)

HDL isolated from each patient was purified in an ÄKTA Pure FPLC system (GE Healthcare) on a SuperdexTM 200 Increase 10/300 GL column (10×300 mm; Cytiva) in EN saline buffer. HDL was loaded into a 500 µL loop and injected at a flow rate of 0.250 mL/min. Elution was then performed with Saline EN buffer at a constant flow rate of 0.300 mL/min, and fractions were collected every 500 µL. Finally, the column was washed with two column volumes at a flow rate of 0.700 mL/min.

Label-free quantification by Nano LC–MS/MS analysis

Label-free protein quantification was performed on HDL at the time of ICU admission from patients who survived their COVID-19 ($n=14$) and on HDL from patients who did not survive their severe COVID-19 ($n=15$).

HDL sample preparation and proteolytic digestion

The concentration of total HDL protein was measured using the BCA assay, with bovine serum albumin serving as the standard. As previously described [23], 5 µg HDL was combined with 25 µL digestion buffer (6 M urea, 50 mM ammonium bicarbonate) and denatured at 80 °C for 10 min for sample preparation. Dithiothreitol was used for sample reduction, followed by iodoacetamide alkylation. Upon alkylation, the samples were diluted with four volumes of 50 mM ammonium bicarbonate to reduce the urea concentration. The HDLs were digested with 3 µg of ditosylphenylalanyl chloromethyl ketone trypsin (TPCK-treated Sigma) overnight at 37 °C. Tryptic digestion was halted with 2% TFA and incubated at 4 °C for 15 min. Subsequently, samples were centrifuged at 14,000 g for 15 min at 10 °C and the desalted peptides in the supernatant were collected on peptide desalting spin columns (Thermo Fisher Scientific Pierce, part number 2162704). Peptides were eluted using a solution of 50% acetonitrile (ACN) and 0.1% trifluoroacetic acid (TFA), followed by drying with a Speed Vacuum. Then, 20 µL of 40% ACN with 0.1% TFA was used to elute the peptide, which was subsequently vacuum-dried at 60 °C prior to mass spectrometric analysis.

Nano LC–MS/MS method

The method was carried out with two solvents, solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in 80% ACN). Digested peptides were solubilized in 20 µL of 4% ACN with 0.1% TFA. Mass spectrometry analysis was performed by nano-LC on a Thermo Fisher Ultimate 3000 Series NCS-3500 RS coupled to a nanospray ionization (NSI)-Q-Orbitrap mass spectrometer (Q Exactive Plus, Thermo Fisher Scientific, Bremen, Germany) [20]. Briefly, 5 µL of samples were injected into a C18 LC-EASY-spray column (2 µm, 100 Å, 75 µm×50 cm, Thermo Fisher Scientific). Peptides were eluted on two linear elution gradients starting from 4 to 25% solution B (0.1% formic acid in 80% ACN) until 103 min, and then from 25 to 40% solution for 20 min. The column was then washed for 5 min with 90% solution B and equilibrated with 4% solution B for 20 min. Solution A was composed of 0.1% formic acid in water. To minimize contamination between samples, two blank runs containing solution A were conducted after every sample run. The flow rate was maintained at 0.300 µL/min, and the

column temperature was maintained at 40 °C. The NSI was set at 1.8 kV, the capillary temperature was set at 275 °C, and the S lens RF level was set at 30% for mass spectrometry parameters.

The mass spectra were obtained in positive ion mode using data-dependent acquisition within the m/z range of 350 to 2,000. Full scan mass spectra were acquired at a resolving power of 70,000 (at m/z 400) using automatic gain control (AGC) at a set value of 5e5 and a maximum injection time (Max IT) of 100 ms. MS/MS spectra of the top ten most intense precursor ions were acquired at a resolving power of 35,000 (at m/z 400) with an AGC set to 1e5 and a maximum IT of 100 ms. Peptide fragmentation was performed by setting the normalized collision energy to 28. Precursor ions were excluded dynamically for 60 s to prevent peptide selection repetition. The Orbitrap's performance was assessed weekly, and the mass spectrometer underwent external calibration before analysis using the LTQ ESI positive ion calibration solution (Pierce™). XCalibur 4.2.47 software (Thermo Fisher Scientific) was used to collect the data.

Protein identification and quantification

Protein identification and quantification were performed automatically from the raw files with Proteome discoverer software (version 2.2.2.0). MS and MS/MS spectra were compared to the Uniprot Human reference proteome database containing only canonical sequences (20,315 sequences; 01 July 2021) using the SEQUEST HT search engine. The following features were defined in SEQUEST HT for identification: variable modifications are methionine oxidation and N-terminal acetylation of proteins, then, a fixed modification sets the carbamidomethylation of proteins and the specific enzyme selected is trypsin with two miscleavage allowed. A mass tolerance of 10 ppm for precursor ions and 0.02 Da for-product ions was used in SEQUEST HT. A decoy database search strategy with target inversion was applied with a false discovery rate (FDR) set at 1% for proteins and peptides. Then, only proteins with at least one unique peptide with a Sequest Score (Xcorr) ≥ 1.5 were used and for Xcorr < 1.5, manual verification of peptide identification was performed on the MS/MS spectrum. Identified peptides were quantified by label-free using the peak intensity of a validated peptide (unique and razor) for a given protein. After quantification, all proteins with more than 60% missing value were not considered in the analysis. For proteins with $\leq 60\%$ missing values, they were replaced by the IQR of the corresponding proteins in the respective groups. The list of identified and quantified proteins and peptides is available in the supplemental table.

