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► **To cite this version:**

Sven-Christian Pawelzik, Hildur Arnardottir, Philip Sarajlic, Ali Mahdi, Claire Vigor, et al.. Decreased oxidative stress and altered urinary oxylipidome by intravenous omega-3 fatty acid emulsion in a randomized controlled trial of older subjects hospitalized for COVID-19. *Free Radical Biology and Medicine*, 2023, 194, pp.308-315. 10.1016/j.freeradbiomed.2022.12.006 . hal-03922206

HAL Id: hal-03922206

<https://hal.science/hal-03922206>

Submitted on 4 Jan 2023

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Decreased oxidative stress and altered urinary oxylipidome by intravenous omega-3 fatty acid emulsion in a randomized controlled trial of older subjects hospitalized for COVID-19

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ARTICLE INFO

Keywords:

Coronavirus disease 2019 (COVID-19)
Eicosanoids
Erythrocyte oxidative stress
Resolution of inflammation
Isoprostanes
Inflammation

ABSTRACT

Proinflammatory bioactive lipid mediators and oxidative stress are increased in coronavirus disease 2019 (COVID-19). The randomized controlled single-blind trial COVID-Omega-F showed that intravenous omega-3 polyunsaturated fatty acids (n-3 PUFA) shifted the plasma lipid signature of COVID-19 towards increased pro-resolving precursor levels and decreased leukotoxin diols, associated with a beneficial immunodulatory response. The present study aimed to determine the effects of n-3 PUFA on the urinary oxylipidome and oxidative stress in COVID-19. From the COVID-Omega-F trial, 20 patients hospitalized for COVID-19 had available serial urinary samples collected at baseline, after 24–48 h, and after completing 5 days treatment with one daily intravenous infusion (2 mL/kg) of either placebo (NaCl; n = 10) or a lipid emulsion containing 10 g of n-3 PUFA per 100 mL (n = 10). Urinary eicosanoids and isoprostanes were analyzed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Erythrocytes obtained at the different time-points from n = 10 patients (n = 5 placebo and n = 5 n-3 PUFA) were used for determination of reactive oxygen species. Intravenous n-3 PUFA emulsion administration altered eicosanoid metabolites towards decreased levels for mediators of inflammation and thrombosis, and increased levels of the endothelial function mediator prostacyclin. Furthermore, non-enzymatic metabolism was skewed towards n-3 PUFA-derived metabolites with potential anti-inflammatory and pro-resolving effects. The oxidative stress marker 15-F_{2t}-isoprostane was significantly lower in patients receiving n-3 PUFA treatment, who also exhibited significantly decreased erythrocyte oxidative stress compared with placebo-treated patients. These findings point to additional beneficial effects of intravenous n-3 PUFA emulsion treatment through a beneficial oxylipin profile and decreased oxidative stress in COVID-19.

1. Introduction

Coronavirus disease 2019 (COVID-19) is characterized by high levels of inflammatory mediators and oxidative stress (OS), which are associated with more severe disease. In addition to cytokines, also lipid mediators of inflammation, e.g. prostaglandins and leukotrienes, are increased in the uncontrolled inflammatory response during a Severe

acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection with acute respiratory distress syndrome (ARDS). Fatty acids are hence crucial in controlling excessive inflammation, which is part of COVID-19 through a balance between proinflammatory and proresolving lipid mediators [1,2]. The formation of toxic oxidation products may contribute to the uncontrolled systemic inflammation in acute as well as to the sustained pathological findings, including endothelial dysfunction [3]. Anti-oxidants have been evaluated for their therapeutic potential to

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Abbreviations

acute respiratory distress syndrome ARDS
 arachidonic acid AA
 eicosapentaenoic acid EPA
 docosahexaenoic acid DHA
 coronavirus disease 2019 COVID-19
 electron paramagnetic resonance EPR
 isoprostane IsoP
 liquid chromatography tandem mass spectrometry LC-MS/MS
 omega-3 polyunsaturated fatty acids n-3 PUFA
 omega-6 polyunsaturated fatty acid n-6 PUFA
 oxidative stress OS
 polyunsaturated fatty acids PUFA
 prostacyclin PGI₂
 Resolving inflammatory storm in COVID-19 patients by Omega-3 Polyunsaturated fatty acids trial COVID-Omega-F
 reactive oxygen species ROS
 specialized proresolving mediators SPM

improve prognosis in acute COVID-19 [4,5] and to alleviate symptoms in long-haul COVID-19 [6].

Oxylipins are bioactive lipids generated from polyunsaturated fatty acids (PUFAs) through both enzymatic and non-enzymatic lipid peroxidation, which may contribute to the inflammatory and OS response. The most studied non-enzymatic lipid oxidation products are isoprostanes derived from the n-6 polyunsaturated fatty acid (PUFA) arachidonic acid and exerting pro-inflammatory and prothrombotic effects. In contrast, isoprostanes and neuroprostanes derived from non-enzymatic oxidation of n-3 PUFA are also associated with anti-inflammatory and pro-resolving responses [7]. n-3 PUFA may in addition act directly as enhancers of the antioxidant defense against reactive oxygen species (ROS) [8]. However, the notion has been raised that n-3 PUFA make cell membranes more susceptible to non-enzymatic oxidation and to the formation of potentially toxic oxidation products and increase the oxidative stress [9].

The n-3 PUFA eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are precursors for an enzymatic biosynthesis of lipid mediators stimulating the resolution of inflammation. Referred to as specialized proresolving mediators (SPM), these bioactive lipids are endogenously formed to dampen systemic inflammation while also improving healing and microbial elimination [10]. Importantly, also oxidative non-enzymatically formed n-3 PUFA metabolites may exert beneficial effects in analogy with the SPM.

