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Oxidative Stress and Lipid Profile During Acute Phase of COVID-19 Infection and After Recovery: Evidence of a Sequel in LDL

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Abstract

This study aimed to investigate the atherogenicity (quality) of LDL particles in patients with acute and recovered from COVID-19 infection. The participants were adults, aged 18 years or older of both sexes. Those with positive RT-PCR results at baseline were included in the Acute COVID-19 group (n = 33), and those with negative RT-PCR six months after acute infection, were included in the Recovered COVID-19 group (n = 30). The LDL quality was evaluated using three validated methods: Z-scan, UV–visible spectroscopy, and Lipoprint system. The Recovered COVID-19 group showed significantly higher numbers of large LDL particles (less atherogenic) than the Acute COVID-19 group ($P < 0.05$). We also found that COVID-19 infection was associated with the oxidative modification of LDL particles. D-dimer and CRP levels were correlated with Z-scan results and antioxidant-amount estimate. Moreover, we noticed that the infection left a sequel in LDL quality, even after six months of recovery. These findings highlight the importance of monitoring lipids during and after recovery from COVID-19 infection, and their potential deleterious effect on the LDL profile might correlate with the progression of atherosclerosis and poor clinical outcomes.

Keywords COVID-19 · Modified LDL · Z-scan · UV–visible spectroscopy · Lipoprint

1 Introduction

COVID-19 and cardiovascular diseases (CVD) share many similar metabolic pathways. Common events observed in CVD, such as thrombosis, dyslipidaemia, inflammation, and oxidation, potentially exert negative impacts on the clinical prognosis of infected COVID-19 patients [1]. Despite worldwide success of vaccine for COVID-19, the transmission and treatment of the COVID-19 long-term morbidities remain a challenge for health system in many countries.

Lipids play a crucial role in viral infection by fusing the viral membrane to the host cell, replicating the virus, and enabling viral endocytosis and exocytosis [1]. Furthermore, viruses like SARS-COV-2 are enveloped by lipid bilayers. Regarding that dyslipidaemia is a common cardiovascular risk factor in about 28%−39% of patients with CVD [2, 3]. Numerous studies have shown that patients with COVID-19 infection have lower levels of total cholesterol, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and apolipoprotein B (Apo B) and AI (Apo AI) [4–10].

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Although some studies showed that patients under lipid-lowering therapy did not show better clinical outcomes than normal or hypercholesterolemic patients [11–13], a recent systematic review with meta-analysis found that statin therapy in-hospital leaded a significant reduction of all-cause mortality in COVI-19 patients [14]. The role of statin on cardiovascular disease risk is not limit to its lipid-lowering effect, but anti-inflammatory, antioxidant and immunomodulatory effect. The last three can impact directly on viral replication [15].

Lipoproteins are complex structures containing different lipids, proteins, density, molecular weight, and minor components (antioxidants, for example, tocopherols and polyphenols) that define their functionality [16, 17]. Therefore, characteristics of lipoproteins other than lipid content may be involved in COVID-19 infection and prognosis. Despite that, the relevance of the quality of lipoprotein in COVID-19 patients were not described yet. It was shown that small and dense LDL ($_{SD}$LDL) particles are more atherogenic than large and dense LDL ($_{LD}$LDL) subfractions [18]. $_{SD}$LDL migrates more quickly to the subendothelial layer, where it associates with proteoglycans undergoing additional oxidative modifications and is uptaken by macrophages [19]. Therefore, qualitative aspects of lipoproteins can be important for COVID-19 infection; however, this needs to be confirmed.

In previous in vitro [20] and in vivo [21–24] studies, we have shown that the nonlinear optical Z-scan experimental technique [25] gives complementary information about the atherogenic profile of the LDL. To the best of our knowledge, the quality (defined hereafter as the better the quality of the LDL, the less atherogenicity of particle) of LDL in COVID-19 patients remains an interesting issue, mainly when different stages of the disease are considered.

Based on this, our aim was to investigate the lipid profile and the quality of LDL particles using three validated methods (Z-scan, UV–visible spectroscopy, and the Lipoprint system), which can improve the traditional lipid profile investigation in patients infected by SARS-CoV2. For that, we monitored independent acute and recovered COVID-19 adult patients who attended a public community hospital in a low-middle-income area in São Paulo, Brazil.

2 Materials and Methods

2.1 Study Population and Design

The study population was derived from a community-prospective Cohort of COVID-19 patients (n = 445) with flu-like symptoms, including more severe cases of pneumonia. These patients were attended in the emergency department care (between February 2020 and March 2021) from a community public secondary hospital, Hospital Universitario (HU) from the University of São Paulo, located in the Butantan region, a low-middle income area in the western region of São Paulo city (Brazil). The Fig. 1 describes the flowchart of study.

All subjects of both sexes and aged 18 years or older with positive COVID-19 confirmed by RT-PCR (n = 445) at hospital admission were potentially considered for inclusion in the present study. Of the 445 patients with acute infection due to COVID-19, 90 had plasma available for additional analysis; however, 57 were excluded due to insufficient