Data processing

Proteome Discoverer (version 2.2.2.2.0) was utilized for the computations. Protein abundance refers to the mean of the protein intensities observed in all samples among each group (COVID-19 survivors versus non-survivors). Protein intensity was defined as the sum of peptide intensities measured for each individual. All protein intensity values were subjected to logarithmic transformation (base 2). The log2 (Fold Change) is determined by taking the base 2 logarithm of the disparity between the average intensities of a specific protein in COVID-19 patients who did not survive and the average intensities of the same protein in COVID-19 patients who did survive.

Statistical analysis

The Shapiro–Wilk test was used to test the normality of the data distributions. Mann–Whitney test or unpaired t-test was then performed, as appropriate, to compare patients who survived COVID-19 with those who did not. A volcano plot was generated by Prism to evaluate differential proteins on the basis of two indicators: the -log10 *p*-value of the Mann–Whitney test and the log2 (protein abundance fold change of non-survivors and survivors). Marker performance was measured using receiver operating characteristic (ROC) curves. A *p-value* < 0.05 is considered to indicate a statistically significant result. Statistical analyses were performed using Prism software (GraphPad Software Inc., San Diego, CA, USA).

Results

Study population

Thirty-seven patients were included in this analysis. Survivors ($n=18$) and non-survivors ($n=19$) were matched based on age and gender. General characteristics of the patient population are presented in Table 1 and a flow chart is expressed on supplemental figure S1.

Comparison of plasma apolipoprotein concentrations between COVID-19 survivors and non-survivors

We used mass spectrometry to measure 14 apolipoproteins and LCAT directly from the plasma of COVID-19 survivors and non-survivors. The following apolipoproteins were measured: Apo(a), Apo As (A-I, A-II, A-IV), Apo B100, Apo Cs (C-I, C-II, C-III), Apo D, Apo E, Apo H, Apo J, Apo L1, and Apo M. The concentration of seven apolipoproteins was significantly reduced in patients who did not survive compared with those who did. These apolipoproteins include Apo A-II (4.68 ± 3.76 *vs.* 2.30 ± 1.51; $p=0.0147$), Apo Cs (C-I (2.29 ± 1.71 *vs.* 0.87 ± 0.62; $p=0.0060$), C-II (1.64 ± 0.99 *vs.* 1.00 ± 0.58; $p=0.0269$) and C-III (0.21 ± 0.18 *vs.*

Table 1 Characteristics and outcomes of the study population in ICU admission

Characterisitics	Study population ($n=37$)	Survivors (INC) ($n=18$)	Non-survivors ($n=19$)	Survivors (INC) vs Non-survivors *p-values*
Age, years, median [IQR]	61 [54; 68]	59 [51; 65]	61 [57; 68]	0.3047 (ns)
Male sex, n (%)	37 (89%)	16 (89%)	17 (89%)	> 0.9999 (ns)
Obesity	13 (35%)	6 (33%)	7 (37%)	> 0.9999 (ns)
BMI, kg/m2, median [IQR]	28 [24; 31]	26 [24; 30]	29 [25; 33]	0.3677 (ns)
High Blood pressure, n (%)	15 (41%)	5 (28%)	10 (53%)	0.1837 (ns)
Diabetes mellitus, n (%)	13 (35%)	5 (28%)	8 (42%)	0.4951 (ns)
Timing between fist symptoms and hospitalization (days), median [IQR]	8 [5; 9]	8 [5; 11]	7 [5; 9]	0.6781 (ns)
Medication				
Statin, n (%)	0	0	0	> 0.9999 (ns)
Corticosteroides, n (%)	0	0	0	> 0.9999 (ns)
Outcomes				
Time between hospitalization and death (days), median [IQR]	13 [8; 27]	N.A	13 [8; 27]	N.A
Clinical features				
TC, mmol/L, median [IQR]	3.45 [3.09; 3.91]	3.28 [2.81; 3.62]	3.62 [3.19; 4.24]	0.0426 (*)
HDL-C, mmol/L, median [IQR]	0.49 [0.40; 0.58]	0.50 [0.43; 0.58]	0.48 [0.40; 0.63]	0.8381 (ns)
LDL-C, mmol/L, median [IQR]	1.64 [1.37; 2.15]	1.98 [1.51; 2.26]	1.46 [1.34; 2.06]	0.1100 (ns)
TG, mmol/L, median [IQR]	1.74 [1.51; 2.06]	1.71 [1.52; 2.17]	1,75 [1.46; 1.95]	0.0554 (ns)
CRP, mg/L, mean [± SD]	136 [± 74.34]	123 [± 86.37]	146 [± 66.14]	0.4976 (ns)

BMI Body mass index, *CRP* C-Reactive Protein, *TC* Total cholesterol, *HDL-C* High-density lipoprotein-cholesterol, *LDL-C* Low-density lipoprotein-cholesterol, *TG* Triglycerides

0.09 ± 0.08; $p=0.0045$), Apo H (11.45 ± 3.45 *vs.* 9.28 ± 2.52; $p=0.0351$), Apo J (13.55 ± 3.99 *vs.* 11.03 ± 2.81; $p=0.0318$) and Apo M (2.90 ± 1.09 *vs.* 2.12 ± 0.63; $p=0.0112$). Furthermore, the concentration of LCAT ($p=0.0137$) is also significantly diminished in patients who did not survive COVID-19. Results are expressed in Fig. 1.

