Table 1. Study participants' characteristics at baseline. Summary statistics are presented as frequency (percentage) or median ± interquartile range.

Variable	Summary Statistics
Sex	Female: 7 (23.33%) Male: 23 (76.67%)
Age (years)	10.68 ± 7.25
BMI (Kg/m^2)	17.70 ± 3.99
BMI z-score	0.03 ± 1.49
HbA1c (%)	5.20 ± 0.20
HbA1c (mmol/mol)	33.00 ± 2.25
FPG (mg/dL)	111.00 ± 31.00
FPI (µU/mL)	21.95 ± 11.50
TG (mg/dL)	190.00 ± 177.25
HOMA-IR index	5.15 ± 5.69
TyG index	9.20 ± 0.73
Total cholesterol (mg/dL)	118.00 ± 72.00
HDL cholesterol (mg/dL)	17.00 ± 21.00
TSH (mIU/L)	2.16 ± 1.81
GGT (IU/L)	26.50 ± 38.75
ALT (IU/L)	31.00 ± 45.50
Creatine kinase (IU/L)	68.00 ± 102.00
Albumin (g/L)	25.50 ± 7.50
Sodium (mEq/L)	132.00 ± 5.00
Potassium (mEq/L)	3.50 ± 0.90
Ferritin (µg/L)	745.00 ± 1259.25
IL-6 (ng/L)	83.00 ± 208.50
C-reactive protein (mg/dL)	236.50 ± 176.00
Procalcitonin (µg/L)	6.2 ± 11.20
NT-proBNP (ng/L)	7554.00 ± 11,143.00

BMI: Body Mass Index; HbA1c: glycated hemoglobin; FPG: fasting plasma glucose; FPI: fasting plasma insulin; TG: fasting triglycerides; HOMA-IR: homeostasis model analysis—insulin resistance index; TyG: triglyceride–glucose index; HDL cholesterol: high-density lipoprotein cholesterol; TSH: thyroid-stimulating hormone; GGT: gamma-glutamyl transferase; ALT: alanine transaminase; IL-6: interleukin-6; NT-proBNP: N-Terminal pro-Brain Natriuretic Peptide.

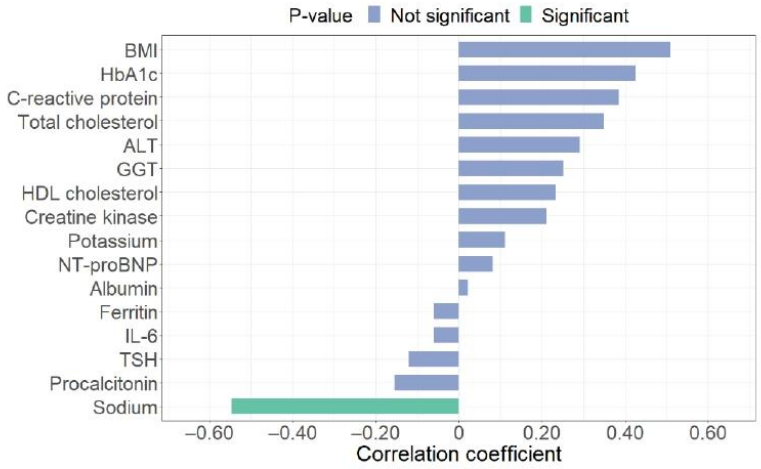

Figure 1. Spearman correlation coefficients between clinical and biochemical parameters and homeostasis model analysis—insulin resistance (HOMA-IR) index. BMI: Body Mass Index; HbA1c: glycated hemoglobin; ALT: alanine transaminase; GGT: gamma-glutamyl transferase; NT-proBNP: N-Terminal pro-Brain Natriuretic Peptide; IL-6: interleukin-6; TSH: thyroid-stimulating hormone.

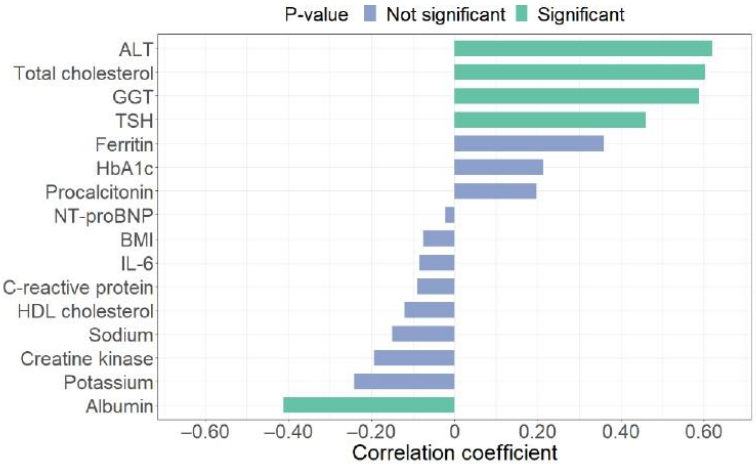

Figure 2. Spearman correlation coefficients between clinical and biochemical parameters and triglyceride–glucose (TyG) index. ALT: alanine transaminase; GGT: gamma-glutamyl transferase; TSH: thyroid-stimulating hormone; HbA1c: glycated hemoglobin; NT-proBNP: N-Terminal pro-Brain Natriuretic Peptide; BMI: Body Mass Index; IL-6: interleukin-6.

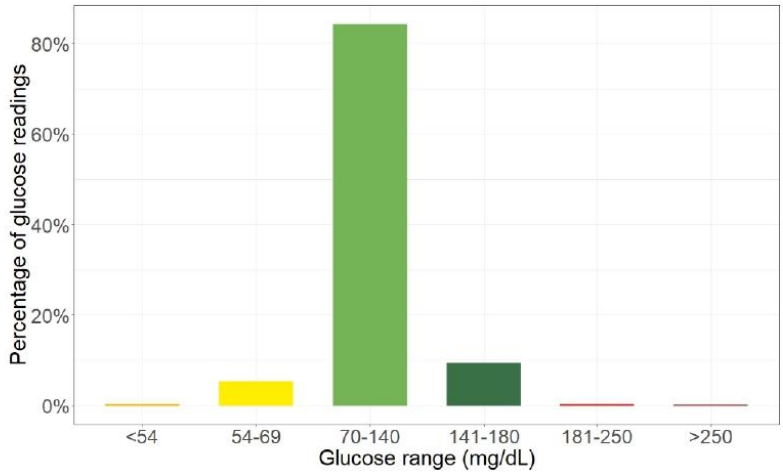

Figure 3. Bar representation of average time in ranges.

Specifically, four patients reveal glycemic fluctuations over the 180 mg/dL threshold of hyperglycemia, as shown by the time series chart in Figure 4.

