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ABSTRACT

Oxylipins are important signalling compounds that are significantly involved in the regulation of the immune system and the resolution of inflammation. Lipid metabolism is strongly activated upon SARS-CoV-2 infection, however the modulating effects of oxylipins induced by different variants remain unexplored.

Here, we compare the plasma profiles of thirty-seven oxylipins and four PUFAs in subjects infected with Wild-type, Alpha (B.1.1.7), Delta (B.1.617.2), and Omicron (B.1.1.529) variants.

The results suggest that oxidative stress and inflammation resulting from COVID-19 were highly dependent on the SARS-CoV-2 variant, and that the Wild-type elicited the strongest inflammatory storm. The Alpha and Delta variants induced a comparable lipid profile alteration upon infection, which differed significantly from Omicron. The latter variant increased the levels of pro-inflammatory mediators and decreased the levels of omega-3 PUFA in infected patients.

We speculate that changes in therapeutics, vaccination, and prior infections may have a role in the alteration of the oxylipin profile besides viral mutations. The results shed new light on the evolution of the inflammatory response in COVID-19.

1. Introduction

In late 2019, the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) appeared in Wuhan, China. Within a few weeks it had spread throughout the world forcing the World Health Organization to acknowledge (March 11, 2020) the novel coronavirus disease 19 (COVID-19) as a global pandemic [1]. So far, SARS-CoV-2 has infected approximately seven hundred million people and killed seven million, thus becoming one of the deadliest public health threats worldwide in recent years (https://covid19.who.int).

Research on the SARS-CoV-2 infection has mainly focused on the cell entry mechanism, however a comprehensive understanding of the viral action and diffusion throughout the body as well as the human host-response is still lacking. Lipids play a central role in viral infection, as viruses interfere with the lipid synthesis and signalling in the host cells to control their entry and replication [2]. Plasmatic levels of lipids in COVID-19 patients have been found to correlate with disease severity, and several lipids have been identified as potential markers of COVID-19 severity [3]. Long chain omega-3 polyunsaturated fatty acids (ω-3 PUFAs) have been shown to play a key role in the inflammation regulation at different stages of SARS-CoV-2 infection [4–7]. The administration of ω-3 PUFAs has also been suggested as a valuable treatment to induce a beneficial immunomodulatory response in COVID-19 patients, as demonstrated by the COVID-Omega-F and VASCEPA-COVID-19 CardioLink-9 trials [8–10].

Lipid metabolism is also an important key factor in the virus life cycle

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[11,12]. In most COVID-19 studies, the over-expression of pro-inflammatory lipid mediators such as prostaglandins (PGs) and thromboxanes (TXs) has been observed in infected subjects compared to controls [13–16]. A clear perturbation in the arachidonic acid (AA) pathway has also been suggested due to an imbalance between pro-inflammatory mid/terminal chain hydroxyeicosatetraenoic acids (HETEs) and anti-inflammatory epoxyeicosatrienoic acids (EETs) [17]. COVID-19 patients with decreased 5-HETE levels have in fact been shown to be at high risk of death [18]. A lipid dysregulation in moderate to severe disease has been broadly observed [19–21]. Severe COVID-19 is characterised by a loss in immune-regulatory PGs and resolvin E_3, as well as an increase in arachidonate 5-lipoxygenase (ALOX-5) and cytochrome P450 (CYP) products [14,21]. The plasma concentration of specialized pro-resolving mediators (SPMs) is downregulated in patients with severe COVID-19 [20,22,23], suggesting a block in the expected shift from the innate immune response to the resolution of inflammation [24].

However, the continuous evolution of SARS-CoV-2, leading to novel variants with spike protein mutations [25], requires further research. A mutation refers to a substitution, deletion, or addition in the virus genetic sequence which makes it different from the Wild-type strain [26]. Only a small number of mutations can affect the virus phenotype and cause changes in pathogenicity, infectivity, antigenicity, and transmissibility. Variants whose impact is significant due to a higher transmissibility, greater disease severity, and lower neutralization by antibodies [27] are defined as Variants of Concern (VOCs) [28]. Since December 2020, five SARS-CoV-2 VOCs have emerged, including the Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), Delta (B.1.617.2) and Omicron (B.1.1.529) variants [29]. The Alpha (B.1.1.7) variant emerged in the United Kingdom in the second half of 2020, spreading globally approximately 50% faster than previously identified variants [30]. Compared to the Wild-type strain, the Alpha (B.1.1.7) variant induced a 3.7-fold increase in infectivity, a 43–90% increase in transmissibility, and an increased risk of hospitalization, severity, and fatality [31]. Meanwhile, the Beta (B.1.351) and Gamma (P.1) variants, which were characterized by two additional mutations at positions E484 and K417, emerged in South Africa and Brazil, respectively [32]. These variants were then replaced by the Delta (B.1.617.2) variant, which emerged in India in December 2020 and spread to more than sixty countries around the world in summer 2021 [33,34]. In comparison to the original virus, the Delta (B.1.617.2) variant showed a 108% higher risk of hospitalization, a 235% higher risk of Intensive Care Unit (ICU) admission, and a 133% higher risk of mortality [35]. In early November 2021, the Omicron (B.1.1.529) variant was first detected in Africa and then rapidly became the most prevalent variant worldwide. In contrast to the eight mutations in the spike protein in the Delta (B.1.617.2) variant, Omicron (B.1.1.529) has more than 30 mutations in the spike protein which have been associated with increased viral replication, transmissibility and immune evasion after previous infection and vaccination [36]. Recently, the Omicron variant was classified into different sublineages, including BA.1, BA.1.1, BA.2, BA.2.12.1, BA.4, and BA.5 [37].