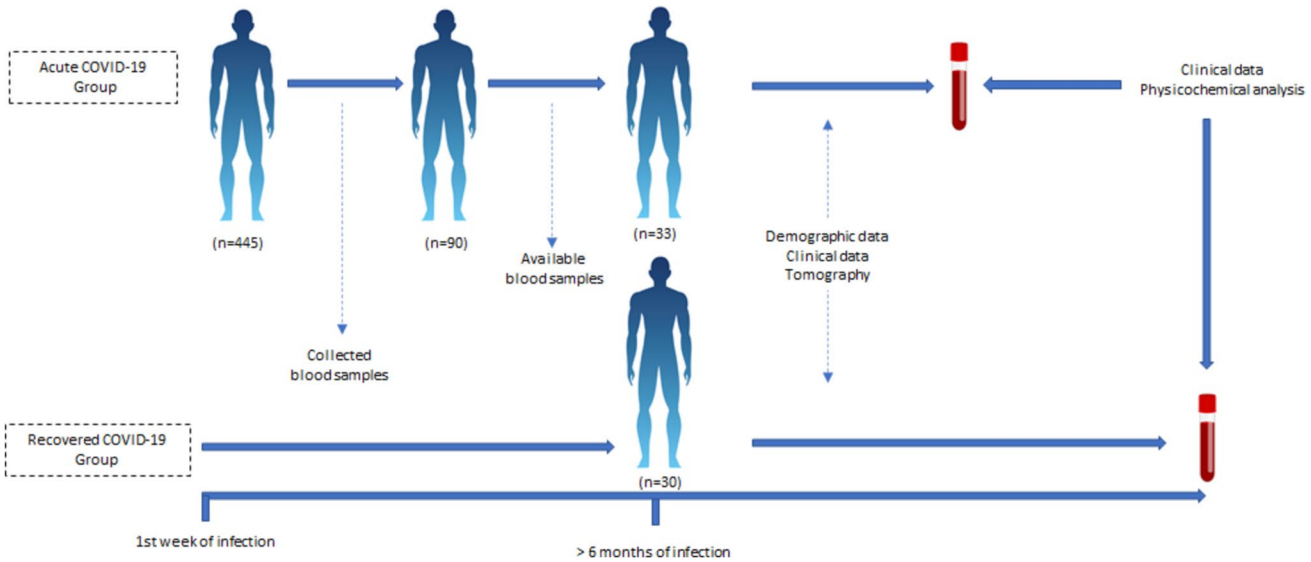

Fig. 1 Flowchart of study

material to analyze LDL quality. Thus, 33 individuals had their LDL quality evaluated during the acute phase (Acute COVID-19 group). Additionally, 30 paired individuals by sex, age and lipid-lowering medication had their lipid quality evaluated six months after acute infection (Recovered COVID-19 group). Therefore, for both groups we enrolled a non-probabilistic sampling of individuals with positive RT-PCR to COVID-19 at acute and recovered phases. Local Research Ethics Committees approved the study (Hospital Universitário, Universidade de São Paulo-HU/USP; CAAE: 59,599,722.9.0000.0076). All procedures followed the ethical principles for medical research involving human subjects as stated in the Declaration of Helsinki and only were performed after the participants signed the informed consent agreement.

2.2 Clinical Characteristics of Individuals

At baseline, clinical information was obtained by direct interview performed by a physician. For Recovered COVID-19 group—6 months after discharge, the patients' clinical characteristics were obtained via telephone and direct interviews performed by a trained interviewer. We collected data on sociodemographic and clinical characteristics (sex, age, race, smoking status, alcohol intake, and previous clinical comorbidities such as diabetes mellitus, cardiovascular diseases, severe acute respiratory syndrome, stroke, subarachnoid hemorrhage, and chronic kidney disease). Regular lipid-lowering drugs were collected from medical records and direct interviews.

2.3 Blood Samples and LDL Purification

Blood samples were collected at hospital (Acute COVID-19 group) or at home (Recovered COVID-19 group) after 12h of fasting in EDTA-2Na tubes, and plasma was obtained after centrifugation (3000 rpm, 4°C for 15 min). Protease inhibitors (10 µg/mL aprotinin, 10 µM benzamidine, and 5 µM phenylmethylsulphonyl fluoride—PMSF) and antioxidant (100 µM butylated hydroxytoluene – BHT) were added to the plasma, and samples were maintained at −80°C until analysis. LDL was obtained from plasma by preparative sequential ultracentrifugation (40,000 rpm, 4°C for 18h) equipped with a fixed-angle rotor (Hitachi Himac CP 70MX, Tokyo, Japan). The total protein level in LDL was determined using a bicinchoninic acid (BCA) protein commercial assay kit (Pierce, Rockford, IL, USA), with bovine serum albumin as the standard. More details about the LDL separation can be found in our previous articles [22, 23, 26]. All experiments were performed by validated methods and instructions of manufacturers for standard and commercial kits.

2.4 Biochemistry Analysis

The concentration of total cholesterol (TC), HDL-C, and triglycerides (TG) was determined using a colorimetric assay using the following kits: Cholesterol Liquiform®, Cholesterol HDL®, and Triglycerides Liquiform®, respectively (Labtest, Minas Gerais, Brazil). The content of cholesterol in LDL was calculated using the formula proposed by Friedewald [27]: LDL-C = (TC – HDL-C) – (TG/5). Additionally, the C-reactive protein (CRP) level was measured by nephelometric technology (BN IITM System, Siemens Healthineers, Germany) and the D-dimer level by a fully automated coagulation analyzer (Siemens Sysmex® CS-2500), following the manufacturer's instructions.

2.5 Lipids Analyses

2.5.1 Z-scan Technique

In order to measure the nonlinear optical properties of LDL samples (1.0 mg/dL), the Z-scan technique was used. A focused Gaussian laser beam, with a wavelength of 532 nm, was used to illuminate the LDL samples. When converting light energy into heat, the sample forms a thermal lens. Several factors determine the strength of a thermal lens, including the thermo-optic coefficient, linear absorption coefficient, and thermal conductivity. A temperature of 37°C was used for all the Z-scan experiments. The amplitude of the thermal lens θ is a dimensionless parameter that measures the strength of the thermal lens within LDL samples and is related to the peak-to-valley amplitude measured from the normalized transmittance as a function of the z-position of the sample characteristic curve [25]. In the Z-scan setup, a mechanical chopper modulated the light intensity with a square pulse (30 ms pulse width). The samples were scanned around the focal point, along the z-direction. The intensity of the transmitted light was measured as a function of the z-position of the sample. The thermal lens strength and, consequently, the LDL particle modifications are proportional to the peak-to-valley amplitude of the characteristic curves.