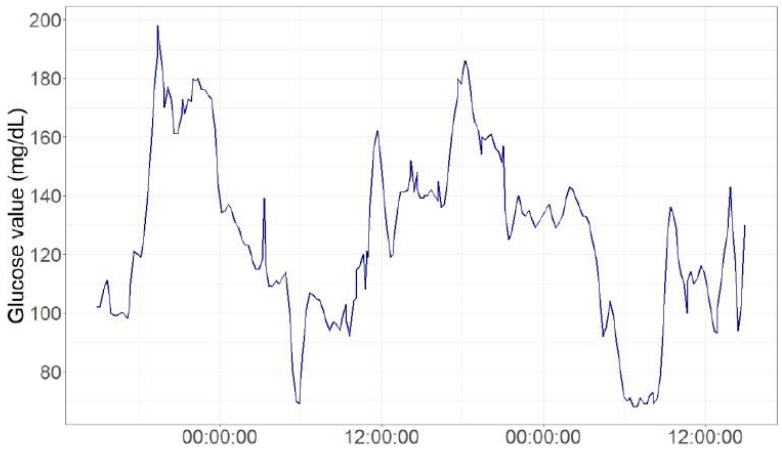

Figure 4. Glycemic fluctuations in a patient 48-h monitoring window.

4. Discussion

MIS-C is a critical illness in children and adolescents, appearing several weeks after initial infection [6–9]. Thus far, no universally agreed upon approach is available for this syndrome [9] and, to the best of our knowledge, no one has previously described glucose-insulin metabolic disorders in a pediatric population affected by MIS-C. Analyzing our cohort of 30 pediatric patients affected by MIS-C, we noted that IR, glycemic fluctuations and/or hyperglycemia can occur.

Alterations in glucose metabolism are common in severely ill patients. As described for other critical illnesses [11,12], a hypermetabolic state also exists in this kind of patient and the adaptive response allows vital organs to conserve energy. The response appears to be driven by counter-regulatory hormones and cytokines, which may be important mediators of IR, and result in mild to moderate hyperglycemia that provides fuel for the brain and immune system after stress conditions. As happened to two of our patients, an insulin therapy may be necessary for limiting glycemic metabolic imbalance [28].

In our children, the high prevalence of pathological values in HOMA-IR and TyG indexes supports a both hepatic and peripheral impaired insulin action. The action of counter-regulatory hormones on IR in skeletal muscles might be mediated through an increase in the circulating free fatty acid level, despite hyperinsulinemia [11,12]. The correlation between IR markers and lipids, hepatic parameters, thyroid values, electrolytes, and albumin may support the predominance of catabolic condition and the impairment of glucose homeostasis within the body.

As reported in the literature, an interaction between COVID-19 and glucose-insulin metabolic disorders is postulated in adults [1,29] and not excluded in pediatrics [30,31]. In particular, the relationship between COVID-19 and type 2 diabetes mellitus (T2DM) has been extensively described in adults [1,2,30] and a relationship between SARS-CoV-2 infection and type 1 diabetes mellitus (T1DM) has also been discussed in children [30–36].

This is possibly the first up-to-date study on the relationship between IR and glycemic fluctuation in normal weight children without glycemic disorders. Usually, IR is noted in children and adolescents who are overweight or have moderate to severe obesity. Our results are not surprising in terms of adaptive metabolic response; nevertheless, in this clinical context, a bidirectional relationship between COVID-19 and glycemic impairment could not be excluded. Pancreatic β cells are permissive to SARS-CoV-2 infection with receptor angiotensin-converting enzyme 2 (ACE2) as its entry [37,38]. As considered by Hoffmann [2], hyperglycemia and glycemic fluctuations could be caused by the inflam-

matory cascade of the attack of SARS-CoV-2 on the pancreas and the potentially impaired β-cell function.

Even though the optimal therapeutic approach to a child with MIS-C has not been defined yet, most patients have been treated with the standard therapeutic protocols including glucocorticoids and intravenous immunoglobulin; thus, the iatrogenic effect on glycemic fluctuation may also be considered. Nevertheless, the evaluation of the HOMA-IR and TyG indexes has been performed at the admission before the start of therapy, as far as possible due to patients' emergency care. Therefore, the hypothesis of the iatrogenic mechanism of IR can be reasonably excluded.

Hyperglycemia is not a physiological or benign condition; clinically, hyperglycemia has been linked to increased incidences of sepsis, longer hospital stays and higher mortality [39–41]. A continuous glucose monitoring may be useful to detect glycemic fluctuation and to prevent metabolic imbalance.

Several limitations should be addressed in this study. Firstly, the small sample size and a male predominance (only seven subjects were females, 23%) might have limited the analyses. Secondly, we were unable to compare isCGM data of the same patients before and after the SARS-CoV-2 infection to obtain an accurate evaluation of the direct influence of COVID-19 on glycemic levels; however, the normal range for HbA1c can support the exclusion of a pre-existing diabetes. Although the gold standard for IR detection is the hyperinsulinemic-euglycemic clamp technique, we used the validated indirect measurement of IR since the clamp in pediatric subjects is cumbersome, time consuming and technically difficult to perform in routine clinical practice. Finally, no genetic susceptibility to type 1 diabetes and/or serum autoantibodies against β-cell antigens were detected. To confirm and strengthen our results, further multicenter collaborative studies are necessary.

5. Conclusions

In this exploratory study, we recorded IR and glycemic fluctuation in pediatric patients with MIS-C. Regular glucose monitoring of both fasting and post-prandial glucose levels may be useful for a better outcome. Moreover, these findings also underscore the concerns for new onset diabetes in the COVID-19 pandemic.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy restrictions.

Conflicts of Interest: The authors declare no conflict of interest.

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5. 3 Manuscript 2

"Blood Fatty acid profile in MIS-C children in acute phase"

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Communication

Blood Fatty Acids Profile in MIS-C Children

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Abstract: MIS-C (multisystem inflammatory syndrome in children) linked to SARS-CoV-2 infection, is a pathological state observed in subjects younger than 21 years old with evidence of either current SARS-CoV-2 infection or exposure within the 4 weeks prior to the onset of symptoms, the presence of documented fever, elevated markers of inflammation, at least two signs of multisystem involvement, and, finally, lack of an alternative diagnosis. They share with adult COVID-19 patients the presence of altered markers of inflammation, but unlike most adults the symptoms are not pulmonary but are affecting several organs. Lipid mediators arising from polyunsaturated fatty acids (PUFA) play an important role in the inflammatory response, with arachidonic acid-derived compounds, such as prostaglandins and leukotrienes, mainly pro-inflammatory and ω3 PUFA metabolites such as resolvins and protectins, showing anti-inflammatory and pro-resolution activities. In order to assess potential alterations of these FA, we evaluated the blood fatty acid profile of MIS-C children at admission to the hospital, together with biochemical, metabolic and clinical assessment. All the patients enrolled showed altered inflammatory parameters with fibrinogen, D-dimer, NT-proBNP, ferritin, aspartate aminotransferase (AST), C-reactive protein (CRP) and TrygIndex levels over the reference values in all the subjects under observation, while albumin and HDL-cholesterol resulted below the normal range. Interestingly, linoleic acid (LA), arachidonic acid (AA) and the ω3 PUFA docosahexaenoic acid (DHA) results were lower in our study when compared to relative amounts reported in the other studies, including from our own laboratory. This significant alteration is pointing out to a potential depletion of these PUFA as a result of the systemic inflammatory condition typical of these patients, suggesting that LA- and AA-derived metabolites may play a critical role in this pathological state, while ω3 PUFA-derived pro-resolution metabolites in these subjects may not be able to provide a timely, physiological counterbalance to the formation of pro-inflammatory lipid mediators. In conclusion, this observational study provides evidence of FA alterations in MIS-C children, suggesting a significant contribution of ω6 FA to the observed inflammatory state, and supporting a potential dietary intervention to restore an appropriate balance among the FAs capable of promoting the resolution of the observed inflammatory condition.