Modulation of HDL subpopulations size between survivors and non-survivors COVID-19 patients

The different HDL subpopulations size (large – intermediate – small) of COVID-19 patients were determined using the Lipoprint HDL tube gel electrophoresis technique. No difference was found between groups (Fig. 2). Similarly, when examining the different HDL subpopulations individually, no significant differences were found between the groups (Figure S2).

HDL proteome analysis of surviving versus non-surviving patients of their COVID-19

A first analysis of the MS/MS spectra by Proteome Discoverer software from the SEQUEST HT database identified 98 proteins that may be associated with HDL from either surviving or non-surviving patients (see Supplemental Table S2). After verification, our inclusion criteria (see Materials and Methods) indicate that 72 proteins are actually associated with patient HDL. To compare COVID-19 survivor and non-survivor groups, we conducted a fold change analysis of intensities associated with HDL proteins prior to log base-2 transformation (Fig. 3). Out of the 72 proteins identified, the relative abundance of 11 proteins was lower in non-survivor HDL as compared to control HDL. These proteins included alpha-1 antitrypsin (AAT), cathelicidin antimicrobial peptide, complement C4 (A, B), serotransferrin, apolipoprotein C-I, heat shock protein HSP90-beta, protein-glutamine gamma-glutamyltransferase E, centrosomal protein, and vitronectin. In contrast, HDL from non-survivors showed an increased abundance of nine proteins, including serum amyloid A-2, CD99 antigen, mitochondria-associated proteins, and cellular debris (Fig. 3).

The intensities of the 72 proteins associated with HDL in the patients were logarithmically transformed to base 2 to facilitate statistical analyses. The results indicate that HDL protein profile displays more significant alterations in non-surviving COVID-19 patients when compared to those who survived. Specifically, there were significant differences in the levels of 24 proteins in the HDL of non-survivors relative to COVID-19 survivors (Fig. 4A). The

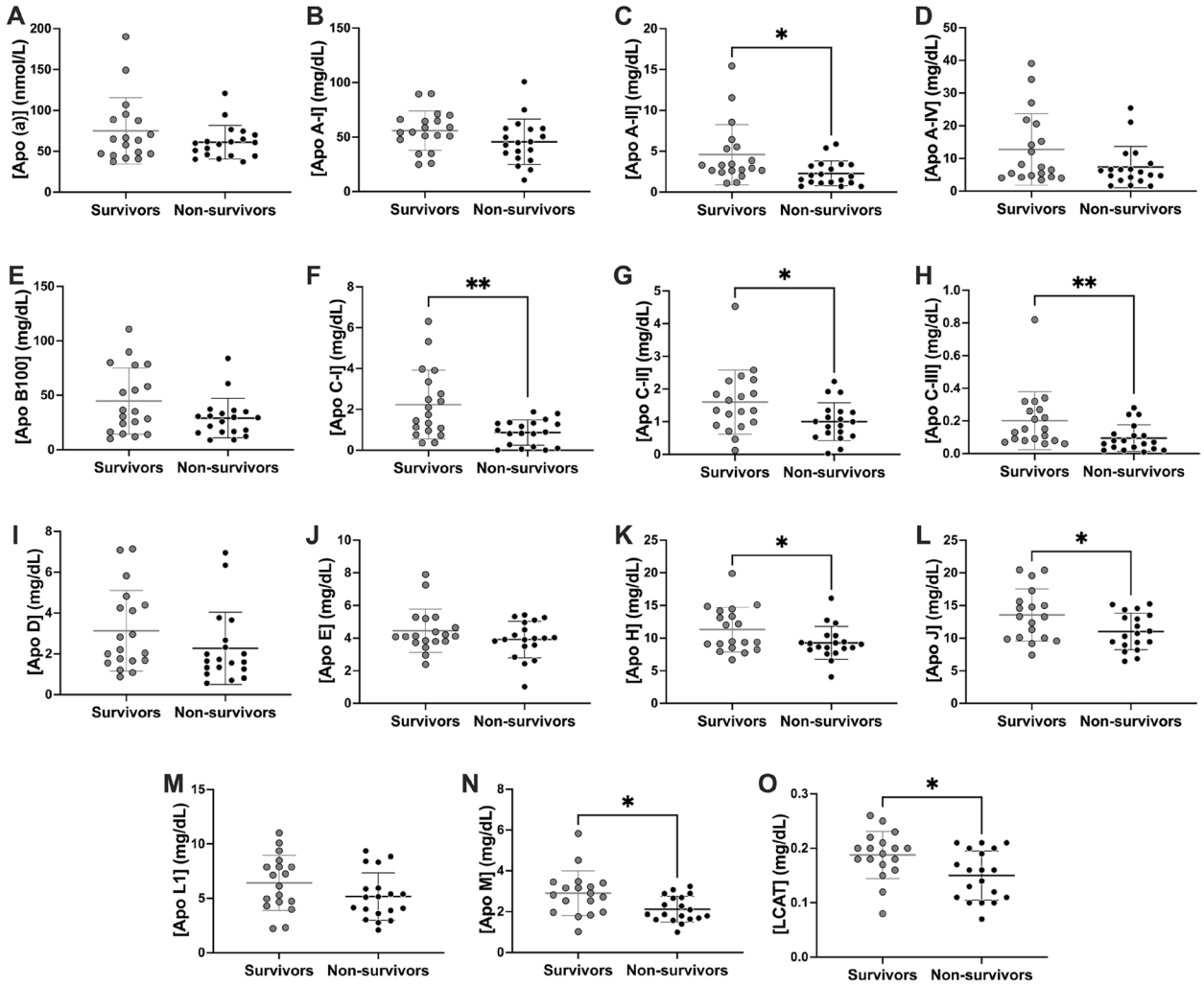

Fig. 1 Apolipoprotein changes in COVID-19 non-survivors in ICU. A panel of 14 apolipoproteins and LCAT (**A-O**) was quantified by mass spectrometry in the plasma of 18 surviving and 19 non-surviving COVID-19 patients. A Shapiro–Wilk normality test was performed to determine whether to use an unpaired t-test for parametric data or a Mann–Whitney test for nonparametric data. *$p < 0.05$, **$p < 0.01$