Much is known regarding the pathogenicity, infectivity, and transmissibility of different variants. Long COVID symptoms have been correlated to the SARS-CoV-2 variant causing the infection [38]. Lewis et al. found that the metabolic dysregulation has changed across waves of infection [39]. Nothing is known, however, about oxylipin synthesis and signalling in the different SARS-CoV-2 variants. Since inflammation is the hallmark of COVID-19, the role of oxylipins in the regulation of the inflammatory insult and its change over the course of the pandemic is of major interest.

In our study, we propose a comprehensive profiling of oxylipins and PUFAs in plasma samples of COVID-19 patients infected with Wild-type, Alpha (B.1.1.7), Delta (B.1.617.2), and Omicron (B.1.1.529) variants. Hypotheses are formulated regarding inflammation regulation depending on the wave of infection.

2. Materials and methods

2.1. Chemicals and materials

Acetonitrile and methanol hypergrade for LC-MS LiChrosolv® (purity ≥ 99.9%), and water for chromatography (LC-MS grade) LiChrosolv® (purity ≥ 99.9%) used for sample treatment and UHPLC-ESI-MS/MS analysis were from EDM Millipore (Milan, Italy). Commercially available oxylipins and PUFAs (purity ≥99%) *i.e.*, 15-F_{2t}-isoprostane, 15-F_{2t}-isoprostane-d4, 15-E_{2t}-isoprostane, 15-E_{2t}-isoprostane-d4, prostaglandin E_2, prostaglandin E_2-d4, prostaglandin D_2, 15-deoxy-$\Delta^{12,14}$-Prostaglandin J_2, thromboxane B_2, leukotriene B_4, lipoxin A_4, lipoxin A_4-d5, lipoxin B_4, resolvin E_1, resolvin D_1, resolvin D_1-d5, resolvin D_2, resolvin D_3, resolvin D_4, resolvin D_5, 17(*R*)-Resolvin-D_1, 17(*R*)-Resolvin-D_1-d5, 17(*R*)-Resolvin-D_3, 17(*R*)-Resolvin-D_4, neuroprotectin D_1 (NPD_1), protectin DX (PDX), maresin-1, maresin-1-d5, 7-epi-maresin-1, maresin-2, 5-hydroxyeicosatetraenoic acid (HETE), 12-HETE, 15-HETE, 20-HETE, 20-HETE-d6, 8,9-DiHETE, 11,12-DiHETE, 14,15-DiHETE, 13-hydroxy-9Z,11E-octadecadienoic acid (HODE), 8,9-epoxyeicosatrienoic acid (EET), 11,12-EET, 14,15-EET, 14,15-EET-d11, adrenic acid (AdA), eicosapentaenoic acid (EPA), alpha-linolenic acid (ALA) docosahexaenoic acid (DHA), arachidonic acid (AA), n-3 docosapentaenoic acid (DPA_{n-3}), linoleic acid (LA) and LA-d4 were from Cayman Chemical (Michigan, USA). Non-commercially available oxylipins *i.e.*, 5-F_{2t}-isoprostane, 5-*epi*-5-F_{2t}-isoprostane, 8-F_{3t}-isoprostane, 8-*epi*-8-F_{3t}-isoprostane, 18-F_{3t}-isoprostane, 20-F_{4t}-neuroprostane, 20-*epi*-20-F_{4t}-neuroprostane, 10-F_{4t}-neuroprostane-d4, 10-*epi*-10-F_{4t}-neuroprostane-d4, 14(*R,S*)− 14-F_{4t}-neuroprostane, 14(*R,S*)− 14-F_{3t}-neuroprostane, 4(*R,S*)− 4-F_{4t}-neuroprostane, C21–15-F_{2t}-isoprostane, tetranor-NPD_1, dinor-NPD_1, 7(*R,S*)-ST-Δ^8-11-dihomo-isofuran, ent-7(*R,S*)− 7-F_{2t}-dihomo-isoprostane, 17-F_{2t}-dihomo-isoprostane, 16(*S*),9(*R*)-linotrin, diH-DPA_{n-3}, diH-DPA_{n-6} and diH-AdA were synthesized at the Institut des Biomolecules Max Mousseron (IBMM) (Montpellier, France), according to procedures reported elsewhere [40–43].

Sterile polypropylene containers were from Eppendorf (Milan, Italy), whereas Phenex™-RC syringe filters (0.2 μm regenerate cellulose, 4 mm of diameter) were from Phenomenex (California, USA). A VELP Scientifica ZX4 Advanced Vortex Mixer (Usmate, Italy) and a Hermle Z-326 K Centrifuge, (Wehingen, Germany) were used for sample vortex-mixing and centrifugation, respectively. The removable needle microextraction by packed sorbent (MEPS) 250 μL syringe for HTA 300APlus (Thermo Scientific & Varian 8400 systems) and MEPS silica-C18 Barrel Insert and Needles (BINs) were purchased from SGE Analytical Science (Melbourne, Australia). The automated HT4000 Series Sample Prep workstation was purchased from HTA S.R.L. (Brescia, Italy).