Keywords: arachidonic acid; docosahexaenoic acid; eicosanoids; specialized pro-resolution mediators; inflammation

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1. Introduction

During the COVID-19 pandemic, children appeared to be less affected than adults, and Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) infections were in most cases asymptomatic or had mild symptoms [1–3]. However, from April 2020, in several western countries, a number of cases of children and adolescents with fever, hypotension, severe abdominal pain and cardiac dysfunction have been reported [4–8].

As stated by the Center of Diseases Control and Prevention (CDC), MIS-C (multisystem inflammatory syndrome in children) linked to SARS-CoV-2 infection requires patients to be less than 21 years old and to have evidence of either current SARS-CoV-2 infection or exposure within 4 weeks prior to the onset of symptoms, the presence of documented fever, elevated markers of inflammation, at least two signs of multisystem involvement, and, finally, lack of an alternative diagnosis (e.g., bacterial sepsis, toxic shock syndrome) [9]. While in Asian countries no cases of MIS-C were reported [10], in Western countries MIS-C represents a critical health condition associated with SARS-CoV-2 infection [11–14].

In COVID-19 adult patients, COVID-19 is typically associated with a significant degree of inflammation with an increase in interleukin 6 (IL6), C-reactive protein (CRP), fibrinogen, and erythrocyte sedimentation rate (ESR). High concentrations of D-dimer have been associated with increased mortality from COVID-19, and evidence in the literature suggest that COVID-19 coagulopathy is largely determined by the host inflammatory response, which in turn causes a pro-thrombotic status [15], leading to severe pulmonary manifestations similar to acute respiratory distress syndrome (ARDS) [16], even if non pulmonary organ damage was also present in the most severe cases [17].

In children with SARS-CoV-2 infections, the pulmonary manifestations are less severe than in adults, possibly because of a lower gene expression of the angiotensin converting enzyme (ACE)-2 receptor [18]. On the other hand, it has been suggested that MIS-C is a delayed immunological phenomenon associated with inflammation (stage-III hyperinflammation phase) following either symptomatic or asymptomatic COVID-19 infection [19], again pointing to a critical role of innate immunity and acute inflammation in the most severe forms of COVID-19.

The acute inflammatory response is associated with the production of several lipid mediators, including eicosanoids, a large family of compounds arising from the oxidative metabolism arachidonic acid known to play a significant role in various vascular and cellular events of the inflammatory response [20,21].

More recently, evidence has emerged that lipid mediators may also play a relevant role in the process leading to the physiological resolution of the inflammatory process, with a new genus of metabolites derived from ω3 polyunsaturated fatty acids (ω3-PUFA) being identified as possessing potent anti-inflammatory and pro-resolution properties [22]. In particular, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) metabolites resulting from the activity of several lipoxygenases have been collectively defined as specialized pro-resolving mediators (SPMs), a family which includes resolvins, protectins, maresins, and maresin-conjugate in tissue regeneration [23].

SPMs produced by the metabolism of ω3-PUFA have been reported to decrease the synthesis of pro-inflammatory mediators and neutrophil recruitment, activating at the same time macrophages with an anti-inflammatory phenotype (M2) and stimulating phagocytosis in a non-phlogistic manner [24]. Given their profile of activities, they clearly operate differently than currently available anti-inflammatory drugs, and do not show immunosuppressive effects [25].

Epidemiological data clearly show that obesity is a risk factor for SARS-CoV-2 infection and a BMI of 30 kg/m^2 increases the risk of infection by 61% in adult patients. Interestingly, it has been hypothesized that a deficiency of SPMs in obese patients may promote severe outcomes during SARS-CoV-2 infection [26].

On the other hand, the use of the available enzyme inhibitor and receptor antagonists targeting 5-lipoxygenase metabolites of AA has been proposed as a strategy to limit the hyperinflammatory response to SARS-CoV-2 [27], in consideration of the evidence available

about the presence of elevated leukotrienes in ARDS [28], and the efficacy of inhibitors and receptor antagonists in relevant preclinical models [29].

Taken together, a large body of evidence supports a potentially critical involvement of PUFA-derived lipid mediators in the evolution of the SARS-CoV-2 infection, and changes in serum lipid species have indeed been reported, with significantly decreased concentrations of AA and AA-containing phospholipids in adults and elderly (30–77 yo) COVID-19 patients [30].

In consideration of the potentially critical role played by PUFA metabolites in the evolution of SARC-CoV-2 infections in both young and adults, we carried out this observational study in order to assess fatty acid profiles in children and adolescents diagnosed with MIS-C while hospitalized at the Vittore Buzzi Children's Hospital in Milan, Italy, during the pandemic.

2. Results

Table 1 summarizes the characteristics of the subjects at the study; children and adolescents after remission from COVID-19 were released from the intensive care unit (ICU) and moved to the pediatric unit at the Buzzi hospital, Milano, Italy. Of the 26 subjects studied, 81% were males; weight, BMI and BMI z score are reported. During ICU care, feeding was minimal, with a few cases requiring parenteral nutrition. The pharmacological regimen consisted in oral or i.v. corticosteroids (26 over 26), enoxaparin (22 over 26), ASA (5 over 26), and immunoglobulins (3 over 26).

Table 1. Anthropometric data of MIS-C children at hospital admission. Values are expressed as mean ± standard deviation (SD).

	Mean ± SD	**Reference Values**
N of subjects	26	
M/F	21/5	
Age (mo)	110.79 ± 50.29	
Height (cm)	135.86 ± 24.24	
Weight (kg)	36.35 ± 16.26	
BMI (kg/m^2)	18.58 ± 3.19	
BMI z score [31]	0.48 ± 1.00	±2 z score obesity and malnutrition (CDC)
Arm circ (cm) [32]	21.16 ± 4.27	<5° or >90° pc (NANHES III)
Waist circ (cm) [32]	66.55 ± 10.48	<5° or >90° pc (NANHES III)
Triceps skinfold (mm) [32]	15.05 ± 7.80	<5° or >90° pc (NANHES III)

The biochemical parameters reported in Table 2 showed that fibrinogen, D-dimer, NT-proBNP, ferritin, aspartate aminotransferase (AST), C-reactive protein (CRP) and TyG index levels were over the reference values in all the subjects (26/26 = 100%). An increase of TnT (68%), procalcitonin (96%), erythrocyte sedimentation rate (ESR) (61%) and IL-6 (89%) was also observed. Some parameters were below the normal values, as was the case for albumin and HDL cholesterol (100% of cases), as well as hemoglobin (72%).

The whole blood fatty acid profiles of these patients (Table 3) showed palmitic (16:0) and oleic (18:1 $n-9$) acids as the most abundant FA, followed by linoleic (18:2 $n-6$) and stearic (18:0) acids. Saturated FA accounted for 41.39 ± 2.39%, while polyunsaturated represented 24.13 ± 2.75 of total FA. The omega 3 index (OI3), calculated as described by Stark [36], ranged from 1.60% to 4.15% with a mean of 2.35%, with only one case where the O3I was higher than 4%, while all others cases were below the value of 3%.