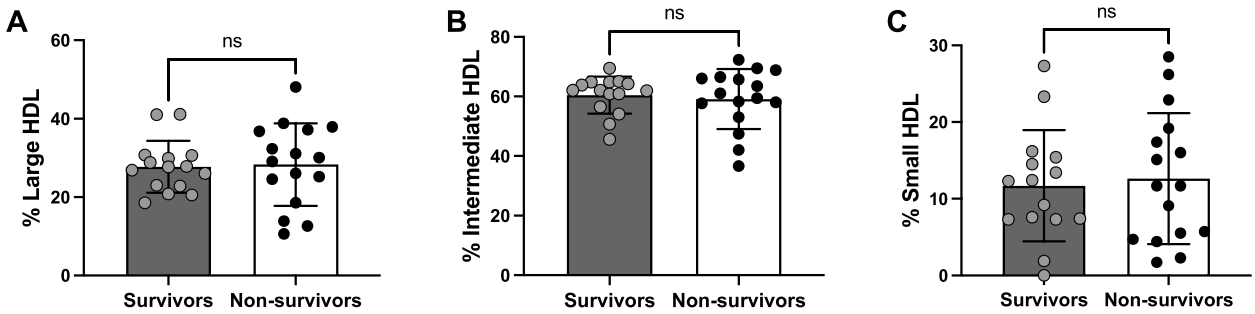

Fig. 2 HDL subpopulations remain unchanged in survivors and non-survivors. The distribution of HDL subpopulations (Large (**A**), Intermediate (**B**) and Small (**C**)) in 15 survivors (gray dots) and 16 non-survivors (black dots) were found to be identical. A Shapiro–Wilk test was conducted to verify the normal distribution of small, intermediate and large HDL, followed by an unpaired t-test for Large HDL and a Mann Whitney test for Intermediate and Small HDL. Each point indicates the percentage of cholesterol per patient. No significant differences were observed in comparisons between survivor and non-survivor groups ($p \geq 0.05$)