2.2. Study design and participants

Adult patients (≥ 18 years) admitted between March 2020 and August 2022 to ASST Sette Laghi – Ospedale di Circolo e Fondazione Macchi with confirmed SARS-CoV-2 infection (laboratory real-time-polymerase chain reaction (RT-PCR) SARS-CoV-2 positivity) were included in the present retrospective study. The study was conducted in accordance with the Declaration of Helsinki (revised version 2000) and was approved by the local Institutional Review Board. The patient's clinical features (mild *vs.* severe) were classified according to the Severity Classification following WHO-Guidelines (https://www.who.int/emergencies/diseases/novel-coronavirus-2019/technical-guidance).

For each patient, information about demographics, clinical history, comorbidities, previous therapies, and physical status was collected.

2.3. Sample collection and storage

Peripheral blood samples were collected from patients during their

hospitalization, as part of the routine clinical activity of the Laboratory of Microbiology of ASST Sette Laghi – Ospedale di Circolo e Fondazione Macchi, Varese (Italy). Whole blood was collected in EDTA tubes (BD Vacutainer®) and centrifuged at 1500 g for 10 min at 25 °C to separate blood cells and plasma. Plasma was removed and stored in aliquots at − 80 °C until analysis. Butylhydroxytoluene (BHT, 15 mg/mL in methanol) was added (1:100 v/v) before storage to preserve PUFAs from *ex-vivo* lipid peroxidation [44]. For safety reasons, plasma samples were checked for the presence of SARS-CoV-2. Positive samples were excluded from chemical analyses.

2.4. Sequence analysis of SARS-CoV-2 variants

Viral nucleic acids were manually extracted from 250 μL of plasma samples using the EXTRA blood kit (ELITechGroup, Turin, Italy) according to the manufacturer's instructions. After the extraction, purified RNA samples were screened by RT-qPCR using the SARS-CoV-2 R-Gene assay (Biomerioux, Marcy-l'Etoile, France) on an ABI 7500 FAST thermocycler (Applied Biosystems). The real-time SARS-CoV-2 R-Gene assay was carried out by two triplex PCRs. The first PCR detected the N and the RdRp genes, whereas the second PCR detected the E gene of the SARS-CoV-2 genome. The assay contains internal controls to check PCR processing, and a cellular control to check sampling for certain results.

2.5. Quantification of plasma oxylipins and PUFAs

Sample treatment and analysis were performed in accordance with an analytical protocol developed in our lab [20,45] which successfully couples micro-extraction by packed sorbent (MEPS) technique to liquid chromatography tandem mass spectrometry for the straightforward analysis of sixty oxylipins and PUFAs in various biofluids [46,47].

Briefly, an aliquot of plasma sample (500 μL) was first added with 20 μL of Internal Standard (IS) mixture (20 ng/mL), and then with salts (*i.e.*, 250 μL of $CuSO_4 \cdot 5\ H_2O$ 10% w/v and 250 μL of $Na_2WO_4 \cdot 2\ H_2O$ 12% w/v) and acetonitrile (500 μL) for protein precipitation. After vortex-mixing (2000 rpm for 1 min), centrifugation (7000 rpm for 5 min), and filtration at 0.2 μm, the supernatant was diluted (1:5 *v/v*) with water and purified by means of MEPS technique. Analytes were eluted from the C18 cartridge with 50 μL of methanol and directly injected into the UHPLC-MS/MS instrument (Agilent 1290 Infinity II LC system coupled to a 6495 Triple Quadrupole mass spectrometer equipped with a Jet Stream electrospray (ESI) ionization source, Agilent Technologies, USA). The chromatographic separation was achieved at 0.7 mL/min using a Polaris 3 C18-A column (50 × 4.6 mm, 3 μm, Agilent Technologies, USA) and a gradient elution with a mobile phase consisting of 0.1% aqueous formic acid (A) and 50:50 *v/v* methanol:acetonitrile (B). The Agilent 6495 Triple Quadrupole mass spectrometer detector operated in ESI negative ionization mode and performed multiple reaction monitoring with unit mass resolution. Mass spectrometer control, data acquisition and data analysis were performed with the MassHunter Workstation software (B.07.00). Each analyte was detected using two specific MRM transitions, the most abundant one was used for quantification (Q) of the target compound whereas the other for its identification (q). A deviation $\leq \pm$ 0.10 min of the expected retention time compared to working standard solutions and a qualifier/quantifier (q/Q) ratio within ± 20% of the ratio measured in working standard mixtures were required for the identification of the analyte in plasma. Detailed chromatographic parameters, and ESI and MRM operating conditions are shown elsewhere [20].

2.6. Statistical analysis

Dataset includes the plasma levels of thirty-seven oxylipins and four PUFAs (Table S1), which were obtained from the analyses of forty-eight samples collected from COVID-19 patients infected with different SARS-CoV-2 variants.

Nineteen out of sixty analytes were excluded from the dataset as the concentrations were below the limit of quantification for more than 50% of samples. A decimal logarithmic transform of all the variables was used to correct for asymmetry [48]. Samples were randomly split into a training (n = 38) and a test set (n = 10): the former was used to build the models and the latter to independently estimate performances and consistency.

Data were analysed by a multivariate exploratory method (principal component analysis, PCA) [49].

In the present study, PCA was applied after column autoscaling to grant the same *a priori* importance to all variables, irrespectively of their magnitude [50].