Table 2. Biochemical data of MIS-C children at hospital admission. Values are expressed as median and 25th–75th percentile. The % of subjects with values outside of the reference intervals is also reported.

Laboratory Markers of Inflammation	Median (25th–75th Percentile)	Reference Values	% Subjects Outside of the Ref Values
Fibrinogen [33]	7 (6.4–7)	<4 g/L	100
D-dimer [33]	2734.5 (1956–4432.5)	<500 µg/L	100
CRP	236.5 (109–291.4)	≤10 mg/L	100
ESR	38 (23–69)	≤30 mm	61
Procalcitonine [33]	6.9 (2.3–24)	<0.5 µg/L	96
IL-6 [33]	75 (10–253)	≤7 ng/L	89
LDH [33]	255 (221.2–287)	180–360 U/L	
Ferritin [33]	875.5 (414.8–1960.2)	<300 µg/L	100
Albumin	25.5 (22.5–29)	35–50 g/L	100
Biochemistry			
Hb [33]	11 (9.2–11.6)	11.5–15.5 g/dL	72
Leukocytes	8.4 (5.1–14.2)	4.5–10 × 10^9/L	40
Platelets	156 (111.5–210.5)	M 155–320, F 169–359 × 10^9/L	44
INR	1.3 (1.2–1.4)	<1.2	76
Ratio aPTT	1.4 (1.2–1.4)	0.84–1.16	86
TnT	47.5 (16–81.5)	≤15 ng/L	81
NT-proBNP	6619 (2621–14,594)	<450 ng/L	100
Creatinine	0.5 (0.4–0.7)	0.15–0.75 mg/dL	
Urea	29 (16.5–44)	19–50 mg/dL	
CK	68 (35.8–147.2)	M 47–322 U/L; F 29–201 U/L	
AST	57 (43–88.5)	11–34 U/L	100
ALT	31 (16.5–63.2)	M ≤ 49U/L, F ≤ 33 U/L	32
GGT	26.5 (20–56.2)	M 12–68 U/L, F 6–40 U/L	20
Na^+ [33]	132 (130–135)	135–145 mmol/L	16
K^+ [33]	3.5 (3–4)	3.5–5 mmol/L	52
TSH [33]	2.3 (1.3–3)	0.5–4.2 mIU/L	
fT3 [33]	2.7 (2–3.6)	3.5–6.3 pmol/L	80
fT4 [33]	12.2 (11.1–14.3)	9–19.3 pmol/L	
Blood lipid and glucose metabolism			
Total cholesterol [33]	120 (85–164)	<170 mg/dL	12
HDL-cholesterol [33]	16 (7–25.2)	>45 mg/dL	100
Triglycerides [33]	190 (124–303.2)	<75 mg/dL 0–9 yo <90 mg/dL age 10–19 yo	52
TyG Index [34]	9.2 (8.8–9.7)	<7.88	100
Glucose [35]	110.5 (95.5–125.2)	70–110 mg/dL	42
HbA1c [35]	33 (32–34.8)	≤39 mmol/mol	

Table 3. Whole blood fatty acid profile in children (n = 26) with MIS-C. Data are expressed as mean ± standard deviation (SD) of FA of the relative percentage (*weight/weight*) of all FA considered, analyzed as described in Methods.

FA	% w/w ± SD	(Min–Max)
16:0	27.99 ± 1.68	(24.65–30.90)
18:0	10.16 ± 1.26	(7.61–13.03)
20:0	0.41 ± 0.10	(0.26–0.62)
22:0	1.05 ± 0.22	(0.67–1.45)
24:0	1.75 ± 0.57	(1.13–3.05)
16:1	3.32 ± 1.09	(1.58–6.44)
18:1 $n-9$	26.87 ± 3.18	(20.30–32.72)
18:1 $n-7$	1.82 ± 0.52	(1.18–3.94)
20:1	0.19 ± 0.10	(0.05–0.63)
22:1	0.09 ± 0.04	(0.02–0.18)
24:1	2.21 ± 0.64	(1.55–3.64)
20:3 $n-9$	0.15 ± 0.10	(0.04–0.44)
18:2 $n-6$	12.39 ± 2.28	(8.29–16.99)
18:3 $n-6$	0.64 ± 0.36	(0.09–1.78)
20:3 $n-6$	1.09 ± 0.26	(067–1.74)
20:4 $n-6$	6.38 ± 1.05	(4.86–8.54)
22:4 $n-6$	0.81 ± 0.21	(0.54–1.35)
22:5 $n-6$	0.42 ± 0.18	(0.27–1.14)
18:3 $n-3$	0.22 ± 0.10	(0.09–0.48)
20:5 $n-3$	0.34 ± 0.09	(0.17–0.49)
22:5 $n-3$	0.48 ± 0.09	(0.28–0.69)
22:6 $n-3$	1.21 ± 0.43	(0.39–2.89)
SAT	41.38 ± 2.39	(37.42–46.37)
MONO	34.50 ± 3.19	(25.88–40.09)
POLY	24.13 ± 2.75	(18.36–31.33)

FA: fatty acids; SD: standard deviation; SAT: saturated FA; MONO: monounsaturated FA; POLY: polyunsaturated FA.

We compared the relative quantities of some representative FA assessed in this study with the relative quantities reported by other studies (Table 4) in which the FA profile was obtained analyzing the whole blood of children and/or adolescents [37–41]. For studies examining differences in FA profile between control and pathological subjects, the values of the control group were considered. To better compare the data with those present in the literature, our subjects were also divided in those under (n = 14) or over 9 years old (n = 12). Levels of linoleic acid (LA), arachidonic acid (AA) and DHA were lower by an average of 38, 35 and 38%, respectively, in MIS-C subjects when compared to values reported for children of similar age in these other studies, including the results of a study carried out in our own laboratory [37]. The EPA that resulted was in line with other values reported, whereas α-linolenic acid (ALA) was higher mainly because of values observed in the group >9 yo.

The possible correlations between relative FA amounts and the inflammatory parameters were also investigated (Table 5), but only a statistically significant positive correlation between ALA and CRP was present, with a Spearman correlation coefficient of 0.508 ($p < 0.05$). It must be noted that samples for the evaluation of inflammatory parameters and for FA analysis were drawn 5–7 days apart from each other.

Table 4. Relative quantities of relevant fatty acids observed in our study in comparison with data present in the literature. Fatty acid data are expressed as mean ± standard deviation of the relative percentage values, or, in the case of [40], the range of percentage values. For studies examining differences in the FA profile between control and pathological subjects, the values of the control group were considered.