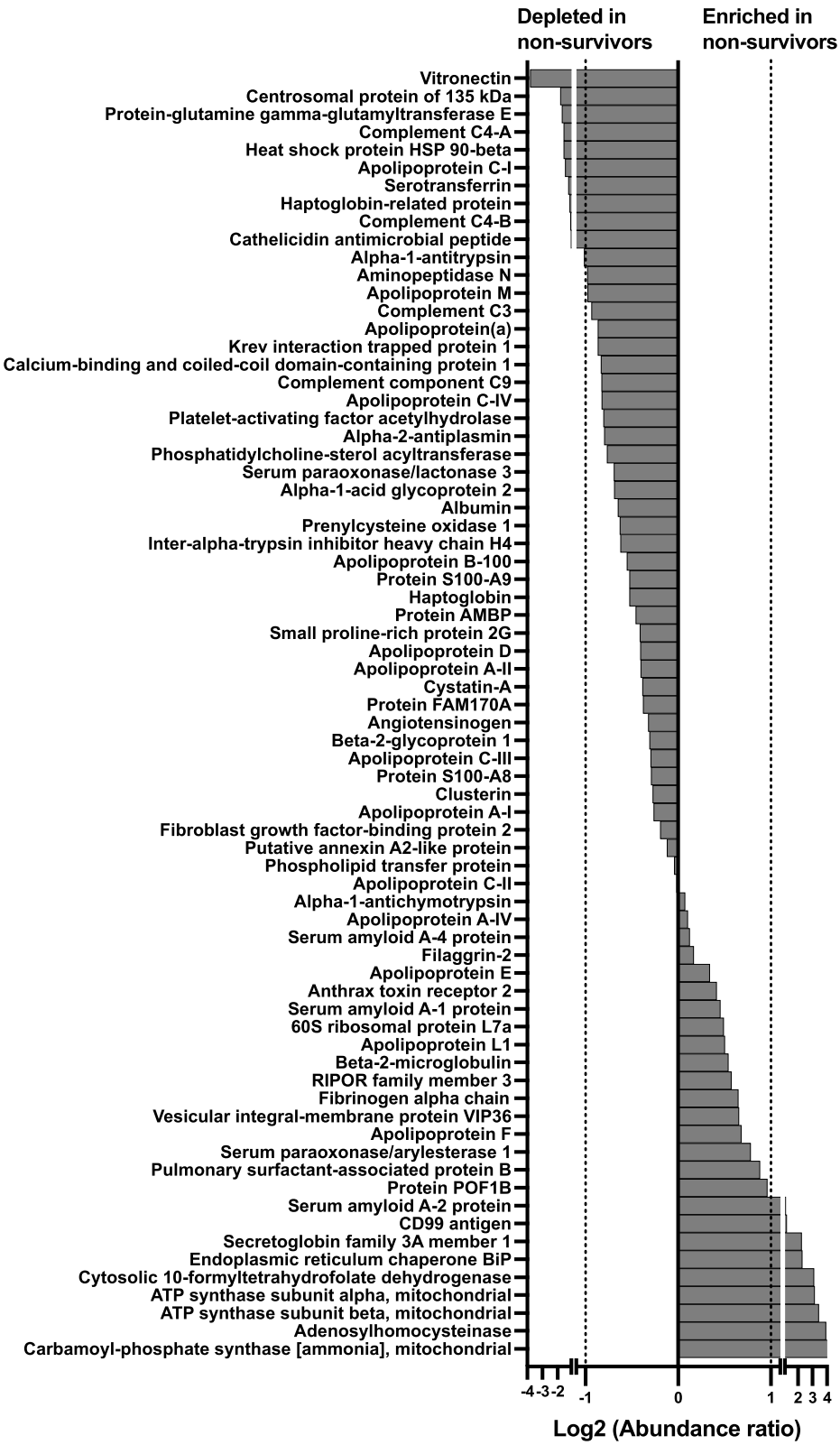

Fig. 3 Relative abundance of proteins identified in HDL isolated from plasma of COVID-19 surviving ($n=14$) and non-surviving ($n=15$) patients. A threshold of Log2(Abundance ratio) > 1 or < -1 is used to indicate statistical significance. Proteins with an abundance ratio meeting these criteria are considered to show significant differences between the two groups

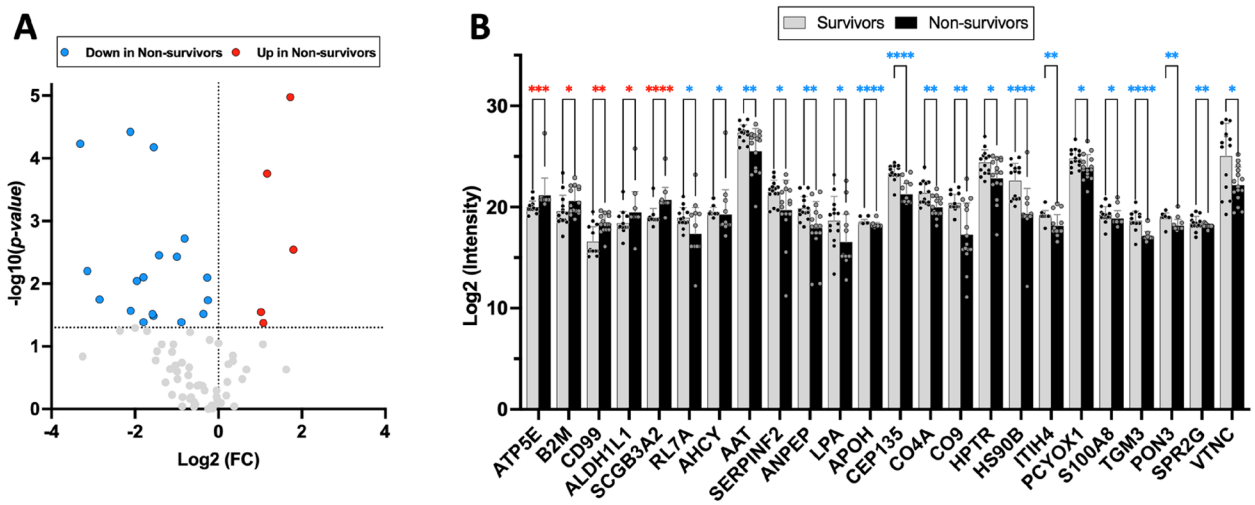

Fig. 4 Altered HDL proteome in COVID-19 patient non-survivors. **A** Volcano plot showing proteins differentially expressed in HDL from survivors ($n=14$) and non-survivors ($n=15$). Red points represent proteins with higher levels in non-survivor HDL, while blue points represent proteins with lower levels in non-survivor HDL. The y-axis represents -log10 (Mann–Whitney *p-value*) and the x-axis represents log2 (P.A.I fold change of non-survivors and survivors). **B** Protein abundance of HDL isolated from the plasma of the 14 survivors and 15 non-survivors of COVID-19. Mann–Whitney test was used to compare HDL protein abundance in survivors and non-survivors. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$

levels of 19 of these proteins were significantly lower in the HDL of the non-survivors. The mentioned proteins comprise ribosomal protein 60S L7a (RL7A), adenosylhomocysteinase (ALDH1L1), alpha-1-antitrypsin (AAT), alpha-2-antiplasmin (SERPINF2), aminopeptidase N (ANPEP), apolipoprotein (a) (LPA), apolipoprotein H (APOH), 135 kDa centrosomal protein (CEP135), complement C4-A (CO4A), complement component C9 (CO9), haptoglobin-related protein (HPTR), heat shock protein HSP 90-beta (HS90B), prenylcysteine oxidase 1 (PCYOX1), protein S100-A8, protein-glutamine gamma-glutamyltransferase E, paraoxonase serum/lactonase 3 (PON3) and vitronectin (VTNC). However, five proteins are significantly elevated in HDL from the non-surviving group. The proteins that have increased are ATP synthase subunit beta mitochondrial (ATP5E), beta-2-microglobulin (B2M), CD99 antigen (CD99), cytosolic 10-formyltetrahydrofolate dehydrogenase (ALDH1L1) and secretoglobin family member 1 3A (SCGB3A2) (Fig. 4B).