Fisher's canonical variable was then computed in the plane described by both PC1/PC2 and PC4/PC5, to identify the direction maximising the ratio between inter-class and intra-class variances [51,52]. This axis represents the most discriminant direction, and its loadings, multiplied by the loadings of the PCs considered, indicate the importance of the original input variables in the differentiation of classes.

Multivariate data processing was performed by in-house MATLAB routines (The MathWorks, Inc., Natick, USA, Version 2019b).

3. Results

Between March 2020 and August 2022, 48 patients (26 male, 22 female) aged 40–80 years were included in our study. They had been infected with the Wild-type (n = 14), Alpha (B.1.1.7) (n = 9), Delta (B.1.617.2) (n = 11), and Omicron (B.1.1.529) (n = 14) SARS-CoV-2 strains.

The clinical characteristics, comorbidities and outcomes of patients are reported in Table 1.

The demographics were generally similar between the different groups, except for a slight prevalence of young people in Delta (B.1.617.2) compared to Omicron (B.1.1.529) population, *i.e.*, the median age of 41 years *vs* 77 years. We observed 40% of severe cases (excluding Alpha), as well as a high survival rate (85–100%) in all the patients regardless of the variant. Only three out of forty-eight patients, all recruited during the first infection wave, were hospitalized in ICU. Different COVID-19 treatments were used. Bamlanivimab/etesevimab was administered only to Alpha (B.1.1.7) patients, whereas casirivimab/imdevimab to 30–40% of both Alpha (B.1.1.7) and Delta (B.1.617.2) patients. About 50% of the Omicron (B.1.1.529) population received remdesivir as a therapy. Patients infected with Alpha and Delta strains were almost unvaccinated (1-dose in 10–20% of the population), whereas 90% of Omicron (B.1.1.529) patients had received at least 3-doses of COVID-19 vaccine. The Omicron (B.1.1.529) group showed a higher incidence (40–50% *vs* 10–20%) of comorbidities, *e.g.* neurological diseases, chronic kidney disease, and cancer, compared to both the Wild-type and Delta (B.1.617.2) groups. This suggests that the most recent wave of infection hospitalized subjects belonging to the most fragile fraction of the population, which develops severe diseases regardless of vaccination and/or previous infection.

No significant correlations were found between comorbidities, clinical characteristics, and drugs and oxylipin levels as clearly shown in the correlation matrix (Table S2) and the correlation map (Fig. S1). Only C-reactive protein showed a slightly positive correlation with oxylipin concentration (correlation coefficient higher than 0.25 for 23 oxylipins and higher than 0.3 for 8 oxylipins), reaching the maximum correlation coefficient of 0.43, reported with 11,12-EET. CKD and Casirivimab/imdevimab therapy were characterized by a weak negative correlation with some oxylipin levels. Casirivimab/imdevimab therapy had a correlation coefficient lower than − 0.25 for 21 oxylipins and lower than − 0.3 for 12 oxylipins, reaching the minimum correlation coefficient of − 0.47 with PGD_2.

The full dataset containing the plasma concentrations of the forty-one oxylipins and PUFAs is reported in Table S1. Data were analysed

Table 1
Demographic and clinical baseline characteristics of enrolled patients infected with Wild-type, Alpha (B.1.1.7), Delta (B.1.617.2), and Omicron (B.1.1.529) variant. Data are represented as median (first and third quartile). Statistics: Kruskal-Wallis test and Chi-Square (χ^2) to compare the prevalence of comorbidities and drug intake between the groups. The significant p-values ($p < 0.05$) calculated from the multiple comparisons between groups are reported in table.

	Wild-type (A) (n = 14)	Alpha (B) (n = 9)	Delta (C) (n = 11)	Omicron (D) (n = 14)	p-value
Comorbidities					
Diabetes (n; %)	0; 0	2; 22	1; 9	0; 0	
Obesity (n; %)	2; 14	2; 22	3; 27	0; 0	C *vs.* D: 0.037
Cardiovascular disease (n; %)	5; 36	3; 33	2; 18	5; 36	
Cancer (n; %)	0; 0	1; 11	0; 0	5; 36	A *vs.* D: 0.014 C *vs.* D: 0.027
Neurological disease (n; %)	2; 14	2; 22	0; 0	5; 36	C *vs.* D: 0.027
Chronic kidney disease (n; %)	1; 7	1; 11	1; 9	7; 50	A *vs.* D: 0.012 C *vs.* D: 0.030
Drugs					
Bamlanivimab/ Etesevimab (n; %)	0; 0	4; 44	0; 0	0; 0	A *vs.* B: 0.006 B *vs.* C: 0.013 B *vs.* D: 0.006
Casirivimab/imdevimab (n; %)	0; 0	3; 33	4; 36	0; 0	A *vs.* B: 0.020 A *vs.* C: 0.014 B *vs.* D: 0.020 C *vs.* D: 0.014
Remdesivir (n; %)	1; 7	0; 0	1; 9	7; 50	A *vs.* D: 0.012 B *vs.* D: 0.011 C *vs.* D: 0.030
Nirmatrelvir+Ritonavir (n; %)	0; 0	0; 0	0; 0	1; 7	
Clinical characteristics					
Age, years	66 (49 – 73)	70 (69 – 76)	41 (38 – 69)	77 (68 – 81)	
Male *vs* Female (M:F)	5:9	6:3	7:4	8:6	
Creatinine, mg/dL	1.0 (0.8 – 1.4)	1.1 (0.9 – 1.3)	1.1 (0.9 – 1.1)	1.2 (1.1 – 1.8)	
While blood cells, cells·10^3/μL	7.7 (6.4 – 8.5)	19 (15 – 24)	7.4 (5.6 – 8.8)	5.3 (3.2 – 8.3)	
Neutrophils, cells·10^3/μL	6.5 (4.2 – 6.9)	18 (13 – 23)	5.9 (2.9 – 6.7)	3.9 (2.0 – 6.8)	
Lymphocytes, cells·10^3/ μL	1.3 (1.1 – 1.6)	1.1 (0.7 – 1.4)	1.5 (0.8 – 1.8)	0.9 (0.6 – 1.2)	
C-reactive protein, mg/ dL	57 (12 – 248)	72 (37 – 107)	40 (10 – 70)	46 (20 – 94)	
D-Dimer, μg/mL	1640 (771 – 3539)	1764 (1030 – 2497)	597 (447 – 878)	1780 (1228 – 4775)	
COVID-19 vaccination					
Vaccinated (n; %)	0; 0	2; 22	1; 9	13; 93	A *vs.* D: < .0001 B *vs.* D: 0.0005 C *vs.* D: < .0001
Disease severity					
Severe (n; %)	6; 43	0; 0	5; 45	6; 43	A *vs.* B: 0.022 B *vs.* C: 0.020 B *vs.* D: 0.022
Outcome					
Survived (n; %)	14; 100	8; 89	11; 100	12; 86	
ICU (n; %)	3; 21	0; 0	0; 0	0;0	