Author (Reference)	Present Study	Risé [37]	Crippa [38]	Bonafini [39]	Van der Wurff [40]	Ryan [41]
Age (yo)	3–18	<9	7–14	7–9	13–15	4
% LA	12.39 ± 2.28		22.54 ± 2.45			
<9 yo	12.72 ± 2.43	17.67 ± 1.92		19.9 ± 2.32		
>9 yo	11.99 ± 2.13					
% AA	6.38 ± 1.05		10.10 ± 0.92			
<9 yo	6.14 ± 1.09	8.33 ± 1.04		12.21 ± 1.67		7.50 ± 1.89
>9 yo	6.66 ± 0.97				11.01–11.33	
% ALA	0.22 ± 0.10					
<9 yo	0.18 ± 0.10	0.15 ± 0.05		0.16 ± 0.08		
>9 yo	0.27 ± 0.09					
% EPA	0.34 ± 0.09		1.13 ± 0.45			
<9 yo	0.32 ± 0.09	0.23 ± 0.08		0.30 ± 0.17		0.30 ± 0.39
>9 yo	0.36 ± 0.08				0.34–0.42	
% DHA	1.20 ± 0.43		1.93 ± 0.53			
<9 yo	1.08 ± 0.28	1.40 ± 0.37		2.92 ± 0.76		1.00 ± 0.34
>9 yo	1.35 ± 0.53				2.49–2.63	

AA: arachidonic acid; ALA: α-linolenic acid; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; LA: linoleic acid.

Table 5. Correlations between some relevant fatty acids and biochemical parameters of inflammation. Statistical significance was estimated using Spearman's non parametric correlation coefficient. * $p < 0.05$.

	LA	AA	ALA	EPA	DHA	O3I
CRP	−0.023	−0.036	0.508 *	0.048	0.149	0.246
IL-6	−0.236	−0.378	−0.092	−0.097	−0.174	−0.117
ESR	0.466	0.054	−0.161	−0.140	0	−0.046

AA: arachidonic acid; ALA: α-linolenic acid; CRP: C-reactive protein; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; ESR: erythrocyte sedimentation rate; IL-6: interleukin-6; LA: linoleic acid; O3I: omega 3 index.

3. Discussion

On May 2020, the Centers for Disease Control detailed the criteria of MIS-C diagnosis, that, together with a previous or current SARS-CoV-2 infection and two signs of multisystem involvement, also includes the presence of altered markers of inflammation. While sharing common features with Kawasaki Disease, toxic shock syndrome, and secondary hemophagocytic lymphohistiocytosis/macrophage activation syndrome [19], MIS-C importantly shares with COVID-19 the presence of laboratory evidence of inflammation, and it has been hypothesized that viral replication affects the production and release of inflammatory mediators, leading to a severe inflammatory reaction both in MIS-C and in COVID-19. Elevated concentrations of cytokines including IL-6, as well as increased CRP and D-dimer concentrations, are common in severe COVID-19 patients [42,43], and the uncontrolled inflammatory response has been deemed responsible for the most severe forms of COVID-19.

Together with cytokines, lipid mediators also play a critical role in the physiological evolution of the acute inflammatory reaction: oxygenated metabolites arising from ω6 PUFA may participate in both the propagation and the resolution of the inflammatory response [44], but they mainly exert potent proinflammatory and prothrombotic activities. Indeed, specifically targeting the production and the activity of AA-derived leukotrienes has been proposed as a novel approach to modulate the hyperinflammatory state in COVID-19 subjects [27], and increased concentrations of LA-derived leukotoxin diols were detected in the plasma of severe COVID-19 patients [45]. On the other hand, ω3 PUFA (i.e., EPA and DHA) are the substrates responsible for the formation of a new genus of anti-inflammatory

and pro-resolution lipid mediators collectively named specialized pro-resolving mediators (SPM) [23,24]. While plasmatic concentrations of SPM did not change upon LPS challenge in healthy volunteers [46], recent reports of their rapid metabolism suggests that plasmatic concentrations may not be predictive of the actual production in vivo [47], as is well known for eicosanoids. Based on their potent biological activities, SPMs may therefore play a critical role in the physiological resolution of the acute inflammatory response, and may be able to counteract the hyperinflammatory status observed not only in severe COVID-19 subjects [26,48], but also in MIS-C children.

In line with previously published results [19,49], clinical and biochemical parameters in the observed subjects support the diagnosis of MIS-C. They have had COVID-19 infection, and present multiorgan system failure, with persistently elevated levels of ferritin, CRP, fibrinogen, IL-6, procalcitonin, and increased ESR, all typical markers of an ongoing inflammatory condition. The presence of glucose metabolism alterations also observed in this sample of children with MIS-C, such as elevated TyG index, suggests that a bidirectional relationship between COVID-19 and glycemic impairment could not be excluded. Indeed, hyperglycemia and glycemic fluctuations could be caused by the inflammatory cascade triggered by SARS-CoV-2 in the pancreas, and the potentially impaired β-cell function associated with the SARS-CoV-2 entry through the ACE2 receptor [49].

The analysis of the whole blood fatty acid profile revealed relative amounts of ω6 PUFA in MIS-C subjects lower than those reported in literature, both for LA and AA. The same holds true upon subdivision of our group of subjects into children <9 and >9 years old, in order to facilitate a direct comparison with literature data relative to children of different age ranges. The levels of ω3 PUFA ALA and EPA were substantially in line with those present in the literature, whereas DHA levels were also lower [37–41]. It is important to note that the FA values observed in normal children by our group and reported in [37] referred to normal weight or overweight children, in line with the characteristics of the subjects observed in this study, whereas Bonafini et al. reported the data of normal weight children only [39]. A few studies suggested the occurrence of an altered FA profile in obese children [50,51], but the statistical analyses of a large number of data processed for the IDEFICS study reported only minor differences in FA levels if obese children were included [52].

These data are in agreement with changes observed in adult severe COVID-19 subjects: serum metabolomic/lipidomic analysis, carried out in a population aged 20–70 years showed not only that AA-containing phosphatidylcholine (16:0–20:4) and AA levels decrease with the severity of the disease [30], but also an increase in lysophospholipids reflecting PLA2 activity leading to significant AA mobilization from phospholipids, as observed in other pulmonary infections [53].

The lower levels of AA in children with MIS-C may therefore be the result of massive release from phospholipid storage followed by metabolic conversion into pro-inflammatory lipid mediators. Increased formation of SPM may also be occurring, as supported by the observed decrease in DHA, but in these subjects the associated formation of SPM did not appear to be enough to stave off the hyperinflammatory state confirmed by the clinical conditions and the altered biochemistry parameters reported.

LA is formally the biological precursor of AA, but only about 2% is converted into AA in humans [54], and in spite of being an essential FA, its current intake with the diet is much higher than the essential levels of linoleate, so that actual depletion is substantially impossible in the absence of an inborn error of metabolism [55]. The decrease of this FA observed in MIS-C children may find a cause very similar to that of AA. In fact, LA is the substrate for the CYP450 enzymes, including CYP2J2, CYP2C8, CYP2C9, and CYP1A1, leading to the formation of linoleic epoxides 9,10-epoxyoctadecenoic acid (9,10-EpOME) and 12,13-epoxyoctadecenoic acid (12,13-EpOME) known as leukotoxin and isoleukotoxin [56]. These epoxides are then metabolized by the soluble epoxide hydrolases (sEH) into the dihydroxyderivatives 9,10-DiHOME and 12,13-DiHOME, with the former known as a major contributor to pulmonary toxicity in acute respiratory distress syndrome (ARDS) [57].