The IMPRECOVID study revealed specific plasma oxylipin profiles to distinguish COVID-19-associated pneumonia requiring intensive care [11]. The randomized controlled single-blind COVID-Omega-F trial showed that intravenous n-3 PUFA administration shifted the plasma lipid signature of COVID-19 patients toward increased proresolving precursor levels and decreased leukotoxin diols [12]. While these plasma analyses provide a snapshot of lipid metabolism in COVID-19, the urinary oxylipidome gives a more complete picture of time-integrated chemically stable metabolites that reflects systemic biosynthesis [13,14,33]. The full urinary oxylipid profile in COVID-19, and the effects of PUFA supplementation have not been previously reported.

The beneficial immune responses after n-3 PUFA administration in the COVID-Omega-F trial included a decreased neutrophil to lymphocyte ratio, preserved leukocyte phagocytic capacity, decreased immunothrombosis and preserved interferon response [12]. The recent VASCEPA-COVID-19 CardioLink-9 trial showed that oral n-3 PUFA treatment with the EPA ethyl ester icosapent ethyl for 14 days improves symptoms and reduces C-reactive protein (CRP) in ambulatory

COVID-19 patients [15]. Similar results have been reported in a smaller study of n-3 PUFA added to hydroxychloroquine-treatment of COVID-19 (performed at the time it was used) [16] and in critically ill COVID-19 patients [17]. However, no previous study has examined the relation of beneficial effects of n-3 PUFA in relation to OS in patients with COVID-19.

The aim of the present study was to determine the effects of n-3 PUFA treatment in patients with COVID-19 on (a) the urinary enzymatic and oxidative oxylipidome, and (b) on the oxidative stress responses in patients with COVID-19.

2. Materials and methods

2.1. Patients

The trial “Resolving inflammatory storm in COVID-19 patients by Omega-3 Polyunsaturated fatty acids” (COVID-Omega-F) was approved by the Swedish Ethical Review Authority (Dnr 2020–02592 and Dnr 2020–06137) and by the Medical Product Agency (Dnr 5.1-2020-42861 and Dnr 5.1-2020-96391). Inclusion criteria were a diagnosis of COVID-19 and a clinical status requiring hospitalization. After signed informed consent, participants were randomized 1:1 to a once daily i. v. infusion (2 mL/kg) of either placebo (0.9% NaCl) or n-3 polyunsaturated fatty acid (PUFA) emulsion containing 0.1 g/mL of fish oil (Omeqaven®; ApoEX, Stockholm, Sweden), including 12.5–28.2 mg/mL DHA and 14.4–30.9 mg/mL EPA for 5 days. The study protocol is registered at clinicaltrials.gov with reference NCT04647604 and has been published [18]. The primary endpoint measures have been reported [12].

2.2. Blood and urine sample collection

Samples were collected at study start before administration of the first dose (baseline), 24–48 h after the first administered dose (early), and within 24 h after the last administered dose (end) as illustrated in the Graphical Abstract. Laboratory measures of blood cell counts and Hb were performed by the Karolinska University Laboratory in accordance with ISO15189. Blood samples were collected by venipuncture into 8 ml sodium heparinized CPT™ Vacutainer® tubes (Becton Dickinson AB, Stockholm, Sweden) for erythrocyte isolation and processed within 2 h from collection. Urine samples for analysis of urinary lipid metabolites (eicosanoids and isoprostanes) were collected in the morning into 10 mL tubes, immediately transferred to 4°C, and aliquots were prepared within 2 h from collection and stored at –80°C.

2.3. Erythrocyte isolation and incubation for ROS measurements

For each time point, erythrocytes were isolated. Whole blood was centrifuged at 1800×g for 15 min at room temperature to separate peripheral blood mononuclear cells from erythrocytes and neutrophils. Erythrocytes were further isolated by sedimentation on 3% dextran at 1×g for 20 min at 4°C and washed 3 times with PBS^{-/-} followed each time by centrifugation at 1000×g for 10 min at 4°C. In a protocol initiated after inclusion of the first 8 patients, erythrocytes derived from 10 of the study participants (n = 5 placebo and n = 5 n-3 PUFA treated subjects) were used to evaluate the production of ROS. Erythrocytes were incubated with the spin probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH; Noxygen Science Transfer & Diagnostics GmbH, Elzach, Germany). In brief, 5 µl of washed erythrocytes were added to 1 mL 200 µM CMH:KREBS/HEPES solution, (1:1, v/v), mixed well, and incubated for 30 min at 37°C with gentle shaking. Incubations were stopped by freezing the samples on dry ice. Samples were stored at –80°C until ROS production was quantified using electron paramagnetic resonance (EPR) spectroscopy on a Bruker E-Scan M system (Bruker, Billerica MA, USA) as previously described [3,19,20]. The following settings were used: center field 1.99 g, microwave power 1 mW, modulation amplitude 9 G, sweep time 10 s, number of scans 10,

field sweep 60 G. The EPR spectrums are expressed as EPR signal intensities (arbitrary units).

2.4. Urinary eicosanoid analysis

300 μ L urine were spiked with 10 μ L of deuterated internal standard mix (29–1429 ng/mL) and acidified with 1500 μ L of 0.12% acetic acid in H₂O. For solid-phase extraction (SPE), a mixed polymer phase (30 mg, ABN Evolute, Biotage Uppsala, Sweden) was conditioned with 1 mL methanol and equilibrated with 0.1% acetic acid in H₂O, the samples were loaded, and the SPE phase was washed with 1 mL 0.1% acetic acid in H₂O, followed by 1 mL 8% methanol in H₂O. Analytes were eluted using a 3:1 mix of methanol and acetonitrile and dried under nitrogen. The dry extracts were reconstituted in 100 μ L 1:1 mix of methanol and H₂O and filtered using a filter plate + (0.22 μ M, Biotage, Uppsala, Sweden). Urinary eicosanoids were analyzed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) on an Acquity UPLC and Xevo TQ-XS system (Waters, Milford, MA, USA) and quantified using two separate LC-MS/MS methods as described before [21]. 7.5 μ L of the reconstituted extracts were separated on a HSS T3 column (2.1 \times 100 mm, 1.8 μ m, Waters, Milford, MA, USA) equipped with a HSS T3 VanGuard column. The remaining extract was incubated with 10 μ L 0.5 g/mL methoxyamine, and derivatized 2,3-dinor TXB₂ and 2,3-dinor-6-keto PGF_{1 α} were quantified as described before [21]. In both methods, mobile phases were 0.1% acetic acid in H₂O (A) and a 9:1 mix of acetonitrile and isopropanol (B). Analytes were quantified in both methods using an external calibration curve with a minimum of 8 points, and three low and high spiked quality control urine samples per plate were measured to assess method performance.