by PCA. The two lowest-order principal components accounted for a total explained variance of about 72% (Fig. 1). PC1 scores provided the main contribution to the strong separation between the Wild-type variant (Fig. 1a, red square) and all the remaining variants *i.e.*, Alpha (B.1.1.7) (black square), Delta (B.1.617.2) (green square) and Omicron (B.1.1.529) (blue square). This pattern was consistent for items of both the training set (full symbols), used to build the model and calculate PCs, and the test set (empty symbols), which were simply projected onto the PC plane for validation purposes [48]. Contrary to the SARS-CoV-2 variant, age, sex, disease severity did not contribute to any differentiation of the samples in the score plot (Fig. S2). Almost all the vaccinated subjects had been infected with Omicron variant. Therefore, it was not possible to effectively assess the role of vaccine on the regulation of plasma oxylipins independently from the variant (Fig. S2).

Wild-type strain samples were located at positive score values of PC1 (Fig. 1b), thus pointing out an over-expression of most investigated oxylipins (*e.g.*, 10- to 100-fold increase in SPMs levels). On the contrary, representatives of the dihydroxy-eicosatetraenoic acid (DiHETE) family (*i.e.*, 8,9-DiHETE, 11,12-DiHETE) were at least 10-fold lower compared to Alpha (B.1.1.7) and Delta (B.1.617.2) strains. These latter were almost superimposed in the PCA plane, with samples spread on the top-left side of the score plot (Fig. 1a). Contrarily, the Omicron (B.1.1.529) population could be distinguished from both Alpha (B.1.1.7) and Delta (B.1.617.2) along PC2. Omicron (B.1.1.529) samples were located at negative scores of PC2, being mainly characterized by an up-regulation of prostaglandins (*i.e.* PGE_2, PGD_2, 5- to 10-fold), neuroprostanes (20-F_{4t}-neuroprostane, 14-F_{3t}-neuroprostane, 2-fold), and isofurans (7(*R,S*)-ST-Δ^8-11-dihomo-IsoF, 3-fold), and a down-regulation of 8,9-DiHETE, 15-E_{2t}-IsoP, and PUFAs, which were approximately 3- to 5-fold higher in both Alpha (B.1.1.7) and Delta (B.1.617.2) variants. This separation was clearly confirmed by computing the most discriminant direction as Fisher's canonical variable (Fig. 2a, full black line) along PC1 and PC2 (total explained variance: 43%, the Wild-type population was excluded). The loadings (more precisely their absolute values, Fig. 2b) were representative of oxylipin contributions to discrimination, with positive and negative weights of the variables referring to "Alpha (B.1.1.7) + Delta (B.1.617.2)" and Omicron (B.1.1.529) population, respectively.

As already observed, the Alpha (B.1.1.7) and Delta (B.1.617.2) variants were almost indistinguishable along PC1/PC2. A partial separation could be obtained by focusing just on these two populations in PC4/PC5 plane (total explained variance 14%, Fig. 3). Contrarily to Alpha (B.1.1.7) variant, Delta (B.1.617.2) was characterised by a slight increase (1.5- to 2-fold) in the levels of most plasma oxylipins, including TXB_2, HETEs, EETs, and lipoxins (lipoxin A_4 and lipoxin B_4) (Fig. 3b). The box plots of the most representative oxylipins and PUFAs are reported in the Supplementary Information file (Fig. S3).