2.5.2 UV–visible Spectroscopy

In the UV–visible spectroscopy, the linear optical absorption of the sample is measured. The linear absorbance spectra of LDL samples were obtained using quartz cuvettes with a 1 cm optical path placed in the UV–visible spectrophotometer. The absorption of the sample was calculated by removing the Rayleigh scattering from the extinction spectra, measured with the spectrophotometer. LDL particles are composed of different molecules which absorb light in particular wavelengths. ApoB-100, cholesterol, α-tocopherol, and phospholipids absorbance mainly

range from 200 to 300 nm. The wavelength of 484 nm corresponds to one of the broad peaks of the absorbance of carotenoids (allowing the antioxidant-amount estimate), which extends to higher wavelengths, reaching 532 nm, used in our Z-Scan experiments. Under these experimental conditions, we are able to evaluate the amount of carotenoids in the LDL particles. All the UV–visible spectroscopy measurements were performed at 37°C.

2.5.3 Lipoprotein Subfractions Analysis

The LDL subfractions were determined using the Lipoprint® System (Quantimetrix Corporation). First, 25 µL of plasma and 200 µL of a gel containing lipophilic dye were pipetted for LDL analysis. After homogenization (7x), the sample were applied to the polyacrylamide gel underwent photopolymerization (30 min), followed by electrophoresis in an electrophoresis buffer. The bands show the relative amount of lipoprotein particles per sample in decreasing order of particle size. One VLDL band, IDL A, B, and C, and seven LDL subfractions were obtained from the LDL subfraction kit. LDL-1 and 2 were classified as larger and less dense particles ($_{LD}$LDL). Small dense particles ($_{SD}$LDL) were identified from the sum of the LDL-3 to 7 subfractions. After the application of the cut-off point based on total LDL size, phenotype A ($\geq$ 26.51 nm—less atherogenic) and phenotype B ($<$ 26.5 nm—atherogenic) were identified. All results are expressed as a percentage of the area under the curve, adjusted by the total cholesterol content. HDL subfractions were also analyzed in the same system. HDL-1 to HL-3 as named as $_{LD}$HDL, HDL-4 to HDL-7 as named as $_{ID}$HDL and HDL-8 to HDL-10 as named as $_{SD}$HDL.

2.6 Statistical Analysis

The statistical strategy was based in primary (differences in new markers—Z-scan, UV–visible spectroscopy, and lipoprotein subfractions) and secondary (traditional markers – lipid profile) outcomes in Acute and Recovered groups. The median and interquartile ranges (IQR), or mean and standard deviation ($\pm$ SD), were used to express continuous variables according to their distribution. The normality of the data distribution was examined using the Shapiro–Wilk test. Depending on the distribution of a variable, the Mann–Whitney U test or t-test was used. The Chi-square and Fisher's exact tests were used to compare categorical variables. Spearman's rank test was used to analyse correlations between continuous variables. Statistical analyses were performed using OriginPro 2021 software. Statistical significance was defined as P-value $<$ 0.05 for all analyses.

3 Results

3.1 Clinical and Biochemistry Parameters

Table 1 shows the results of the clinical parameters of all patients. The mean time of recovering was 26.8 months (min. = 10 months – max. = 32 months), in which only 4 (16%) were two or more reinfection episodes during this period of time. Between 6–12 months of follow-up, we observed that 24% (n = 8) patients in Acute group death. Individuals in both the Acute and Recovered COVID-19 groups had similar profiles, except for D-dimer (1,362; IQR = 579–2,201 ng/mL versus 656; IQR = 451–1,529 ng/mL; P = 0.0248), respectively. Together, CRP and D-dimer levels in both groups indicated high cardiovascular risk at baseline time. More than 60% of patients in both groups presented SARS (P = 0.41) during acute phase. Although 24% (n = 8) of patients in Acute COVID-19 group and 3% (n = 1) in Recovered COVID-19 needed ICU admission, only 9% (n = 3) and 3% (n = 1), respectively, were submitted to tracheal intubation. Almost 67% of the computed tomography analyses in both groups were compatible with COVID-19 infection, in which more than 48% and 37% of lung were affected in Acute and Recovered groups, respectively. Tiredness/shortness of breath (60%) were the most frequent symptoms in Acute COVID-19 group and similar profile was cited by 27% patients in Recovered group after 10 months of discharge.

3.2 Lipid Profile

Total cholesterol in Acute COVID-19 group was significantly lower than Recovered COVID-19 group (TC = 200.7; IQR = 185.9–213.1 mg/dL) versus TC = 215.4; IQR = 208.0–220.2 mg/dL; P = 0.002), and similar characteristics were observed for LDL-C (101.6 $\pm$ 24.4 mg/dL versus 115.6 $\pm$ 18.1 mg/dL; P = 0.021). Triglycerides (TG = 138.5; IQR = 93.5–173.1 versus 109.3; IQR = 73.9–171.4 mg/dL; P = 0.121) and HDL-C (70.4 $\pm$ 21.6 versus 72.3 $\pm$ 11.3 mg/dL; P = 0.693) showed similar profile between the Acute and Recovered COVID-19 groups, respectively (Fig. 2). Additionally, non-HDL levels in both groups were similar (Acute group = 109.6 $\pm$ 64.7 mg/dL and Recovered group = 123.4 $\pm$ 37.7 mg/dL; P = 0.067).

3.3 Z-scan and UV–visible Results

Figure 3 shows a patient's typical UV–visible spectroscopy and Z-scan results from the Acute and Recovered

Table 1 Characteristics of individuals at baseline and after 6 months, according to the Acute and Recovered COVID-19 groups