2.5. Urinary isoprostane analysis

Urinary isoprostane profiling was performed using a micro-LC-MS/MS equipment after extraction of lipids from the biological matrix. 1 mL of urine was added to 1 mL of 40 mM formic acid (pH 4.5) including 4 μ L internal standard mix (1 ng/ μ L of each IS) and vortexed for 30 s. The samples were then extracted by SPE using Oasis Max cartridges (30 μ m, 60 mg, Waters, Milford, MA, USA). Cartridges were conditioned with 2 mL of methanol followed by 2 mL of 20 mM formic acid. After loading of the samples, they were washed with 2 \times 1 mL of four successive solvent mixtures: 2% NH₃, methanol/20 mM formic acid (30:70, v/v), hexane, and hexane/ethyl acetate (70:30, v/v). The metabolites were eluted with hexane/ethanol/acetic acid (70:29.4:0.6, v/v/v), concentrated to dryness under a nitrogen stream, and reconstituted in 100 μ L of mobile phase (water/acetonitrile, 83:17, v/v, with each containing 0.1% formic acid). The metabolites were analyzed by microLC-MS/MS on an Eksigent chromatographic system (Sciex Applied Biosystems, Framingham, MA, USA) coupled to a QTRAP 5500 mass spectrometry system (Sciex, Concord, ON, Canada) as previously described [22]. The MS/MS was operated in electrospray ionization (ESI) negative mode. Detection of the fragmentation ion products from each deprotonated molecule [M – H]⁻ was performed in the multiple reaction monitoring mode (MRM). Concentration of the analytes was obtained by calibration curves calculated by the area ratio of analytes and IS. Data was processed using MultiQuant 3.0 software (Sciex Applied Biosystems). The obtained IsoP levels were normalized to urinary creatinine concentrations, which were determined using a standard colorimetric assay (Cayman Chemicals, Ann Arbor, MI, USA).

2.6. Statistical analysis

To determine the effect of time and the n-3 PUFA intervention, a mixed analysis of variance model was implemented in which time was defined as a within-subjects effect and the intervention as a between-subjects effect. Moreover, the interaction between these effects was measured. All significance tests were two-sided and findings with $p <$

0.05 were regarded as statistically significant. The above-mentioned analyses were performed in Prisma 8, version 8.4.3 (erythrocyte ROS) or R version 4.1.1 (R Core Team (2021)). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL <https://www.R-project.org/> [23].

3. Results

3.1. Patient characteristics

3.1.1. Patient characteristics

All subjects were included from June to December 2020, before COVID-19 vaccination was available, at a first COVID-19 infection. The COVID-Omega-F trial was completed for 22 older subjects (mean age 81 \pm 6.1 years). Of these, serial urine samples were available from 20 participants, which did not exhibit any significant differences in baseline characteristics between the groups (Table 1). The severity of COVID-19 was evaluated at admission before initiation of treatments by CRP, which was 71.6 \pm 46.5 pg/mL (n = 20).

3.1.2. n-3 PUFA administration alters the urinary eicosanoid profile in COVID-19 patients

The urinary profile of enzymatically synthesized eicosanoids and their metabolites (Table 1) revealed a significant increase in the prostacyclin (PGI₂) metabolite, 2,3-dinor-6-keto PGF_{1 α} , at the early time

Table 1
Baseline characteristics.

	All	Placebo	n-3 PUFA	P
n	20	10	10	
Age - years	80.7 \pm 6.2	80.7 \pm 5.8	80.7 \pm 7.0	1.0
Female sex – no. (%)	11 (55%)	6 (60%)	5 (50%)	1.0
Body Mass Index - kg/m ²	25.6 \pm 4.1	24.9 \pm 3.9	26.3 \pm 4.4	0.47
Current smoker – no. (%)	2 (10%)	0 (0%)	2 (20%)	0.47
Days since symptom start	7.4 \pm 3.7	7.5 \pm 3.6	7.0 \pm 3.1	0.87
Days since COVID-19 diagnosis	3.9 \pm 2.2	4.2 \pm 2.7	3.7 \pm 1.3	0.83
White Blood Cells				
Leukocytes (x10 ⁹ /L)	7.3 (5.9–9.6)	6.1 (4.1–8.0)	8.1 (6.1–11)	0.06
Monocytes (x10 ⁹ /L)	0.60 (0.46–0.74)	0.52 (0.27–0.77)	0.65 (0.50–0.85)	0.25
Neutrophils (x10 ⁹ /L)	5.4 (4.2–7.0)	4.4 (2.8–6.0)	5.6 (4.1–9.2)	0.09
Lymphocytes (x10 ⁹ /L)	1.1 (0.9–1.4)	0.98 (0.71–1.2)	1.15 (0.78–1.6)	0.20
Red Blood Cells				
Erythrocytes (x10 ¹² /L)	3.9 (3.6–4.3)	4.1 (3.5–4.6)	4.0 (3.4–4.3)	0.45
EVF	0.36 (0.33–0.38)	0.35 (0.31–0.40)	0.36 (0.34–0.39)	0.89
Ery-MCH	29 (28–31)	29 (26–31)	31 (29–32)	0.13
Ery-MCV	90 (86–94)	87 (80–94)	93 (90–96)	0.09
Hb (g/L)	116 (102–129)	114 (99–130)	120 (106–129)	0.80