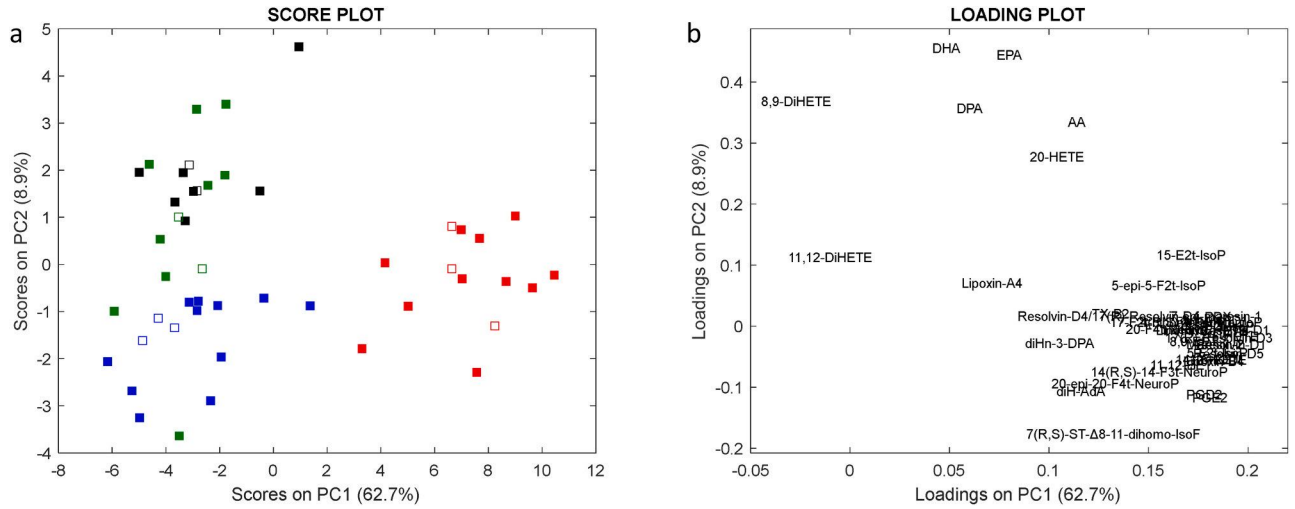

Fig. 1. Oxylipin and PUFA score plot (a) and loading plot (b). Square symbols represent samples collected from patients infected with different SARS-CoV-2 variants: Wild-type (red); Alpha (B.1.1.7) (black), Delta (B.1.617.2) (green), and Omicron (B.1.1.529) (blue). Full and empty symbols belong to the training and test sets, respectively.

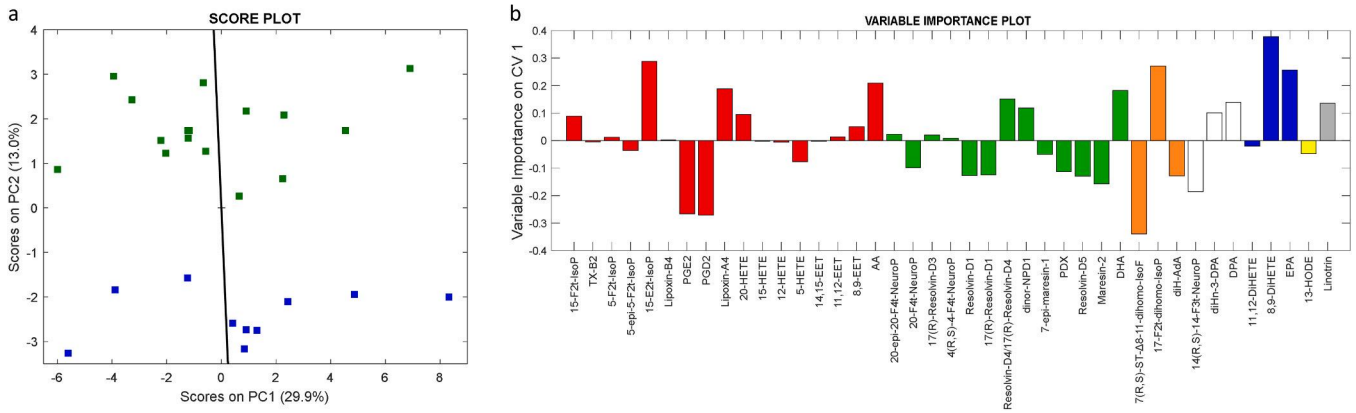

Fig. 2. (a) Fisher's linear discriminant analysis performed on the training set and based on the two lowest-order PCs. Classes: Alpha (B.1.1.7) +Delta (B.1.617.2) (green) and Omicron (B.1.1.529) (blue). The full black line represents Fisher's canonical variable, *i.e.*, the direction of maximum discrimination between the two classes. (b) Loadings of Fisher's canonical variable indicating the importance of the input variables in discriminating between the two classes. Colours refer to different PUFAs as oxylipin precursors: red – arachidonic acid (AA); green –docosahexaenoic acid (DHA); blue – eicosapentaenoic acid (EPA); orange – adrenic acid (AdA), grey – alpha-linolenic acid (alpha-ALA), white – n-3 docosapentaenoic acid (DPA_{n-3}); yellow – linoleic acid (LA). Positive and negative weights of the variables refer to the Alpha (B.1.1.7) +Delta (B.1.617.2) and Omicron (B.1.1.529) population, respectively.

4. Discussion

Inflammation-based immunity is a frontline defence aimed at preventing pathogen infection [53]. Targeted and untargeted mass spectrometry has been widely used to characterize lipidome alteration upon coronavirus infection [54]. Lipid metabolism has been found to be altered for twelve years upon SARS infection [55]. Recently, several studies have reported the involvement of lipids and lipid metabolism in COVID-19 [3,56]. However, to date, the levels and modulating effects of lipids in COVID-19 across the different infection waves remain largely unexplored.