	Baseline		> 6 months		
	Acute COVID-19 group (n = 33)	Recovered COVID-19 group (n = 30)	Recovered COVID-19 group (n = 30)	P*	P**
Age, years (mean, SD)	60 ± 19	53 ± 15	-	0.101	-
Sex, male (n, %)	21 (63)	13 (43)	-	0.451	-
Race, white (n, %)	25 (75)	20 (66)	-	0.890	-
Smoking (n, %)	2 (6)	3 (10)	-	0.662	-
Alcoholism (n, %)	1 (3)	0 (0)	-	1.000	-
Physical activity, yes (n, %)					
Less	-	-	15 (60)	-	-
Similar	-	-	3 (12)	-	-
High	-	-	7 (28)	-	-
Changes in medications (n, %)					
Lipid-lowering	-	-	7 (28)	-	-
Anti-hypertensive	-	-	11 (44)	-	-
Anti-hyperglycaemia	-	-	9 (37.5)	-	-
SARS	28 (84)	20 (66)	-	0.411	-
SAH	12 (36)	13 (43)	-	0.952	-
Comorbidities (n, %)	20 (61)	18 (60)	18 (60)	0.998	1.000
DM	9 (27)	6 (20)	6 (20)	0.933	1.000
Stroke	1 (3)	0 (0)	0 (0)	1.000	1.000
Dyslipidaemia	1 (3)	0 (0)	7 (28)	1.000	**0.032**
CVD	1 (3)	0 (0)	0 (0)	1.000	1.000
COVID-19 infection (n, %)					
≤ 1	33 (100.0)	30 (100.0)	26 (84)	1.00	0.076
> 1	0 (0.0)	0 (0.0)	4 (16)	-	-
CRP (mg/L)	92 (49–195)	87 (56–175)	-	0.840	-
D-dimer (ng/mL)	1,362 (579–2,201)	656(451–1,529)	-	**0.025**	**-**
Sequaeles, yes (n, %)					
Tiredness/Shortness of breath	20 (61.0)	16 (53.3)	6 (27.3)	0.067	**0.001**
Forgetfulness/Dizziness	3 (9.0)	0 (0.0)	5 (22.7)	0.089	**0.048**
Anosmia	6 (18.0)	7 (23.3)	2 (9.1)	0.124	**0.042**
Others	33 (100.0)	30 (100.0)	3 (13.6)	**0.244**	**< 0.010**

Results are shown in median (IQR), n (%) or mean ± SD. *DM*, Diabetes Mellitus; *SARS*, severe acute respiratory syndrome; *SAH*, subarachnoid haemorrhage; *CVD,* cardiovascular diseases; *CRP*, C-reactive protein. Categorical variables were compared using the Chi-square test or Fisher's Exact test, and continuous variables using Mann–Whitney test or T-test according to the normality distribution. Significant level adopted ($P < 0.05$). *Acute COVID-19 group vs Recovered COVID-19 group at baseline. **Acute COVID-19 group vs Recovered COVID-19 group after 6 months.

COVID-19 groups. The Acute COVID-19 group shows smaller light absorbance peaks at the visible wavelengths (Fig. 1a) and smaller peak-to-valley amplitude (Fig. 1b) when compared to the Recovered COVID-19 group.

In Fig. 4a, the box plot presents the measurements of the phase shift θ from the Acute and Recovered COVID-19 groups. We observed a significant difference ($P < 0.001$) in the median value in the Acute COVID-19 group (median $\theta = 0.011$; IQR = 0.004–0.017) in comparison to the Recovered COVID-19 group (median $\theta = 0.027$; IQR = 0.014–0.039). Figure 4b shows the box plot for optical absorbance at wavelength 484 nm in both groups. Median values of this parameter are also significantly lower in the Acute COVID-19 group (median absorbance = 0.076; IQR = 0.054–0.147 *versus* median absorbance = 0.195; IQR = 0.121–0.315; $P < 0.001$) than in the Recovered COVID-19 group.

Figure 5a-b shows a significant negative correlation between CRP level and carotenoids estimated by absorbance at wavelength 484 nm ($r = -0.58$; $P < 0.001$), and phase shift θ ($r = -0.37$; $P = 0.042$) for the Acute COVID-19 group. CRP level was consistently correlated with

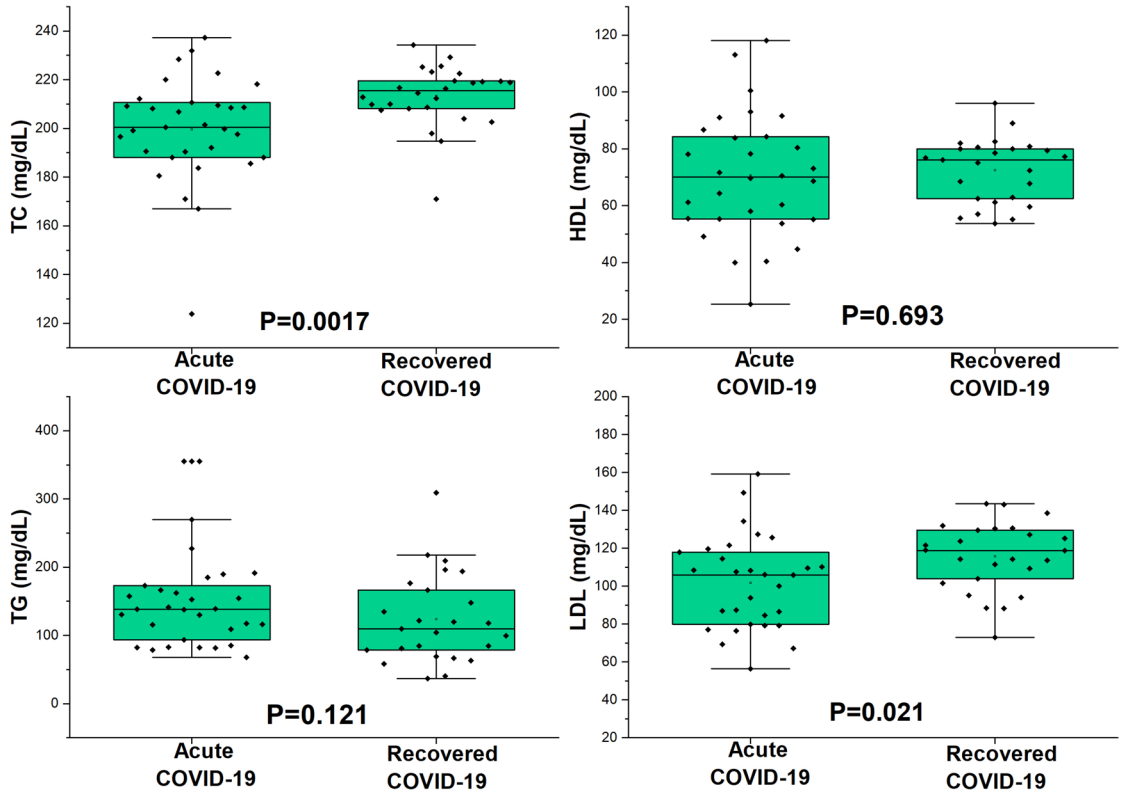

Fig. 2 Acute and Recovered COVID-19 groups lipid profiles (TC, HDL, TG, and LDL)