Demographic data are expressed as either mean \pm standard deviation or absolute numbers and percentage. Laboratory measures are expressed as mean (95% CI). Statistical analyses were performed by either a Student's t-test (continuous variables) or a Fisher Exact Test (categorical variables) **Abbreviations:** EVF, erythrocyte volume fraction; Ery-MCH, erythrocyte mean corpuscular hemoglobin; Ery-MCV, mean corpuscular volume.

point in the n-3 PUFA group compared with the placebo group (Fig. 1, left panel; Table 1). The increase in the thromboxane (TX) A_2 metabolite, TXB₂, observed in the placebo-group at the end of the treatment was not observed in the n-3 PUFA treated group, but failed to reach significance (Fig. 1, middle panel). There was also a trend towards decreased levels of the urinary leukotriene metabolite LTE₄ in the n-3 PUFA treated compared with the placebo group (Fig. 1, right panel). The urinary concentrations of the urinary metabolites of PGE₂, PGD₂, and PGF_{2 α} were not significantly altered over time or between the groups (Table 2).

3.1.3. Altered urine autooxidation lipid profile by n-3 PUFA administration in COVID-19

A targeted urinary lipid metabolite analysis was performed for a panel of non-enzymatically oxidized PUFA metabolites. The urinary concentrations of 15-F_{2t}-isoprostane (15(RS)-15-F_{2t}-IsoP) were increased in the placebo-group, whereas n-3 PUFA-treated patients exhibited a time-dependent decrease of 15(RS)-15-F_{2t}-IsoP, resulting in significantly lower urinary 15(RS)-15-F_{2t}-IsoP levels in n-3 PUFA-treated patients compared to placebo treated patients at end of study (Fig. 2, left panel). In contrast, the n-3/n-6 ratio for all oxidative urinary metabolites was significantly increased by n-3 PUFA treatment, which reflected the increased n-3 PUFA metabolite F_{3t}-IsoP observed for the n-3 PUFA compared with placebo treatment (Fig. 2). The complete results of the oxidative PUFA metabolite profiling are shown in Table 3.

3.1.4. Reduced oxidative stress in erythrocytes by n-3 PUFA administration in COVID-19

Urinary 15-F_{2t}-IsoP is an oxidative stress marker. To examine whether the significant 15-F_{2t}-IsoP decrease observed after n-3 PUFA treatment (Fig. 2) reflected a beneficial effect on OS burden, EPR spectroscopy using the spin probe CMH was performed on erythrocytes. The results demonstrated significantly lower levels of ROS in erythrocytes derived from n-3 PUFA compared with placebo-treated COVID-19 patients (Fig. 3, n = 5 in each group).

4. Discussion

The urinary oxylipidome after i. v. n-3 PUFA treatment of patients hospitalized with COVID-19 revealed altered PUFA metabolism, affecting eicosanoids involved in inflammation, thrombosis, and endothelial function. Furthermore, non-enzymatic metabolism was skewed towards n-3 PUFA-derived metabolites with potential anti-inflammatory and pro-resolving effects. In line with those findings, the OS marker 15-

F_{2t}-isoprostane was significantly lower in patients receiving n-3 PUFA treatment, which was also detected through decreased erythrocyte OS. These findings point to additional beneficial effects of n-3 PUFA treatment through a reduced OS response in COVID-19.

Most lipid mediators are, in line with their biological role as local autacoids, rapidly inactivated through enzymatic and non-enzymatic degradation to several metabolites [33]. Therefore, the plasma metabolome of these molecules is dynamic, and its plasma analysis provides only a snapshot of the metabolic situation *in vivo*. In contrast to the plasma metabolome of PUFA metabolites, the analysis of the urinary oxylipidome yields a more complete picture of time-integrated chemically stable metabolites that reflects systemic biosynthesis [13,14,33]. In addition to the separately reported plasma analyses [12], we here analyzed the urinary eicosanoid metabolites.

The urinary eicosanoid profiling revealed a significant transient increase in the urinary prostacyclin metabolite 2,3-dinor-6-keto PGF_{1 α} by n-3 PUFA treatment. Endothelial dysfunction is a hallmark of COVID-19 [24], which potentially can be improved by increased prostacyclin formation. In contrast to the increase in the urinary prostacyclin metabolite, a trend towards lower thromboxane formation was accompanied by significantly decreased urinary concentrations of the isoprostane 15-F_{2t}-IsoP, which transduces prothrombotic responses by means of the thromboxane prostanoid (TP) receptor. Taken together, these findings reinforced that the thrombotic balance is tipped in a beneficial direction through a decrease in ligands for the pro-aggregatory TP receptor and increased anti-aggregatory prostacyclin.

Importantly, eicosanoids and isoprostanes may contribute to the uncontrolled inflammatory response in COVID-19 [18]. The trend towards decreased urinary LTE₄ by i. v. n-3 PUFA emulsion to the possible therapeutic anti-inflammatory effects of leukotriene receptor antagonists have been evoked for treatment of COVID-19 [25] in addition to specific cytokine antibody treatment [26,27].

The isoprostane 15-F_{2t}-IsoP, which is an established OS marker formed through non-enzymatic oxidative metabolism of the n-6 PUFA arachidonic acid (AA), was significantly lower compared with placebo after n-3 PUFA treatment. Previous results from this trial revealed that plasma levels of EPA and DHA were significantly increased by n-3 PUFA treatment, whereas AA levels were not significantly altered [12]. The increased n-3 PUFA plasma concentrations may, however, skew the enzymatic and non-enzymatic metabolism.