Diagnostic performance via receiver characteristic (ROC) curve

ROC curves were used to assess the diagnostic accuracy of diverse lipid markers (Fig. 5). Except for TC (AUC = 0.71 ± 0.09, $p=0.0418$), the regular lipid markers had areas under the curve (AUC) lower than 0.7 and a *p-value* over 0.05 (Fig. 5A-D). Plasma apolipoproteins and LCAT provide a AUC score of greater than 0.7 for all except Apo H (AUC = 0.68 ± 0.09, $p=0.0056$) and Apo J (AUC = 0.69 ± 0.09, $p=0.0483$), which are unreliable indicators of patient outcomes (Fig. 5E-L). Among the potential markers identified in HDL particles from COVID-19 patients are Apo H (AUC = 0.94 ± 0.06, $p<0.0001$) and the 135 kDa CEP135 protein (AUC = 0.91 ± 0.05, $p=0.0001$), all of which have shown significant effectiveness in predicting survival, with an AUC greater than 0.90. When using patient HDL proteins as markers, ATP5E, CO4A, PON3 and ITIH4 demonstrate effectiveness in predicting patient survival, while CO9 display lesser effectiveness with a lower AUC (Fig. 5M-T).

Discussion

In this present study, we have therefore shown that while lipid profiles on admission to ICU for COVID-19 cannot be used to stratify patients who died from those who survived, plasma analysis of certain apolipoproteins and proteomic analysis of HDL seem to be able to do so. Especially, APOH and CEP135 are potentially novel biomarkers for COVID-19 severity linked to HDL proteins.

Lipid metabolism plays a key role in SARS-CoV-2 infection. Several hypotheses have been proposed to explain the changes in lipid metabolism, including viral use and modification of lipids to support replication, hemodilution, and increased vascular permeability [24, 25]. Lowering cholesterol levels in lipoproteins is also aided by the cytokine storm [26]. However, while in bacterial sepsis HDL-C concentrations are associated with patient outcome and in particular mortality, these links were not found in COVID-19 [13, 14, 20, 27–31]. In this present study, we confirmed that HDL-C, LDL-C, and