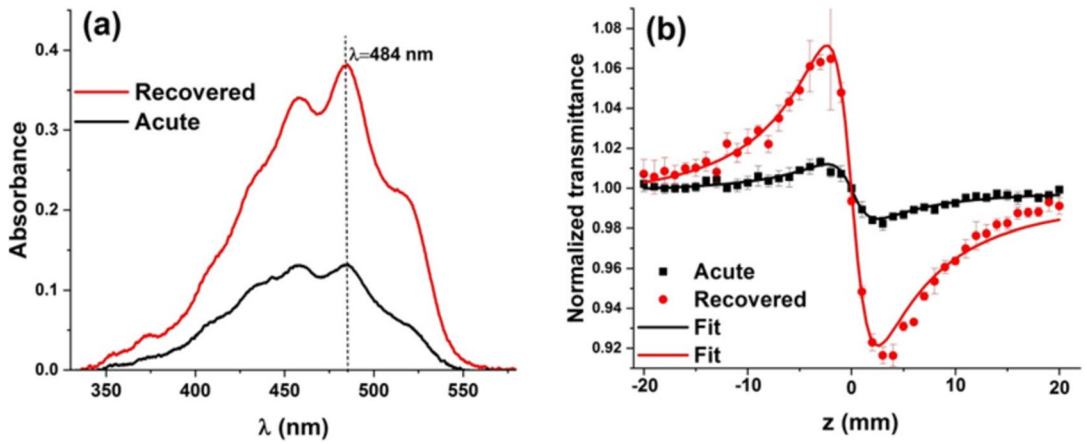

Fig. 3 The mean of UV–visible results (**a**), with the absorbance as a function of wavelength; and Z-scan results (**b**), with the normalized transmittance as a function of the sample z-position from Acute and Recovered COVID-19 groups

fibrinogen in Acute COVID-19 (r = 0.826; p = 0.001) and Recovered groups (r = 853; p = 0.007). Figure 5c shows a significant negative correlation between D-dimer and carotenoids estimated in the Acute COVID-19 group (r = −0.36; P = 0.039). For the Acute and Recovered COVID-19 groups (Fig. 5d), a significant positive correlation between the phase shift (θ) and carotenoids estimated (r = 0.84; P < 0.001) was observed. The lipid profile was not correlated with θ and carotenoid levels in the LDL particles. Additionally, we found a significant positive correlation between age and CRP level (r = 0.42; P = 0.019) in the Acute COVID-19 group; and a significant negative correlation between age and phase shift θ (r = −0.59; P < 0.001).

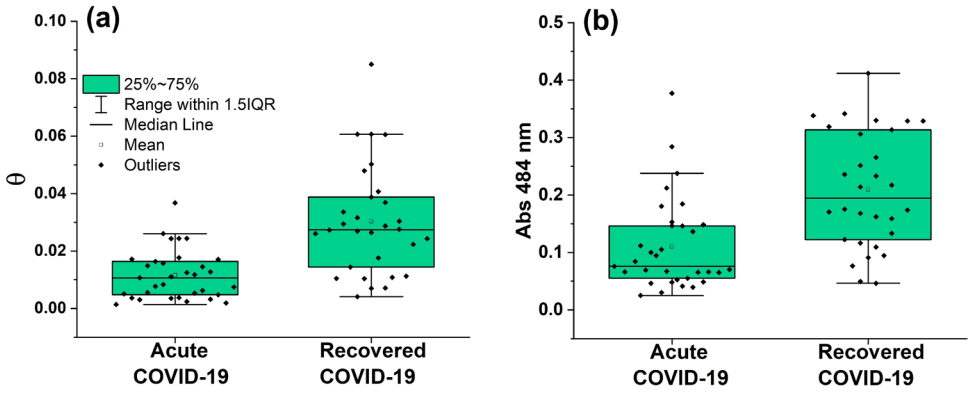

Fig. 4 Box plot showing the significant differences between the Acute and Recovered COVID-19 groups for (**a**) phase shift θ (**a**) and absorbance at wavelength 484 nm (**b**)