Although few of the individual metabolites were significantly altered by n-3 PUFA treatment, a skewing of the oxidative PUFA metabolism was reflected by the significantly increased n-3/n-6 ratio for the

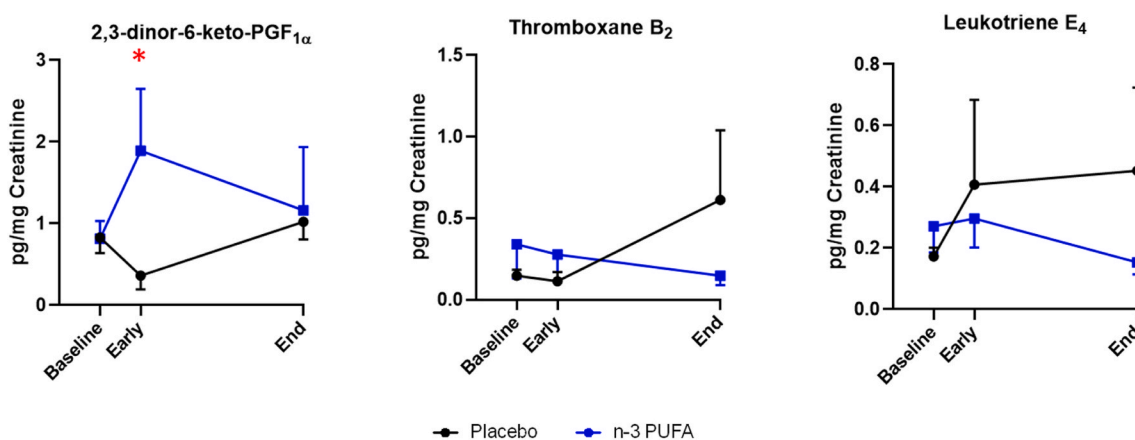


Fig. 1. Mass spectrometry analysis of urinary eicosanoids. Urinary levels of the prostacyclin urinary metabolite 2,3-dinor-6-keto-PGF_{1 α} (left panel), the thromboxane A₂ metabolite thromboxane B₂ (middle panel), and the cysteinyl-leukotriene E₄ (right panel). Urine was collected from patients at baseline, 24–48 h (Early) and after treatment (End) following intravenous infusion (2 mL/kg) of either placebo (NaCl; black symbols, n = 12) or n-3 PUFA emulsion containing 10 g of n-3 polyunsaturated fatty acid emulsion per 100 mL (blue symbols, n = 10) for 5 days. Results (mean \pm SEM) are expressed as pg/mg creatinine. Statistical analyses were performed with 2-way ANOVA for repeated measures and post hoc testing. *p < 0.05 compared to baseline and #p < 0.05 between placebo and n-3 PUFA treatment. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Urinary eicosanoids.

	n-PUFA			Placebo			P		
	Baseline	Early	End	Baseline	Early	End	Interv	Progress	Interv:Progr
Leukotriene E₄									
LTE ₄	0.17 ± 0.03	0.41 ± 0.28	0.45 ± 0.27	0.27 ± 0.09	0.3 ± 0.09	0.15 ± 0.04	0.450	0.332	0.072
Prostaglandin D₂ metabolites									
Tetranor-PGDM	3.65 ± 1.13	3.62 ± 0.89	3.84 ± 0.71	3.35 ± 0.55	4.65 ± 1.09	4.36 ± 1.16	0.561	0.942	0.640
Tetranor-PGJM	2.75 ± 1.01	3.04 ± 0.78	3.3 ± 0.6	2.83 ± 0.54	4.12 ± 1.02	3.75 ± 1.13	0.543	0.911	0.504
2,3-dinor-11-β-PGF _{2α}	0.79 ± 0.43	0.42 ± 0.13	0.35 ± 0.05	0.41 ± 0.07	0.41 ± 0.08	0.51 ± 0.17	0.509	0.917	0.509
	0.11 ± 0.05	0.17 ± 0.09	0.19 ± 0.08	0.12 ± 0.04	0.12 ± 0.04	0.11 ± 0.02	0.467	0.957	0.359
Prostaglandin E₂ metabolites									
PGE ₂	59.7 ± 20.5	40.1 ± 9.81	55.0 ± 13.2	82.7 ± 24.3	75.0 ± 14.5	82.6 ± 17.6	0.107	0.213	0.678
Tetranor-PGAM	0.21 ± 0.06	0.27 ± 0.09	0.52 ± 0.16	1.28 ± 0.59	0.81 ± 0.22	0.87 ± 0.45	0.203	0.448	0.638
Tetranor-PGEM	1.93 ± 0.61	1.29 ± 0.29	1.48 ± 0.3	2.71 ± 0.89	2.55 ± 0.54	2.4 ± 0.69	0.090	0.960	0.667
Tetranor-PGE ₁	57.4 ± 20.0	38.3 ± 9.7	52.6 ± 12.9	77.9 ± 23.4	69.9 ± 13.6	78.8 ± 17.1	0.123	0.178	0.745
	0.11 ± 0.04	0.18 ± 0.09	0.38 ± 0.2	0.73 ± 0.25	1.7 ± 1.07	0.52 ± 0.16	0.163	0.419	0.257
Prostaglandin F_{2α} metabolites									
PGF _{2α}	3.52 ± 0.77	2.64 ± 0.70	3.01 ± 0.55	3.61 ± 0.47	2.95 ± 0.47	2.76 ± 0.34	0.955	0.850	0.569
13,14-dihydro-15-keto PGF _{2α}	1.06 ± 0.09	1.25 ± 0.18	1.8 ± 0.41	1.66 ± 0.21	1.45 ± 0.34	1.17 ± 0.16	0.481	0.643	0.158
Tetranor-PGFM	1.42 ± 0.15	1.79 ± 0.23	2.24 ± 0.31	1.87 ± 0.39	1.54 ± 0.44	1.24 ± 0.26	0.141	0.713	0.095
	2.46 ± 0.74	1.39 ± 0.65	1.2 ± 0.31	1.95 ± 0.4	1.5 ± 0.28	1.59 ± 0.26	0.612	0.887	0.636
Prostacyclin metabolite									
2,3-dinor-6-keto PGF _{1α}	0.83 ± 0.19	0.36 ± 0.17	1.02 ± 0.22	0.81 ± 0.22	1.89 ± 0.76	1.16 ± 0.78	0.310	0.882	0.009*
Thromboxane A₂ metabolites									
TXB ₂	4.69 ± 1.02	4.16 ± 0.86	18.9 ± 14.5	4.45 ± 1.25	6.19 ± 2.53	4.89 ± 1.59	0.404	0.356	0.273
2,3-dinor TXB ₂	0.15 ± 0.04	0.11 ± 0.06	0.61 ± 0.43	0.34 ± 0.21	0.28 ± 0.11	0.15 ± 0.06	0.511	0.375	0.139
11-dehydro TXB ₂	1.07 ± 0.2	1 ± 0.27	4.16 ± 3.18	0.85 ± 0.25	1.27 ± 0.53	0.63 ± 0.15	0.306	0.424	0.232
11-dehydro-2,3-dinor TXB ₂	2.32 ± 0.52	2.29 ± 0.46	8.25 ± 5.99	2.32 ± 0.76	3.01 ± 1.31	3.28 ± 1.53	0.488	0.326	0.367
	1.15 ± 0.41	0.76 ± 0.17	5.91 ± 4.91	0.94 ± 0.24	1.63 ± 0.69	0.85 ± 0.16	0.384	0.367	0.225