Hospitalized COVID-19 patients with severe pulmonary involvement presented increased plasmatic amounts of regioisomeric leukotaxin diols [45], suggesting that the formation of these LA metabolites could be associated with the most severe forms of COVID-19. Again, the altered FA profile observed in MIS-C patients seems to point to a massive formation of pro-inflammatory lipid mediators potentially leading to decreased relative amounts of their precursor FAs.

No statistically significant correlation was found between inflammation markers and relative amounts of relevant FA (i.e., LA, AA, EPA and DHA), while a direct correlation between relative ALA quantities and CRP concentration was present. ALA has been shown to represent the precursor of ω3 PUFA, but its conversion is unreliable in humans [58], and its association with CRP may even indicate that a restricted conversion from ALA to DHA may be leading to a decreased formation of pre-resolution SPM and increased inflammatory biomarker(s). It must be noted that sampling for the evaluation of inflammatory parameters and for FA analysis was carried out at 5–7 day intervals.

Inverse associations between the LA, total ω6 PUFA, EPA/AA and DHA/AA ratios and hs-CRP were reported in a study of children [59], with differences emerging between boys and girls. Nevertheless, in that work, the population examined was composed of healthy young children in the absence of pathological states and therefore with very low CRP concentrations which were in some cases not detectable. This could explain the differences with our data, where the average CRP was in the range of 200 mg/L.

O3I, a recognized good biomarker of ω3 PUFA status, has also been reported as being inversely associated with some inflammatory biomarkers such as IL-6 and CRP [60]. In our study O3I did not associate with IL-6, CRP or ESR, but it must be stressed that typical association studies, including González-Gil et al. and Fontes et al., have been examining populations of subjects in "normal" conditions, i.e., in the absence of an acute inflammatory state such as the one reported in our study. A pilot study investigating the possible association between O3I and risk of death in COVID-19 patients (n = 100), found that patients with a O3I equal or greater than 5.7% were at lower risk of death (about 75%) compared to patients with an O3I lower than that value [61]. In light of our interpretation of the ω6 and ω3 PUFA values observed in MIS-C children, the O3I values obtained in hospitalized COVID-19 subjects may reflect the severity of the inflammatory status, with higher values in milder cases, and vice-versa, resulting from the use of EPA and, in particular, DHA to synthesize SPM in order to counterbalance the massive AA-derived pro-inflammatory metabolite formation resulting from SARS-CoV-2 infection.

The western, modern diet is a diet rich in ω6 PUFA with low levels of ω3 PUFA, a condition that causes a "chronic state of low-grade inflammation" [62], and therefore the baseline status of ω6 and ω3 PUFA, as has been proposed for cardiovascular, neurodegenerative, or autoimmune diseases with a critical inflammatory component, may be also of relevance for COVID-19 as well as for MIS-C subjects with respect to their ability to cope with the hyperinflammatory state induced by SAR-CoV-2.

Based on the potential anti-inflammatory and pro-resolution effects of ω3 PUFA and their endogenously generated metabolites (SPM) [22–24], it has been hypothesized that ω3 PUFA supplementation could be beneficial in COVID-19 patients [63], and parenteral infusion of pure fish oils for hospitalized patients with COVID 19 has also been proposed to attenuate respiratory failure and to reduce infection, sepsis rate and hospital length of stay, due to its proven clinical efficacy in patients with ARDS [64]. Interestingly, the results of randomized, double-blinded clinical trials have indeed recently appeared in the literature, showing that ω3 PUFA supplementation with one capsule of 1000 mg ω3 daily containing 400 mg EPA and 200 mg DHA for 14 days improved the levels of many parameters of respiratory and renal function as well as the survival in critically ill adult COVID-19 patients [65].

Our work, to our knowledge, is the first reporting the whole blood FA profile of children with MIS-C, showing a lipidic dysregulation similar to that observed in COVID-19 adults, with decreased levels of LA, AA and DHA as a possible result of increased metabolic

use for the formation of the lipid mediators participating in the hyperinflammatory inflammatory status observed. This observational study comes with several limitations, including the relatively small number of subjects enrolled, resulting in the imbalance between the number of boys and girls, while the absence of a control group is partially compensated by the fact that the published data relative to FA profiles in children used to compare the values observed in this study with normal values were indeed generated in our laboratory, and therefore used the same analytical approach. The observation of a potentially significant contribution of lipid mediators to the MIS-C phenotype also suggests that a possible dietary intervention could aim at tilting the balance between ω6 and ω3 PUFA, helping steer patients toward the final formation of anti-inflammatory, pro-resolution lipid mediators. Nevertheless, proving that a dietary intervention is beneficial in these patients will be difficult, in consideration of the small number of children affected. To date, there are no clinical trials enrolling children and adolescents with SARS-CoV-2 infection aimed at assessing the effects of DHA or other ω3 PUFA supplementation. The promising activities of ω3 PUFA notwithstanding, more experimental, randomized control trials and epidemiological research is warranted before recommending this approach.

4. Materials and Methods

4.1. Subjects

A group of 26 children and adolescents with MIS-C, as defined according to the CDC classification [9], were enrolled at the Pediatric Department of Children's Hospital Vittore Buzzi in Milan, Italy, between 1 December 2020 and 12 February 2021.

For all patients, a clinical and biochemical assessment was recorded on admission. Moreover, anthropometric measurements were collected at time of admission and before hospital discharge.

After 5–7 days from admission, a drop of blood was collected on Guthrie Test paper from each patient and stored in a refrigerator until analysis as described below. At this time, the drug therapy of children was also recorded.

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Review Board of the hospital (protocol number 2021/ST/004). Children's caregivers gave their written consent for inclusion after being informed about the nature of the study.

4.2. Anthropometric and Blood Measurements

Physical examination included anthropometric measurements of weight and height, Body Mass Index (BMI) calculation and evaluation of the pubertal stage was made for each patient. Weight and height were measured using a mechanical column scale with altimeter (Seca 711 and Seca 220), arm and waist circumferences were measured with atape measure (Seca 201) and tricipital skin-folds were measured using a caliper (Holtain 610). BMI (kg/m2) and BMI Z-SCORE were established according to CDC growth chart reference values [31,32].

The diagnostic procedure for confirming the MIS-C diagnosis involved a complete blood count and measurements of C-reactive protein (CRP), procalcitonin, ferritin, cardiac troponin T (cTnT), N-terminal pro-brain natriuretic peptide (NT-proBNP), coagulative parameters, creatine kinase, electrolytes, and interleukin-6 (IL-6). These measures were compared to our clinical laboratory's normal range values.

Additionally, the metabolic profile including total and high-density lipoprotein cholesterol (HDL), fasting plasma glucose (FPG), insulin and triglycerides (TG) was acquired from a blood sample obtained in a fasting state between 8:30 and 9:00 a.m. Insulin was measured using the electrochemiluminescence immunoassay (ELCIA) method.

The triglyceride–glucose (TyG) index as a surrogate for insulin resistance was calculated as [ln(fasting triglycerides (mg/dL) × fasting plasma glucose (mg/dL)/2)] [66,67]; the cutoff point for pathological IR was set at 7.88 [35,68].

Guthrie Test paper was used to collect whole blood for the analysis of fatty acids (FA) 5–7 days from admission, during the acute phase of the disease, for each patient.