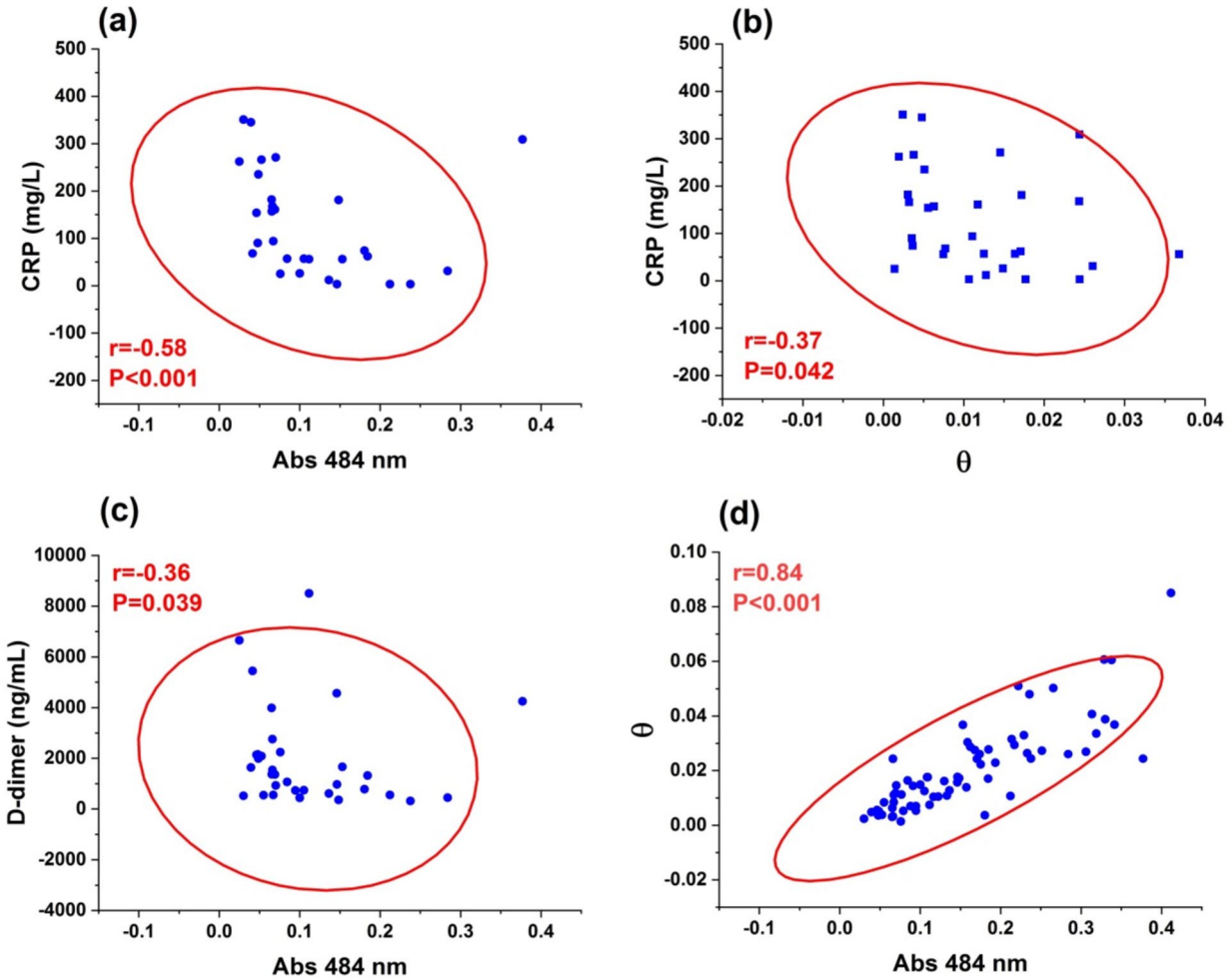

Fig. 5 Correlation between (**a**) CRP level and antioxidant-amount estimate (absorbance at wavelength 484 nm), (**b**) CRP level and phase shift θ, (**c**) D-dimer level and antioxidant-amount estimate, and (**d**) phase shift θ and antioxidant-amount estimate

3.4 Lipoprotein Subfractions Analysis

The results of LDL subfraction analysis are shown in Table 2. The Recovered COVID-19 group showed significantly higher values for $_{LD}$LDL (% and mg/dL) (P = 0.025 and P = 0.040, respectively), corresponding to LDL-1 (P = 0.009) and LDL-2 (P = 0.008) when compared to the Acute COVID-19 group. The mean LDL size did not differ between the Acute and Recovered COVID-19 groups (P = 0.336), impacting similar phenotype A (43% versus

Table 2 Low-density lipoprotein subfractions, according to the Acute and Recovered COVID-19 groups

Variables	Acute COVID-19 (n = 33)	Recovered COVID-19 (n = 30)	P
VLDL (%)	25.4 (0.0–35.5)	27.8 (0.0–58.4)	0.875
IDL-C (%)	15.0 (7.2–22.2)	13.5 (5.7–28.1)	0.124
IDL-B (%)	6.8 (0.2–15.7)	8.2 (0.0–22.7)	0.417
IDL-A (%)	5.7 (0.0–11.7)	6.4 (0.0–19.8)	0.099
LDL-1 (%)	14.1 (2.6–26.5)	17.0 (2.0–31.7)	0.266
LDL-2 (%)	8.9 (0.0–23.0)	12.9 (0.0–24.7)	**0.008**
LDL-3 (%)	1.8 (0.0–9.4)	3.1 (0.0–12.5)	0.060
LDL-4 (%)	0.0 (0.0–4.7)	0.0 (0.0–8.4)	0.261
LDL-5 (%)	0.0 (0.0–1.2)	0.0 (0.0–5.0)	**0.046**
LDL-6 (%)	0.0 (0.0–2.2)	0.0 (0.0–2.2)	0.502
LDL-7 (%)	0.0 (0.0–30.6)	0.0 (0.0–11.0)	0.657
$_{LD}$LDL (%)	23.1 (2.6–39.0)	29.9 (2.0–50.2)	**0.025**
$_{SD}$LDL (%)	3.2 (0.0–32.8)	4.8 (0.0–25.1)	0.153
$_{LD}$LDL/ $_{SD}$LDL	7.9 (0.3–37)	11.5 (0.2–50)	0.291
VLDL (mg/dL)	53.6 (26.4–71.9)	58.8 (0.0–117.5)	0.324
IDL-C (mg/dL)	25.9 (7.3–59.7)	28.5 (60.1–13.5)	0.340
IDL-B (mg/dL)	13.2 (3.9–26.2)	17.6 (0.0–51.9)	0.089
IDL-A (mg/dL)	10.6 (4.0–55.0)	13.8 (0.0–45.0)	0.147
LDL-1 (mg/dL)	23.9 (2.5–58.2)	35 (4.5–70.1)	**0.009**
LDL-2 (mg/dL)	24.6 (0.0–47.6)	27.1 (0.0–53.2)	0.489
LDL-3 (mg/dL)	8.1 (0.0–39.8)	6.3 (0.0–28.1)	0.548
LDL-4 (mg/dL)	0.0 (0.0–13.9)	0.0 (0.0–17.8)	0.753
LDL-5 (mg/dL)	0.0 (0.0–3.4)	0.0 (0.0–10.7)	**0.039**
LDL-6 (mg/dL)	0.0 (0.0–0.0)	0.0 (0.0–4.3)	0.195
LDL-7 (mg/dL)	0.0 (0.0–8.5)	0.0 (0.0–25.2)	**0.029**
$_{LD}$LDL (mg/dL)	49.4 (2.5–94.6)	60.9 (4.5–108.3)	**0.040**
$_{SD}$LDL (mg/dL)	8.3 (0.0–53.7)	10.1 (0.0–53.6)	0.595
$_{LD}$LDL/ $_{SD}$LDL	5.9 (0.2–21.8)	11.9 (0.2–54.0)	**0.051**
LDL size (nm)	267 (205–276)	266 (201–275)	0.336
Phenotype A (%)	15 (45)	11 (36)	0.996

Variables are expressed as median (min–max) and n (%). Variables compared between groups using two samples T-test, Mann–Whitney tests or qui-square test. Bold numbers: significant difference ($P < 0.05$).