Results are presented as pg/mg creatinine (mean ± SD).

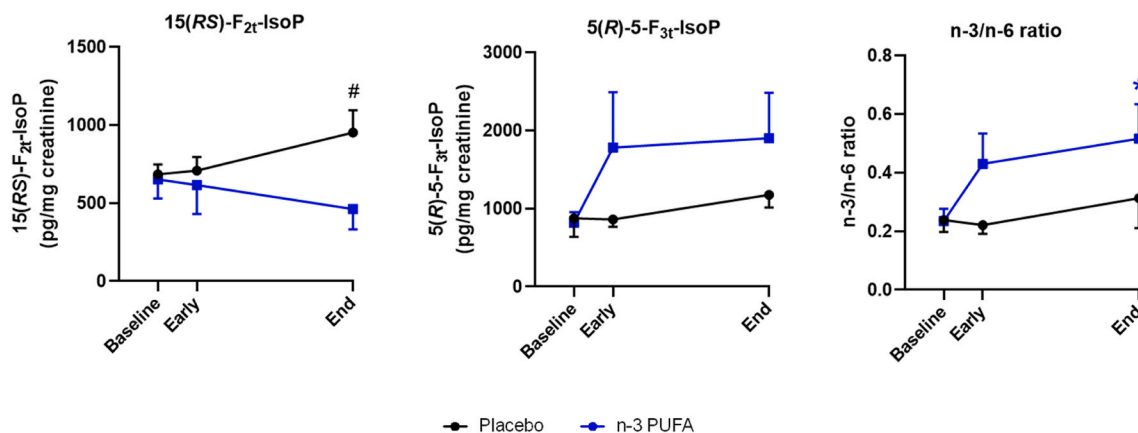


Fig. 2. Mass spectrometry analysis of urinary isoprostanes (IsoP) derived from polyunsaturated fatty acids (PUFA). The left panel shows the n-6 PUFA-derived isoprostane 15(RS)-15-F_{2t}-IsoP and the middle panel shows the n-3 PUFA-derived isoprostane 5(R)-5-F_{3t}-IsoP. Results (mean ± SEM) are expressed as pg/mg creatinine. The right panel shows the ratio (mean ± SEM) between total urinary IsoP derived from n-3 and n-6 PUFA. Urine was collected from patients at baseline, 24–48 h (Early) and after treatment (End) following intravenous infusion (2 mL/kg) of either placebo (NaCl; black symbols, n = 10) or n-3 PUFA emulsion containing 10 g of n-3 PUFA per 100 mL 100 mL (blue symbols, n = 10) for 5 days. Statistical analyses were performed with 2-way ANOVA for repeated measures and post hoc testing. *p < 0.05 compared to baseline and #p < 0.05 between placebo and n-3 PUFA treatment. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

complete non-enzymatic urinary oxylipidome after i. v. n-3 PUFA treatment of COVID-19. Within the n-3 PUFA metabolome, the n-3 PUFA isoprostane F_{3t}-IsoP is part of the neuroprostane family of proresolving mediators [7]. EPA and DHA are enzymatically metabolized into SPM, which directly can contribute to the observed anti-oxidative effects [28]. n-3 PUFA are also subjected to non-enzymatical oxidative metabolism, yielding additional mediators of the resolution of inflammation [7].

Results from EPR spectroscopy using the spin probe CMH for high precision quantification revealed a significantly lower ROS production in erythrocytes derived from n-3 PUFA- compared with placebo-treated

COVID-19 patients in the present study, which may reflect established direct antioxidant effects of n-3 PUFA as well as the skewed lipid metabolism away from n-6 isoprostanes. Furthermore, elevated ROS production in erythrocytes from patients with COVID-19 induce endothelial dysfunction *ex vivo* [3]. Therefore, treatment with n-3 PUFA might improve vascular function in patients with COVID-19 by means of increasing prostacyclin and restoring the redox balance in erythrocytes. A high neutrophil to lymphocyte ratio (NLR) reflects neutrophil-induced oxidative stress in COVID-19, which contributes to disease severity in terms of involvement in tissue damage, thrombosis, and red blood cell

Table 3
Urinary oxidation metabolites.