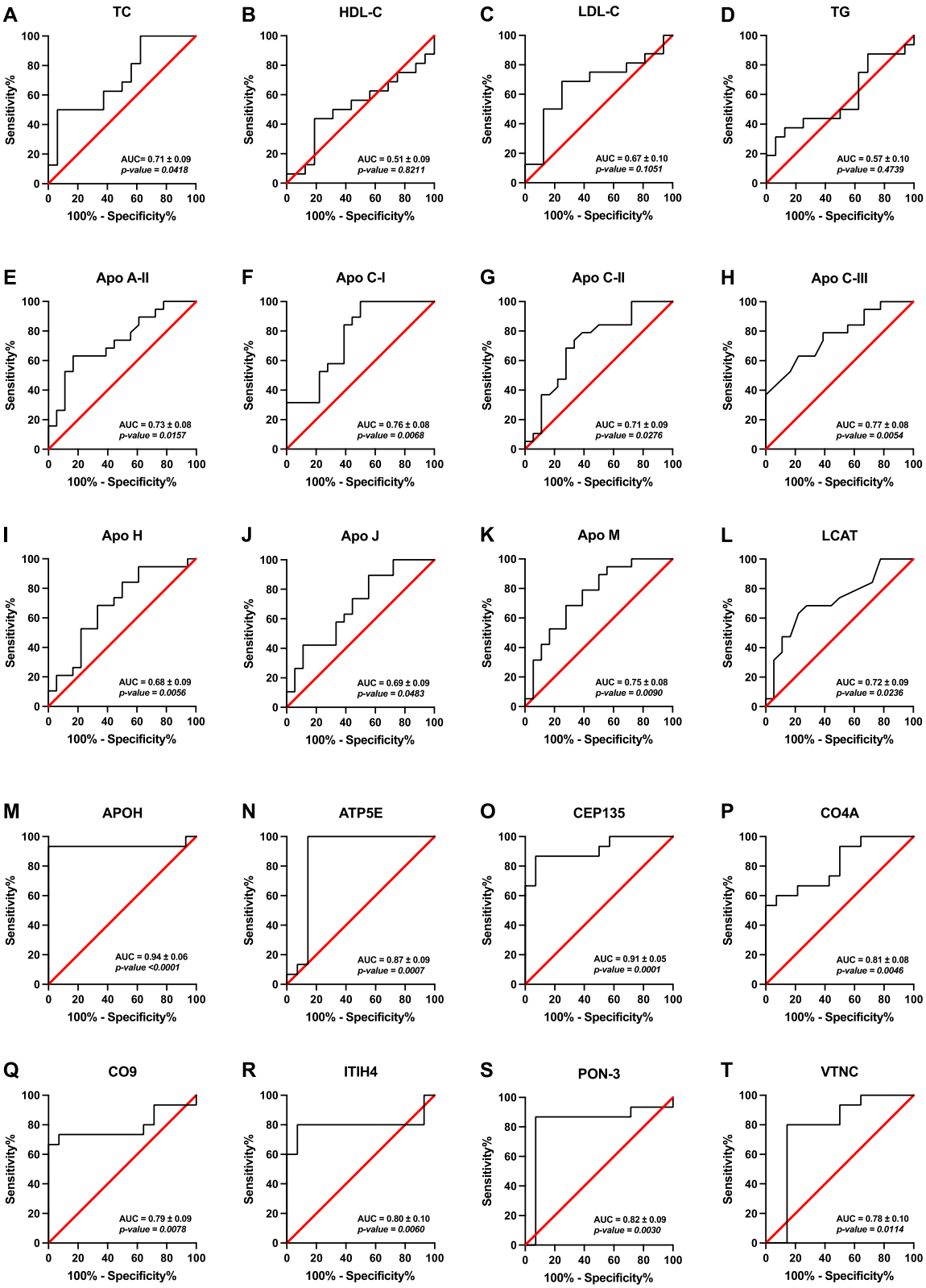

Fig. 5 Receiver Operating Characteristic (ROC) curve. ROC curve of the routine lipid markers (**A**-**D**). ROC curve of plasma apolipoproteins varying significantly between COVID-19 survivor vs. non-survivor groups (**E**-**L**). ROC curve of altered proteins in the HDL proteome of patients surviving vs. not surviving their COVID-19 (**M**-**T**). The red line corresponds to random classifier

TG concentrations are similar between survivors and non-survivors COVID-19 patients.

We have also shown that a crude concentration of HDL-C does not appear to be sufficient to stratify patients during COVID-19. Thus, the first step was to characterize the plasma apolipoproteins which are key regulators of lipid metabolism and have anti-inflammatory, antioxidant and endothelial protective properties [9, 32]. The mechanisms between apolipoproteins and the severity of COVID-19 are not yet fully understood. However, multiple studies have demonstrated a negative correlation between Apo A-I and Apo B levels and the severity of COVID-19 [16, 33, 34]. In our previous studies, we demonstrated that COVID-19 patients exhibit diminished levels of nine Apo forms, such as Apo A-II, C (I, II), H, J, M, as well as LCAT, alongside elevated levels of Apo E as compared to controls [13]. Our study confirms the previous one and highlights several apolipoproteins that are significantly more reduced on admission to ICU in patients who will die secondarily. Most of these apolipoproteins are linked to reduced macrophage function [35]. Cholesterol efflux by macrophages induces production of Apo C through Liver X Receptor (LXR) activation [36]. Reduced Apo C concentrations indicate a decrease in macrophage cholesterol efflux function.

Interestingly, we observed a decreased in Apo A-II but not in Apo A-I concentrations among non-survivors of COVID-19 when compared to survivors. This observation differs from most of the studies reporting essentially a decrease in Apo A-I concentration with an association with worth outcome [33, 34]. This difference can be attributed to the sample size, which is significantly larger than ours. Moreover, corticosteroid therapy modifies lipid profile. Our findings show a trend towards reduced Apo A-II concentrations. Our previous studies, however, observed apo A-II concentrations in both HDL and plasma of COVID-19 patients [13, 20]. Additionally, other research has also seen lower Apo A-II levels in COVID-19 patients [35, 37]. This decrease in Apo A-II suggests a potential alteration in cholesterol efflux, as well as a decrease in the anti-inflammatory and antioxidant properties of HDL [38, 39].

It is interesting to note that ApoB100 and LCAT were associated with mortality in our last study [13] but not in this work. The role of Apo H, also known as beta-2-glycoprotein 1, is not well understood. According to earlier research [40], it has been observed that Apo H levels decrease in COVID-19 patients. This decrease in Apo H could potentially create a prothrombotic environment in COVID-19, leading to antiphospholipid syndrome. This decrease in Apo H could potentially create a prothrombotic environment in COVID-19, due to an antiphospholipid syndrome. Furthermore, Apo H seems to capture the SARS-CoV-2 virus, leading to the hypothesis that the virus utilizes Apo H [41]. Clusterin, also known as Apo J, acts as an inhibitor of the complement system [42]. Reductions in the concentration of Apo J led to complement system activation, which then causes a prothrombotic environment [43, 44]. Our study indicates also that Apo M levels were decreased in COVID-19 patients who did not survive. A study conducted by Marfia et al. [45] showed that the concentration of sphingosine-1-P (S1P) was closely related to the concentration of Apo M and was able to accurately predict the prognosis of COVID-19 patients. Our findings reaffirm that Apo M can serve as a potential marker for assessing COVID-19 severity. Furthermore, Apo M depletion is associated with loss of anti-inflammatory and anti-atherogenic functions of S1P/ApoM complex [46, 47]. Therefore, deficiency of these apolipoproteins promotes an overall thromboinflammatory environment [48].

The size of lipoproteins can be significantly altered in response to acute infections such as that caused by the SARS-CoV-2 virus. A pilot study using nuclear magnetic resonance (NMR), conducted by Ballout et al., demonstrated a marked reduction in high-density lipoprotein (HDL) particles in patients infected with SARS-CoV-2, suggesting significant remodeling of the lipid profile under the effect of the disease [49]. In the present study, the Lipoprint gel tube technique was employed for the analysis of HDL [50]. Although this method is effective in identifying structural changes in HDL, it lacks the benefit of reference values, which may limit inter-study comparisons. However, this technique has already been used to assess changes in the distribution of HDL subpopulations in the context of sepsis, where an increase in large HDL and a reduction in small HDL have been observed [51]. In contrast to the observations made in sepsis, our analysis of patients with COVID-19 in the intensive care unit revealed no significant differences in HDL size between survivors and non-survivors. This lack of variation may indicate that the acute inflammation associated with SARS-CoV-2 infection causes global, homogeneous HDL remodeling, thereby making it difficult to detect specific differences between groups. These results suggest that changes in HDL size are related more to the generalized inflammatory state than to patient survival.