4.3. Fatty Acid Analysis

The FA profile was evaluated in a drop of blood collected on a Guthrie paper embedded with butylated hydroxy toluene (BHT) as antioxidant. After direct transmethylation, FA methyl esters were analyzed by gas chromatography using a GC-2100 (Shimadzu Italia S.r.l., Milano, Italy) equipped with a 15 m capillary column (DBB Agilent), PTV injector and FID detection [69,70]. Relative percentages were used to report 23 FA; total saturated FA (SAT), monounsaturated FA (MUFA) and PUFA were also reported. In addition, the omega 3 index (O3I) was calculated in accordance with Stark et al. [36].

4.4. Statistical Analysis

A descriptive statistical analysis was performed using IBM SPSS statistics version 27, whereas Spearman's correlation coefficients were estimated for biochemical parameters and FA.

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Informed Consent Statement: Children's caregivers gave their written consent for inclusion after being informed about the nature of the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available for privacy.

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5.4 Manuscript 3

"Omega3 Index in MIS-C children: possible correlation with length of stay and PICU admission?"

Introduction

SARS-CoV-2 infection in children and adolescents has different consequences from those observed in adults. In most cases they develop mild symptoms, but a small number of infected subjects develop severe symptoms, more frequently in the presence of comorbidities such as diabetes, obesity, or cardiovascular diseases. In less than 1% of children who contract SARS-CoV-2 a hyper-inflammation state has been observed, both during the infection and approximately 4 to 6 weeks afterwards.

In 2020, the World Health Organization (WHO) defined this hyper-inflammation state as the Multisystem Inflammatory Syndrome in Children (MIS-C). This disease results in multiple organ failure, with the involvement of gastrointestinal, cardiovascular, haematological, cutaneous, and respiratory systems. The syndrome is post-infectious rather than related to the acute phase of the infection. Thus, it has been hypothesized that it is a delayed immunological phenomenon associated with the hyperinflammatory phase following symptomatic or asymptomatic SARS-CoV-2 infection[9].

The infection appears to trigger activation of macrophages followed by stimulation of T-helper cells. This leads to the release of cytokines, stimulation of macrophages, neutrophils, and monocytes. B-cells and plasma cells are also activated, with the production of antibodies leading to a hyperimmune response. This immune deregulation is associated with MIS-C syndrome in these children[11].

The blood fatty acid profile in children affected by MIS-C was altered compared to that of healthy children. AA (20:4 n-6), LA (18:2 n-6), and DHA (22:6 n-3), were 38%, 35% and 38% lower, respectively. In contrast, the levels of ALA (18:3 n-3) and EPA (20:5 n-3), were in line with those found in the literature [133]. This alteration is the possible result of an increased metabolism of FA that leads to the formation of lipid mediators that participate in the hyperinflammatory state observed. The lower levels of AA in children with MIS-C seem to be the result of its marked release from accumulation in phospholipids followed by its conversion into pro-inflammatory lipid mediators[133]. In fact, oxygenated metabolites derived from ω-6 may participate in both the propagation and resolution of the

inflammatory response, but they exert mainly potent pro-inflammatory and pro-thrombotic activities[134].

In contrast, from ω-3 PUFA, specialized pro-resolving mediators (SMPs) can be produced. SMPs are metabolites derived from reactions mediated by lipoxygenases starting from DHA and EPA, and essential ω-3 PUFAs. SMPs are powerful anti-inflammatory agents, so they can promote the physiological resolution of the inflammatory process[74,128]. In 2021 a pilot study with 100 adult patients positive for SARS-CoV-2 was conducted to test the hypothesis that EPA and DHA levels – expressed as Omega-3-Index (O3I) – were inversely associated with the risk of death. The results showed that patients with an O3I of 5.7 % or higher had an approximately 75% lower risk of death than those with a lower O3I value. This difference in mortality risk is not statistically significant, however, it is a strong trend suggesting the existence of a relationship [104]. These findings support the idea that EPA and DHA have anti-inflammatory properties that could contribute to reducing morbidity and mortality in SARS-CoV-2 infection. As the above study is currently the only one in the literature regarding a possible correlation between ω-3 fatty acid levels and the outcome of COVID-19 disease caused by SARS-CoV-2, we decided to investigate this possible correlation in children.

Aim

We aimed to assess the fatty acid profile in whole blood in 51 children diagnosed with MIS-C admitted to Vittore Buzzi Hospital in Milan (Italy), to calculate the O3I, and to assess its possible correlation with days of hospitalisation and whether the children were admitted to the paediatric intensive care unit (PICU).

Subjects and methods

A group of 51 children (2-18 years old) with MIS-C, as defined according to the CDC classification[10], were enrolled at the Paediatric Department of the Vittore Buzzi Children's Hospital in Milan, Italy, from 21 December 2020 to 31 March 2022 .For all patients, a

clinical and biochemical assessment was performed on admission. In addition, anthropometric measurements and the drug therapy of the children were also reported.

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Review Board of the hospital (protocol number 2021/ST/004). Children's caregivers gave their written consent for inclusion after being informed about the nature of the study.

Fatty Acid Analysis

A few days after admission, a drop of blood was collected on a card embedded with butylated hydroxy toluene (BHT) as an antioxidant, and stored in a refrigerator until analysis.

The fatty acids (FA) profile was evaluated after direct transmethylation. FA methyl esters were analysed by gas chromatography using a GC-2100 (Shimadzu Italia S.r.l., Milan, Italy) equipped with a 15 m capillary column (DBB Agilent), PTV injector and FID detection, as reported previously [135,136]. A total of 22 FA were considered and reported as relative percentages. FA classes, i.e. total saturated FA (SAT), monounsaturated FA (MUFA) and PUFA and FA series (ω-6 and ω-3) were also reported. In addition, the O3I was calculated in accordance with Stark et al. applying the suggested equation, O3I = 1.1 * (EPA+DHA in WB) + 0.65, to convert EPA and DHA sum from whole blood to O3I in red blood cells [137].

Statistical Analysis

The statistical analyses were performed using IBM SPSS statistics v. 18.01 for non-parametric tests (Spearman correlation coefficient for FA versus days of hospitalization and Kruskal-Wallis test to compare different groups). A chi-squared test was administered for possible associations between O3I and categorical variables. The analyses and differences were considered significant for $p<0.05$.

Results

Table 3 summarizes the characteristics of the sample. The mean age of the subjects (n=51) was 8.6±3.7 years, and 74.5% of them were males. Hospitalization ranged from 6 to 27 days, with a mean of 14 days. The subjects were also divided in two groups: the first included those children who had been admitted for a few days in the paediatric intensive care unit (PICU), and the second, children who had not been in the PICU (No PICU). No significant differences were found between these groups in terms of age and days of hospital stay.