	n-3 PUFA			Placebo			P		
	Baseline	Early	End	Baseline	Early	End	Interv	Progress	Interv: Progr
AA oxidation	21.10 ± 3.790	20.30 ± 5.190	16.90 ± 2.720	18.50 ± 1.78	22.40 ± 2.890	25.60 ± 4.45	0.308	0.984	0.144
5(RS)-5-F _{2t} -IsoP	1.50 ± 0.270	1.33 ± 0.280	1.19 ± 0.230	1.42 ± 0.190	1.68 ± 0.220	2.02 ± 0.310	0.114	0.404	0.052
5-F _{2c} -IsoP	13.80 ± 2.780	13.70 ± 4.32	11.00 ± 2.370	12.30 ± 1.100	16.30 ± 2.210	17.60 ± 3.170	0.284	0.675	0.221
15(RS)-F _{2t} -IsoP	0.65 ± 0.120	0.62 ± 0.180	0.46 ± 0.120	0.66 ± 0.062	0.68 ± 0.081	0.93 ± 0.130	0.134	0.627	0.042
15-F _{2t} -IsoP	0.24 ± 0.047	0.22 ± 0.074	0.17 ± 0.047	0.24 ± 0.031	0.22 ± 0.026	0.31 ± 0.045	0.282	0.542	0.046
15-epi-15-F _{2t} -IsoP	0.41 ± 0.081	0.40 ± 0.110	0.29 ± 0.086	0.42 ± 0.041	0.46 ± 0.060	0.61 ± 0.087	0.101	0.717	0.046
15-F _{2t} -IsoP Metabolites	4.60 ± 0.761	4.16 ± 0.730	3.77 ± 0.339	3.81 ± 0.649	3.38 ± 0.518	4.45 ± 1.013	0.957	0.469	0.127
2,3-dinor-15-F _{2t} -IsoP	2.26 ± 0.340	2.08 ± 0.336	1.91 ± 0.197	1.94 ± 0.329	1.78 ± 0.299	2.29 ± 0.504	0.926	0.476	0.163
2,3-dinor-15-epi-15-F _{2t} -IsoP	1.94 ± 0.367	1.66 ± 0.329	1.53 ± 0.153	1.46 ± 0.298	1.25 ± 0.199	1.71 ± 0.400	0.745	0.383	0.125
ent-2,3-dinor-5,6-dihydro-15F _{2t} -IsoP	0.41 ± 0.069	0.42 ± 0.083	0.33 ± 0.051	0.41 ± 0.065	0.35 ± 0.056	0.46 ± 0.136	0.786	0.903	0.103
15-A ₂ -IsoP	0.52 ± 0.100	0.43 ± 0.064	0.46 ± 0.056	0.27 ± 0.039	0.34 ± 0.062	0.61 ± 0.132	0.770	0.014	0.046
EPA oxidation	3.05 ± 0.497	4.71 ± 0.962	4.91 ± 1.161	3.29 ± 0.739	2.85 ± 0.320	5.10 ± 1.667	0.529	0.208	0.288
5(R)-5-F _{3t} -IsoP	0.818 ± 0.130	1.78 ± 0.707	1.90 ± 0.581	0.82 ± 0.220	0.84 ± 0.086	1.17 ± 0.144	0.187	0.391	0.687
5(S)-5-F _{3t} -IsoP	1.71 ± 0.388	2.48 ± 0.513	2.63 ± 0.716	2.18 ± 0.470	1.62 ± 0.258	3.52 ± 1.550	0.992	0.219	0.291
DHA oxidation	1.37 ± 0.503	1.63 ± 0.542	2.81 ± 1.660	1.03 ± 0.182	1.49 ± 0.316	1.36 ± 0.206	0.488	0.378	0.270
4(RS)-4-F _{4t} -NeuroP	0.80 ± 0.011	0.10 ± 0.010	0.10 ± 0.020	0.09 ± 0.005	0.12 ± 0.015	0.11 ± 0.019	0.648	0.645	0.856
14(RS)-14-F _{4t} -NeuroP	1.28 ± 0.500	1.53 ± 0.550	2.71 ± 1.660	0.941 ± 0.170	1.38 ± 0.309	1.25 ± 0.200	0.483	0.374	0.271
ALA oxidation	1.11 ± 0.129	1.49 ± 0.320	1.25 ± 0.330	1.87 ± 0.690	1.34 ± 0.170	2.81 ± 1.120	0.318	0.262	0.128
ent-16-F _{1t} -PhytoP	0.35 ± 0.052	0.41 ± 0.130	0.31 ± 0.130	0.98 ± 0.560	0.45 ± 0.078	1.29 ± 0.606	0.151	0.230	0.133
ent-16-epi-16-F _{1t} -PhytoP	0.32 ± 0.043	0.34 ± 0.420	0.35 ± 0.100	0.39 ± 0.077	0.37 ± 0.061	0.76 ± 0.230	0.130	0.134	0.145
9-F _{1t} -PhytoP	0.14 ± 0.032	0.22 ± 0.035	0.16 ± 0.032	0.13 ± 0.018	0.20 ± 0.023	0.32 ± 0.160	0.431	0.665	0.293
9-epi-9-F _{1t} -PhytoP	0.12 ± 0.021	0.19 ± 0.056	0.15 ± 0.052	0.14 ± 0.036	0.16 ± 0.033	0.24 ± 0.110	0.736	0.616	0.169
ent-16-B ₁ -PhytoP	0.15 ± 0.043	0.28 ± 0.102	0.25 ± 0.098	0.20 ± 0.049	0.14 ± 0.040	0.17 ± 0.043	0.299	0.902	0.410
ent-9-L ₁ -PhytoP	0.03 ± 0.005	0.05 ± 0.002	0.04 ± 0.001	0.03 ± 0.005	0.03 ± 0.007	0.03 ± 0.005	0.472	0.375	0.396
18(S)-18-F _{3t} -IsoP	0.52 ± 0.130	0.45 ± 0.089	0.38 ± 0.110	0.29 ± 0.098	0.39 ± 0.052	0.42 ± 0.089	0.892	0.783	0.532

Results are presented as pg/mg creatinine (mean ± SD).

dysfunction. Importantly, i. v. n-3 PUFA emulsion treatment significantly reduces the NLR in COVID-19 [12]. The present study hence extends the potential therapeutic effects of anti-oxidative treatments in COVID-19 [29] by showing reduced OS by n-3 PUFA.