In this study, a multivariate analysis of the plasma levels of thirty-seven oxylipins and four PUFAs enabled the differentiation of the Wild-type, Alpha (B.1.1.7), Delta (B.1.617.2), and Omicron (B.1.1.529) SARS-CoV-2 variants. The analysis of the relative contributions of individual oxylipins and their precursors to the separation of the different variants may provide new insights into COVID-19 pathophysiology and its evolution over time.

Among the investigated variants, the Wild-type strain population showed a significant up-regulation in the concentration of the overall plasma oxylipins and PUFAs, thus being strongly separated in the PCA score plot. The marked increase (10- to 100-fold) in both pro-inflammatory and pro-resolving mediators suggests the occurrence of a major inflammatory storm, which is typical of several inflammatory conditions (*i.e.*, sepsis, acute respiratory distress syndrome, and viral infection), and reflects the activation of compensatory mechanisms tuned by the host response to infection [57]. This finding is consistent with the severity of the illness in patients infected during the first wave of the pandemic [58]. In fact, these patients were generally characterised by severe lung injury, lymphocytopenia, as well as a decrease in complement C3 and C4 levels which are strongly associated with increased disease severity and mortality [59]. However, the Wild-type strain may be more aggressive and harmful to humans because of the improved immune response across populations due to vaccination or prior infections [60]. DiHETEs were found to be the only down-regulated mediators in the Wild-type samples. These eicosapentaenoic acid (EPA)-derived diols are the least active hydrolysed form of the epoxy-metabolites, *i.e.* epoxyeicosatetraenoic acids (EpETEs). The EPA oxygenation pathway has been demonstrated to be fundamental in maintaining human health and tissue homeostasis [61]. Specifically,

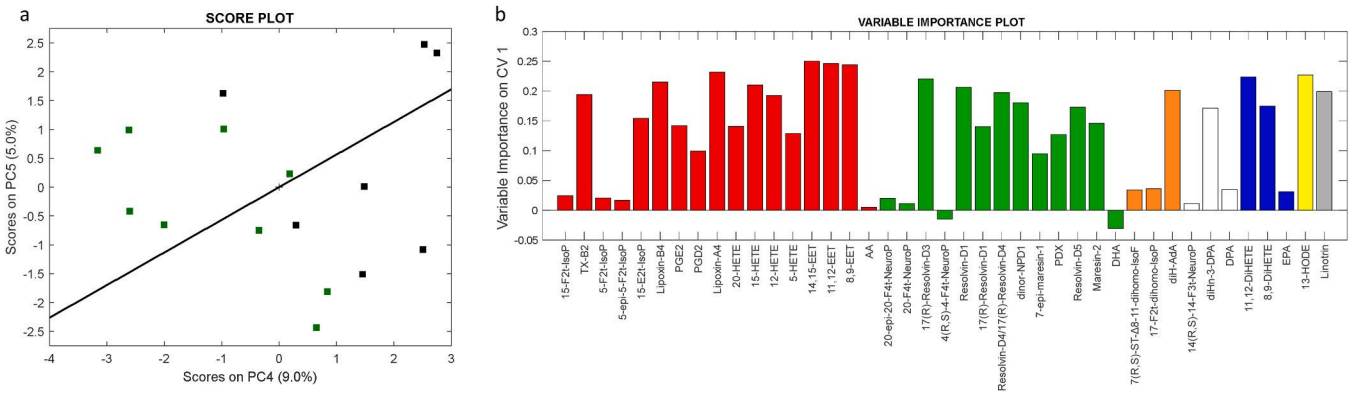

Fig. 3. (a) Fisher's linear discriminant analysis performed on the training set and based on PC4 and PC5. Classes: Alpha (B.1.1.7) (black) and Delta (B.1.617.2) (green). The full black line represents Fisher's canonical variable, *i.e.*, the direction of maximum discrimination between the two classes. (b) Loadings of Fisher's canonical variable indicating the importance of the input variables in discriminating between the two classes. Colours refer to different PUFAs as oxylipin precursors: red – arachidonic acid (AA); green –docosahexaenoic acid (DHA); blue – eicosapentaenoic acid (EPA); orange – adrenic acid (AdA), grey – alpha-linolenic acid (alpha-ALA), white – n-3 docosapentaenoic acid (DPA_{n-3}); yellow – linoleic acid (LA). Positive and negative weights of the variables refer to the Delta (B.1.617.2) and Alpha (B.1.1.7) population, respectively.

EpETEs attenuate the inflammation by inhibiting the production of cytokines and airway mucin in epithelial cells and by inhibiting neutrophil functions through the cell surface receptor GPR40 [62]. EpETEs can inhibit lipopolysaccharide (LPS)-induced airway inflammation in the nose and lung [62]. Thus, the inhibition of diol formation, as well as the increase in EpETE epoxide formation, generally show beneficial effects in response to lung injury as likely observed in our case.