Prior research, most notably that of Davidson et al. [52], has identified a multitude of proteins associated with HDL particles, underscoring their involvement in a myriad of biological processes, including lipid transport, inflammatory response, and coagulation. Among these proteins, several have been identified as being modulated in pathological contexts. In the present study, a significant alteration in the protein profile of HDL was observed in non-survivor patients with COVID-19, with

a decrease in 19 proteins and an increase in five proteins. These proteins, which have previously been associated with HDL, were also identified in our previous studies of patients with severe forms of the infection [20]. This reinforces the hypothesis that perturbations in the HDL proteome contribute to the pathophysiological mechanisms observed in severe forms of the infection. Among the proteins increased in the HDL of COVID-19 patients, the CD99 antigen plays a key role in the T-cell adhesion process. CD99 is mainly localized on T lymphocytes and endothelial cells. The level of this particular protein is lower in COVID-19 patients with severe symptoms, indicating reduced endothelial integrity and recruitment of monocytes, neutrophils and T cells [53]. A hypothesis to explain its increase in HDL from non-survivors of COVID-19 is that it is captured by the latter. HDL levels in non-survivors are found to be enriched with mitochondrial ATP synthase subunit beta, which interacts with the ecto-F1-ATPase receptor on the cell surface [54]. SARS-CoV-2 is able to interact with ecto-F1-ATPase and ATP synthase [55, 56]. An elevated beta-2-microglobulin has been identified as a potential biomarker of COVID-19 severity [57, 58], which may explain the elevated levels in the HDL of non-surviving COVID-19 patients. We observed an increase in PON-3 in the HDL of non-surviving COVID-19 patients. Although PON-3 is less abundant than PON-1 [59], most studies have focused on the latter paraoxonase. Indeed, our previous study [20], have demonstrated a decrease in PON-1 levels in the HDL of COVID-19 patients. Furthermore, PON-1 activity has been associated with antiviral properties against SARS-CoV-2 [60], highlighting its potential role in the immune response to this infection. These observations suggest that although PON-3 may be increasing, the decrease in PON-1 may have significant clinical implications for the pathophysiology of COVID-19. Interestingly, the HDL proteins of non-survivors of COVID-19 exhibit a decrease in AAT, I'TIH4, Complement C4A, and C9, which are normally increased in plasma as COVID-19 severity increases. This increase could be explained by an increase in the compounds and proteins of the acute phase complement and a decrease in the proteins involved in the coagulation cascade [61, 62]. Among the coagulation-related proteins, the levels of beta 2 glycoprotein 1 (Apo H) are reduced in the HDL of COVID-19 non-survivors, suggesting a disturbance in the coagulation cascades [35, 62]. A previous study examining HDL remodeling as a function of COVID-19 severity revealed a decrease in Apo M in the HDL proteome, as well as an increase in serum amyloid proteins (SAA) [63]. However, in our study, these differences were not observed. It is possible that the selection of our patients, all of whom were admitted to the ICU, reflects a more advanced or systemic alteration of HDL lipoproteins, which could mask some of the specific variations observed at less critical stages of the disease. This more profound alteration of the lipid profile could explain the lack of correlation with previously reported results. The remodeling of high-density lipoprotein in patients during the COVID-19 pandemic suggests a loss of high-density lipoprotein (HDL) functionality [20].

Our study is the first to investigate LDL and HDL distribution changes during COVID-19 using tube gel electrophoresis (Lipoprint) among patients who survived or did not survive the disease. The distribution of lipoproteins was similar regardless of patient outcome. COVID-19 brings about a modification in the lipoprotein subpopulations' distribution. This change is characterized by a reduction in the concentrations of small, intermediate, and large subpopulations of HDL and large LDL, accompanied by an escalation in small and dense LDL concentrations [49].

Finally, routine lipid markers in our study demonstrated poor accuracy in assessing disease severity. It is surprising to observe this in light of the association between HDL-C and disease severity [64]. In contrast, apolipoproteins have superior performance to routine lipid assays in assessing patient outcome. This is expected since various studies have shown that apolipoproteins are decreased in COVID-19 [13, 24, 33–35].

The primary obstacle we encountered was the small number of available samples, which hindered our ability to conduct experiments on a representative sample of the population under study. Nevertheless, our preliminary results are promising. This study is focused on a modest sample of 37 patients during the initial wave of COVID-19, providing supplementary information to the current knowledge on COVID-19. Another limitation is the low levels of HDL volume of patients included in our study. It would be pertinent to evaluate the lipoproteins' functionality to ascertain the effect of changes in their proteome. Further comparative studies carried out in various centers can help confirm and validate the conclusions presented in our research.

In conclusion, in a cohort of patients hospitalized in ICU for severe COVID-19, we demonstrated major differences in plasma apolipoproteins and in protein composition of HDL by comparing surviving and non-surviving patients. These markers, measured on admission to ICU, also appear to be more predictive of mortality than BASIC lipid concentrations such as HDL-C. Nevertheless, these results should be interpreted with caution given the small number of patients included in this study. In this context, it is important to conduct a high-powered multicenter study in the future.

Supplementary Information

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Supplementary Material 1.

Supplementary Material 2.

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Authors' contributions
OM and ST designed the study. FB, BV acquired the data. FB, OM, ST analyzed the data. FB, ST, OM wrote the manuscript. PR, OM, ST, AF and SJ critically reviewed the manuscript. All authors reviewed the manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Competing interests
The authors declare no competing interests.

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