This is the first report of effects of i. v. n-3 PUFA emulsion on the oxidative PUFA metabolism in COVID-19. There are, however, limitations that should be acknowledged. Since placebo was NaCl, it cannot be determined which of the constituents of the n-3 PUFA emulsion were active. It should furthermore be pointed out that the emulsion contains the anti-oxidant dl- α -tocopherol (0.02–0.03 g/100 mL). However, the notion that the altered oxylipidome being dependent of a rapid increase in DHA and EPA [12], was further reinforced by the present study, showing an increased ratio between the urinary isoprostanes derived from n-3 and n-6 PUFA. The PUFA substrate availability in cells and tissues was not determined in this study. The low number of participants is also a limitation, and larger studies are needed to determine the relation of the observed beneficial effects of i. v. n-3 PUFA emulsion on oxidative stress to clinical outcomes in COVID-19. Finally, the older study population may limit the extrapolation of the results to younger subjects.

5. Conclusions

In summary, the present study shows beneficial effects on OS exerted by i. v. n-3 PUFA emulsion treatment of COVID-19 through (a) altered levels of eicosanoids and isoprostanes toward a beneficial profile for inflammation, thrombosis, and endothelial function, (b) reduced urinary levels of the oxidative stress marker 15-F_{2t}-isoprostane and decreased erythrocyte OS, and (c) a skewed non-enzymatic metabolism toward n-3

PUFA-derived oxylipin metabolites with potential anti-inflammatory and proresolving effects. Meta-analyses have shown significantly shorter length of intensive care hospital stay and reduced infections by n-PUFA lipid emulsion in parental nutrition [30,31]. The use of n-3 PUFA emulsions as treatment of COVID-19 [32] induced beneficial immunomodulation [12]. In conclusion, the present trial identified additional beneficial effects of n-3 PUFA treatment on the OS response in COVID-19.

Funding sources

The study received a research grant from King Gustaf V and Queen Victoria Freemason Foundation. The investigators were supported by the Swedish Research Council [Grant number 2019-01486], the Swedish Heart and Lung Foundation [grant numbers 20180571, 20190625; 20190196; 20200693; 20210519], Cayman Biomedical Research Institute, CABRI, and Stiftelsen Professor Nanna Svartz Fond. The sources of funding had no access to the study data and no role in the design, implementation, or reporting.

Registration in trial registries

This trial “Resolving Inflammatory Storm in COVID-19 Patients by Omega-3 Polyunsaturated Fatty Acids - A single-blind, randomized, placebo-controlled feasibility study” (COVID-Omega-F) is registered in the European Union Drug Regulating Authorities Clinical Trials (EudraCT) database with number 2020-002293-28 and at Clinical Trials.gov with number NCT04647604.

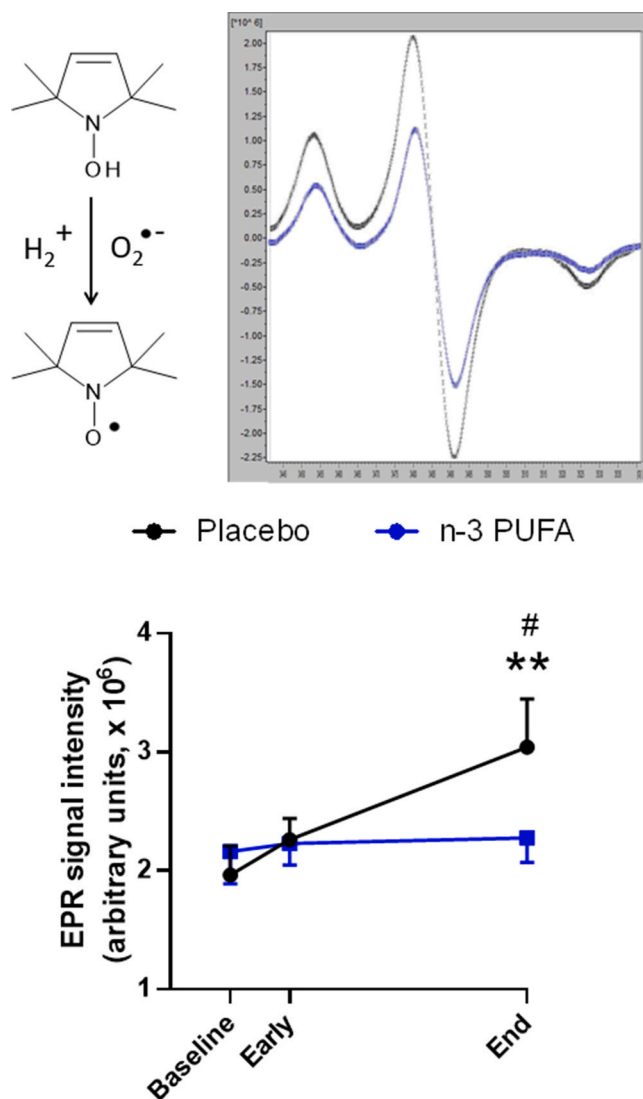


Fig. 3. Erythrocyte oxidative stress. The upper panel illustrates the oxidation reaction of the CMH-hydrochloride probe by ROS into EPR visible species (left) and shows representative spectra obtained by EPR spectroscopy from the measurement of ROS produced by erythrocytes derived from patients randomized to either placebo (black) or i. v. n-3 PUFA treatment (blue). The lower panel shows the absorbance (mean \pm SEM) for measurement of ROS production following CMH-hydrochloride incubation of erythrocytes derived from patients at baseline, at 24–48 h (Early), and after treatment (End) with i. v. infusion (2 mL/kg) of either placebo (black symbols, NaCl; n = 5) or n-3 PUFA emulsion containing 10 g of fish oil per 100 mL (blue symbols; n = 5). Statistical analyses were performed with 2-way ANOVA for repeated measures and post hoc testing. **p < 0.01 compared to baseline and #p < 0.05 between placebo and n-3 PUFA treatment. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Data availability

Individual participant data that underlie the results reported will be shared, after de-identification, with researchers who provide a methodologically sound proposal.

Time frame

Beginning 9 months following article publication and finishing 36 months following article publication.

Access criteria

Investigators interested in data should contact the corresponding author.

Declaration of competing interest

None.

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