It was possible to distinguish the Omicron (B.1.1.529) population from both the Alpha (B.1.1.7) and Delta (B.1.617.2) populations on the PC2 of the score plot (Fig. 1). The separation is mainly driven by the up-regulation of cyclooxygenase (COX) metabolites (PGE_2, PGD_2, and TXB_2, 5- to 10- fold) and ROS-mediated F_4 and F_3-neuroprostanes (2-fold), as well as the down-regulation of ω-3 PUFAs (*e.g.* EPA, docosahexaenoic acid (DHA), 3- to 5-fold). PGE_2 modulates the tissue influx and the functions of neutrophils, macrophages, and mast cells [63]. Along with TXB_2, PGE_2 is released at the very beginning of the inflammatory insult, which is characterized by the prevalence of COX-2 and 5-LOX activity [64]. TXB_2 is the biological inactive catabolite of TXA_2, which is found to activate leukocytes and platelets, thus inducing bronchoconstriction, thrombosis, and microcirculatory damage in COVID-19 patients [15]. As in our previous work [20], the presence of ROS-derived mediators, such as DHA-derived F_4-neuroprostanes and DPA-derived F_3-neuroprostanes, clearly suggests that a strong oxidant insult supports the inflammation upon viral infection. F_4-neuroprostanes have been found to prevent the oxidation of the ryanodine receptors (RyR) and the leak of Ca(II), thus regulating calcium homeostasis [65, 66]. High levels of neuroprostanes may thus exert a protective role against severe and long-term COVID-19 symptoms.

The lipid mediator switching from prostanoids and leukotrienes to SPMs is critical for the inflammation resolution [67–69]. EPA and DHA contribute to the synthesis of SPMs [70,71], which mediate endogenous resolution and promote tissue healing by stimulating macrophage phagocytosis of cellular debris as well as by inhibiting cytokine and chemokine production [72–75]. The activation of the pro-inflammatory axis with the production of TNF-α and IL-6 can induce AA, EPA, and DHA shortage at the cellular level and a lower-rate synthesis of SPMs [76], as observed in the Omicron population. In this case, a PUFA supplement may compensate for the shortage and aid in accelerating the resolution phase [77].

The oxylipin profiles were found to be similar in the Alpha (B.1.1.7) and Delta (B.1.617.2) strains. Alpha (B.1.1.7) and Delta (B.1.617.2) samples were superimposed in the PCA score plot (Fig. 1a). A slight difference between the Alpha (B.1.1.7) and Delta (B.1.617.2) variants only occurred when focusing on these two populations along the PC4 and PC5. The Delta (B.1.617.2) strain is characterised by a slightly marked activation of the AA cascade. As shown in our results, the COX-generated prostaglandins and thromboxanes are increased as part of the pro-inflammatory response activated immediately upon SARS-CoV-2 infection. COX and 5-LOX pathways are then countered the CYP pathway which is principally responsible for EET synthesis [78]. EETs promote clearance of cellular debris and trigger anti-inflammatory programs to inhibit pro-inflammatory cytokines [79]. EETs also induce the formation of important SPMs, such as lipoxins [80,81], by shifting arachidonic acid metabolism to foster inflammation resolution [82]. The slightly marked activation of the AA-cascade in Delta (B.1.617.2) rather than Alpha (B.1.1.7) patients was consistent with its virological characteristics. Delta (B.1.617.2) has been found to cause more severe illness, 2-fold higher hospitalization rate, increase in oxygen supplementation and mortality [83], thus a stronger alteration in the oxylipin profile was expected.

5. Limitations

The present work has some limitations. Firstly, the number of subjects we were able to enrol in the study is quite low, and this could affect the statistical solidity of its conclusions. Although large numbers would have been desirable (we are actively working in this direction for a follow-up study), we speculate that our data could in any case be useful to the scientific community and inspire other research.

Secondly, a better characterization of patients' conditions would also have been advisable as changes in therapeutics, vaccination, and prior infections could modify the oxylipin profile besides viral mutations. In a previous paper, we showed the presence of major differences in the oxylipin profile of COVID-19 patients (Wild-type infection) hospitalized in wards or in ICUs [20]. This issue is somehow connected to the previous one, as a stratification of patients in different subgroups to assess the role of concurring factors would have required a number of patients far larger than 48. We feel that it would have been particularly interesting to compare profiles of unvaccinated and vaccinated patients infected with different variants, as this would have given information about the level of protection provided from vaccines. A longitudinal study showing the evolution of the oxylipin profile over time in patients would also be of interest, particularly in long COVID patients.

6. Conclusions

In this work, we have clearly shown that lipid metabolism is strongly activated upon SARS-CoV-2 infection. Oxylipin synthesis and release

suggest a strong activation of oxidative stress and inflammation in subjects infected with Wild-type, Alpha (B.1.1.7), Delta (B.1.617.2), and Omicron (B.1.1.529) variants. Both the level and type of oxylipins and PUFAs changed significantly across the different COVID-19 waves. This could reflect differences in virological characteristics of the variants, such as viral load, infectivity, and pathogenicity. Immunity from vaccination or prior infection may also have a role in modulating the driving changes of the oxylipin and PUFA profile over time. Overall, the results shed new light on the evolution of the inflammatory response in COVID-19.

Author contribution

D.B., S.G., T.L., F.D.F., F.M.: Conceptualization; D.B., S.G., A.L.: Methodology; P.O.: Formal analysis; D.B., T.L., S.G.: Investigation; D.B.: Writing - original draft preparation; D.B., S.G., T.L., A.B., F.V.: Writing - review and editing; T.D., C.O., J-M.G., L.B., A.B., D.D.G., F.D.F., F.M.: Resources; A.B., D.D.G.: Data curation; T.L., F.D.F.: Supervision.

Conflicts of Interest

The authors declare no competing interests.

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Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version

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