41%; P = 0.996). Although $_{SD}$LDL was similar between groups, individuals in the Recovered COVID-19 group had higher LDL-5 (% and mg/dL), (P = 0.046 and P = 0.039, respectively) and LDL-7 (mg/dL) (P = 0.029) than the Acute COVID-19 group. The $_{LD}$LDL to $_{SD}$LDL ratio in Recovered COVID-19 group was higher, suggesting positive net quality in individuals after at least 10 months after acute phase. The $_{LD}$LDL to $_{SD}$LDL ratio showed a tendency to high in Recovered COVID-19 group (P = 0.051). In Recovered COVID-19 group, the $_{LD}$LDL to $_{SD}$LDL ratio (% and mg/dL) were negatively correlated with CRP (r = −0.517; p = 0.020 and r = −0.563; p = 0.010, respectively) (data not shown).

Lipoprotein subfractions and size were not correlated with θ and the carotenoid estimate; however, a positive correlation between D-dimer and LDL-2 (r = 0.35; P = 0.043) was observed in the Acute COVID-19 group. Moreover, this LDL subfraction was positively correlated with CRP level (r = 0.37; P = 0.044).

4 Discussion

COVID-19 and lipid metabolism have an interesting relationship that can modify the disease's acute phase and prognosis. Here, we expand the state-of-the-art addition to traditional lipid profile, data on quality of LDL subfractions, using validated and innovative methods.

Our results showed that the TC and LDL-C levels in the Recovered COVID-19 group were higher than those observed in the Acute COVID-19 group, confirming previous studies on the hypocholesterolaemia response associated with COVID-19 infection [1, 4–7]. During the acute phase of infection, the intense viral replication requires cholesterol for the synthesis of new cell membranes [1]. Cholesterol is the main energy source for many viruses, including COVID-19 [1, 28]. The study by Fan et al. [5], based on a similar design but including lipid profile prior to the COVID-19 infection, described lower TC and LDL-C levels during the acute phase. Interestingly, in the same study, patients with poor outcomes had lower lipid levels than those observed in the previous stages of infection. Regarding the close relationship between COVID-19 and lipid levels, the potential impact of lipid-lowering drugs has been hypothesized. According to Gil and Ambrose [29], the intensity of COVID-19 infection can be reduced in individuals with lower TC levels. Type of medication and time under therapy can affect the lipid profile and its relationship with COVID-19 infection. Patients in our study were under lipid-lowering medication, but the frequency of these drugs was similar in both groups. Furthermore, the impact of lipid-lowering in quality of lipoprotein is not clear in literature. The clinical protocol used for patients in our study not preview statin use. Therefore, the significant differences observed in quality of LDL in our study cannot be explained by statin therapy.

Changes in lipid levels are part of an inflammatory storm typical of COVID-19 and a widespread event in other viral infections related to SARS [4]. Although we did not perform a wide cytokine panel, the CRP and D-dimer levels used to monitor acute inflammation response and thrombotic risk, respectively, confirmed the proinflammatory status of the Acute COVID-19 group. We observed that these markers did not correlate with both groups' TC and LDL-C levels. The inflammatory process is a positive stimulus for oxidative stress in different molecules, including LDL particles [30–32]. For the last twenty years, we [23, 33, 34] and other

groups [35, 36] have investigated the relationship between modified LDL levels and diseases using different methods. For the first time in the literature, we demonstrated that COVID-19 infection is associated with oxidative modification of LDL using the nonlinear optical Z-scan technique (measuring the amplitude of the thermal lens formed when the LDL interacts with the light from a laser – θ) and antioxidant-amount estimate, in addition to the changes in classical lipid markers. In fact, the Recovered COVID-19 group, when compared with individuals during the acute COVID-19 phase had higher θ values and antioxidant-amount estimate. A positive correlation between them confirmed this complementary profile. Inflammatory processes and oxidative stress may play a relevant role in the severity of COVID-19 [37]. Coronavirus-infected host cells produce more free radicals during infection, which results in severe inflammation [38, 39]. The lipid molecules present in LDL are oxidized by these toxic free radicals [10, 40].

Previously, we demonstrated in a transversal study that patients with acute periodontitis had improved LDL quality after treatment, as evaluated by increased θ in the Z-scan results [24, 26]. More recently, we observed that diabetes patients under nutritional supplementation based on green banana biomass (rich in fibers and antioxidants) showed a significant improvement in antioxidant-amount estimate [22]. Low levels of lutein/zeaxanthin, α- and β-carotene, and total carotenoids are strongly related to higher oxidative stress and inflammation [41, 42]. Despite the potential mechanisms related to and promisors' results [43, 44], there is a gap in controlled and randomized clinical trials testing the supplementation of antioxidant nutrients for the prevention and treatment of COVID-19.

In order to broaden our understanding of the relationship between lipids and COVID-19, we monitored individuals according to LDL subfractions in addition to the oxidative aspects of LDL. The Recovered COVID-19 patients had higher $_{LD}$LDL than those with acute COVID-19 did. Large, dense LDL are less atherogenic than smaller LDL because they transport fewer oxidized lipids and migrate less to the subendothelial space [45, 46]. Additionally, large LDL have high α-tocopherol content and lipid peroxidation evaluated by TBARS, as previously demonstrated, suggesting a non-atherogenic profile compared to smaller particles [47]. LDL subfractions were not significantly correlated with θ and antioxidant-amount estimate; however, LDL-2 subfraction and oxidized LDL were independently correlated with inflammation (CRP) and prothrombotic event risk (D-dimer). Based on this, we propose that the Z-scan technique and the Lipoprint test can be used as adjuvant methods to understand better the cardiovascular disease risk of COVID-19 in patients with unbalanced lipid metabolism. Small dense LDL was investigated in the Multi-Ethnic of Atherosclerosis (MESA) and The Atherosclerosis Risk in Communities (ARIC) Study and associated with increased cardiovascular risk [48, 49]. Small LDL was also found to be involved in the inflammatory pathway in other outcomes, such as diabetes patients [50]. In the context of COVID-19, it is essential to remember that some patients evolve to thrombotic events; however, the specific risk factors for that are unclear. Previously, a case–control study including acute ischemic stroke (AIS) matched by healthy individuals verified that an adverse lipoprotein subfraction profile ($_{SD}$LDL and $_{SD}$HDL) was a predictor of stroke and mortality [51], and based on that, we speculate that LDL subfractions can improve the traditional estimate of stroke risk in COVID-19 patients. Small LDL (% and mg/dL) were similar in both the Acute and Recovered COVID-19 groups; however, when we evaluated smaller LDL, the LDL-5 and LDL-7 in Recovered COVID-19 group were lower than those in the Acute COVID-19 group, suggesting a lower thrombotic risk.

A striking finding of our study is that the quality of LDL particles is higher in patients after recovery. In the Recovered COVID-19 group, there is a significant increase in the value of the parameter θ, concerning the value from the Acute COVID-19 group. This result reveals that the LDL particles from patients in the Recovered COVID-19 group are less modified and better protected against oxidation. This last conclusion comes from the higher number of carotenoids in LDL particles evaluated in patients in the Recovered COVID-19 group.

At this point, an interesting question could be proposed: Is there a sequel due to COVID-19 infection concerning the quality of the LDL particles in patients diagnosed with severe COVID-19? To shed some light in this direction, one possibility is to compare our present values of θ with those obtained from a group of "healthy" individuals (no diabetes mellitus, no history of cardiovascular diseases, no hypertension, and no smoking), which were investigated in a previous study [24]. As this study was conducted in 2009, the selected individuals were not infected with SARS-CoV-2. The amplitude of the parameter θ (median) from healthy individuals, normalized to the same experimental conditions of the present study, is $\theta_H = 0.047$ (0.028–0.078). The same parameters for the Acute COVID-19 and Recovered COVID-19 groups are $\theta_A = 0.011$ (0.004–0.017) and $\theta_R = 0.027$ (0.014–0.039), respectively. The difference between these three groups is also significant ($P < 0.001$, Kruskal–Wallis ANOVA test, post hoc Dunn's test (θ_A versus θ_H; $P < 0.001$ and θ_R versus θ_H; $P = 0.024$)). These numbers bring interesting information about the quality of the LDL particles among these three groups: $\theta_A < \theta_R < \theta_H$. Since the quality of the LDL particles in patients recovered from severe COVID-19 infection, after 6 months from the PCR-RT negative result, did not reach the typical value of that characteristic of healthy individuals, a

sequel of the infection seems to be present. The follow-up of these patients with time may inform about their recovery for this aspect.

This study has some limitations. First, the cross-sectional design not allow to establish a causality relationship between quality of LDL and clinical parameters. Despite that, both groups were paired by demographic and clinical data. Second, multivariable regression models were not tested because some relevant variables not full fill statistical assumption such as collinearity, correlations ($p > 0.2$) and/or for small sample size. Third, although all comparisons were based in adequate statistical tests, we cannot avoid the false-positive p-values that can occur in a simple comparison analysis. Although of the innovative and validated methods applied represent strengths of this study and expand the current view about COVID-19 and cardiovascular disease risk, the reduced sample size needs further confirmation in a large population and therefore, current results must be interpreted with caution. Furthermore, the simultaneous investigation of traditional and emerging markers of lipoprotein quality expands the classical approaches applied to COVID-19 and cardiovascular disease risk.

5 Conclusions

In conclusion, the quality of LDL particles in COVID-19 patients improved after recovery. The significant increase in physical parameters (θ and absorbance at 484 nm) after recovery of COVID-19 patients and the increasing large LDL subfraction observed by Lipoprint experiment supports this finding. Moreover, comparing our present results with those from previous experiments, we noticed that the infection left a sequel in the quality of LDL, even after 6 months of recovery. These findings highlight the importance of monitoring lipids during and after recovery from intense inflammatory states, such as COVID-19 infection, due to potential deleterious effects in the LDL profile that might corroborate the progression of atherosclerosis, resulting in poor clinical cardiovascular outcomes.

Author Contributions Z.L. Made the absorption, Z-scan measurements, and data analysis. Participate in the discussion of the results and write the manuscript. Performed the statistical analysis. L.S.N. Participate in the absorption and Z-scan measurements, collection of blood, separation of the LDL, and discussion of the results. A.F.M. Participate in the design of the experiment and collection of blood separation of the LDL. M.C.P.F. and F.C.C. Performed the Lipoprint measurements and participated in the blood collection. A.C.V. Collected data and built the dataset, approved the final version. P.A.L. Developed the design and concept of the study, dataset supervision, and approved the final version of the manuscript. A.C.G. Participate in the design of the experiment, responsible for the clinical data and cohort. Participate in the data analysis and writing of the manuscript. N.R.T.D. Participate in the design of the experiment, responsible for the Lipoprint experiments, discussion of the results, and writing of the manuscript. Participate in the data analysis and writing of the manuscript. J.B.A. Participate in the collection of blood samples, design of the experiments, and cohort. R.A.F. Participate in the collection of blood samples, design of the experiments, and cohort. A.M.F.N. Responsible for the absorption and Z-scan experiments, participated in the design of the experiment, discussion of the results, and writing of the manuscript.

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

Declarations

Institutional Review Board Statement The study was approved by the local Research Ethics Committees (Hospital Universitário, Universidade de São Paulo-HU/USP; CAAE: 59599722.9.0000.0076). All procedures followed the ethical principles for medical research involving human subjects as stated in the Declaration of Helsinki and were performed only after the participants signed the informed consent agreement.

Informed Consent Not applicable.

Conflicts of Interest The authors declare no competing interests.

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