Table 3. **Characteristics of the sample**

	All subjects n=51	**PICU n=30**	**No PICU n=21**
Sex-male n, %	38 (74.5%)	24 (80%)	14 (67%)
Age *y* mean±SD, (min-max)	8.6±3.7 (3-17)	9.2±4.1 (3-17)	7.8±2.9 (3-14)
Days in H mean±SD, (min-max)	14.1±4.2 (6-27)	14.7±4.6 (6-27)	13.3±3.5 (10-26)

ICU, intensive care unit

The levels of ω-3 PUFAs are reported in Table 4. A comparison of PICU group with No PICU group showed no significant differences concerning FA levels, nor for the O3I which was 2.27% in ICU versus 2.34% in No ICU. The mean value of O3I in all subjects was 2.30±0.51. As these patients were diagnosed with MIS-C and had an altered FA profile with respect to the control subjects of the same age. O3I values in the control groups were searched for in the literature.

Table 4. Whole blood ω-3 fatty acid levels, expressed as relative percentages, in patients admitted or not to the intensive care unit.

Fatty acids	**All subjects n=51**	**PICU n=30**	**No PICU n=21**
ALA	0.20±0.09	0.21±0.09	0.19±0.09
EPA	0.38±0.09	0.39±0.10	0.36±0.08

DPA	0.44±0.16	0.42±0.12	0.47±0.21
DHA	1.12±0.46	1.08±0.47	1.17±0.45
Total ω3	2.14±0.63	2.11±0.58	2.19±0.72
O3I	2.30±0.51	2.27±0.49	2.34±0.55

The values reported are the mean ± standard deviation (SD). ALA, alpha linolenic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; O3I (calculated as reported in the Methods section); PICU, paediatric intensive care unit.

Table 5 shows articles in which the FA profile of healthy children was reported [98,138–147]. The analyses were conducted in different specimens (i.e. whole blood, red blood count, plasma) and thus when possible, the O3I was calculated as reported in the Methods. Figure 16 shows the O3I values from all the studies mentioned, from the Italian literature alone and from the current study with significant differences.

Table 5. O3I in healthy children and adolescents, based on literature data.

Author, year of publication	Country	Age in years	Specimen	O3I
Ryan AS, 2008	USA	4	WB	2.08
Burrows T, 2011	Australia	5-12	RBC	5.00
Risé P, 2013	Italy	2-9	WB	2.44
Van der Wurff, 2016	Netherlands	13-18	WB	3.92
Crippa A, 2018	Italy	7-14	WB	4.02
Al-Ghannami SS, 2018	Oman	9-10	RBC	4.10
Crippa A, 2019	Italy	7-14	WB	3.39
Van der Wurff, 2019	Netherlands	13-18	WB	3.93
Bonafini,2020	Italy	9-10	WB	4.19

Murphy A, 2021	USA	3-5	plasma	2.36
		6-11		2.47
		12-19		2.46
Syrèn ML, 2022	Italy	2	WB	3.26
		2-10		3.03
		10-19		2.99

The O3I are those reported in the mentioned papers or calculated on the basis of the EPA+DHA levels in other specimens than RBC, as reported by Stark et al.[148].

The correlations between the ω-3 FA levels of all the patients and days of hospitalization (DoH) were investigated, and the only correlations found were a negative correlation for docosapentaenoic acid (DPA) and DoH (p=0.013) (Table 6). In a further assessment, O3I values were divided into quartiles as follows: Q1 (<1,88%), Q2 (1.88≤O3I<2.29%), Q3 (2.29%≤O3I<2.51%), and Q4 (O3I≥2.51%) (Table 7). Concerning age and days of H, no significant differences among quartiles were found. On the other hand, for the upper quartile Q4 versus the other three together Q1-Q3, the days of H were significantly lower in Q4 with respect to Q1-Q3. The chi-squared test revealed that there was no association between PICU and O3I quartiles, nor in Q4 versus Q1-Q3 analyses.

Table 6. Correlations between ω-3 fatty acid levels and days of hospitalization.

Fatty acids	**R=Spearman coeff**	**P-value**
ALA	-0.025	0.863
EPA	0.209	0.141
DPA	***-0.347***	***0.013***
DHA	-0.241	0.089
Total ω 3	-0.234	0.099
O3I	-0.178	0.212

Fatty acid levels are expressed as relative percentages. The statistical significance was estimated administering the two-tailed Spearman's nonparametric test. ALA, alpha linolenic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; and O3I (calculated as reported in the Methods).

Table 7. Unadjusted associations of O3I quartiles and different variables

Categorical by O3I quartile	**Age mean±SD**	**Sex -Male% (X/N)**	**Days of H mean±SD**	**PICU% (X/N)**
Q1: O3I<1.88%	8.00±3.10	72.73% (8/11)	12.58±4.29	45.45% (5/11)
Q2: 1.88%≤O3I<2.29%	8.31±3.40	75.00% (12/16)	14.88±4.29	65.20% (10/16)
Q3: 2.29%≤O3I<2.51%	8.64±4.30	63.64% (7/11)	15.91±5.84	54.54% (6/11)
Q4: O3I≥2.51%	8.79±4.33	84.62% (11/13)	11.21±3.95	53.85% (7/13)
p-value*	0.79	0.704	0.07	0.855
Comparison Q1-Q3 vs Q4				
Q1-Q3: O3I<2.51%	8.32±3.51	71.05% (27/38)	14.84±4.15	60.53% (23/38)
Q4: O3I≥2.51%	8.79±4.33	84.62% /11/13)	11.21±3.95	53.85% (7/13)
p-value*	0.367	0.333	***0.031***	0.67

O3I, ω-3 index; H, hospitalization; PICU, intensive care unit

*The p-value was calculated administering the non-parametric Kruskal Wallis test for age and days of H, and the chi-squared test for sex and PICU

Discussion

Several reviews have suggested that during the pandemic ω-3 PUFA supplementation may have been able to regulate and ameliorate the inflammatory status of patients [109,110]. In fact, EPA and DHA are the precursors of anti-inflammatory compounds, i.e. resolvins, maresins, and protectins, which are involved in the resolution of inflammation unlike those compounds derived from arachidonic acid [67–69]. In fact, we previously reported an altered FA profile in children diagnosed with MIS-C. LA, AA and DHA levels were lower than those of control children, suggesting the conversion of these FAs to molecules with pro and anti-inflammatory activities in order to counteract the hyper inflammation status [133]. The present work reports data concerning FAs and O3I in children, hospitalization, and admission in PICU. In general, the FA profile of these children is altered (not shown) confirming our previous findings [133], and also the levels of O3I are significantly lower than those of healthy children.

As mentioned, we focused on ω-3 PUFA and found that their profile was similar in the PICU group versus the NO PICU group, as was the O3I. In none of the subjects were correlations found with the days of hospitalization, except for ω-3 DPA (negative correlation). In addition, O3I quartiles were not associated with age, sex, admission in PICU and days of hospitalization, whereas in the comparison of the upper quartile (Q4:O3I ≥2.51%) versus the others (Q1-Q3 < O3I 2.51%), a significant relation with days of hospitalization was found.

Asher et al. were the first to investigate a possible association between O3I and the mortality of Covid-19 in a group of 100 hospitalized adults and, although not significant, a strong, negative trend was found[104]. The patients with severe COVID-19 had low O3I values that were inversely associated with the major clinical endpoints (mechanical ventilation, death) of the disease [101], and high levels of O3I were associated with a 48% reduction in the risk of severe pathology [149] .

A recent retrospective study confirmed that low ω-3 levels and O3I, are associated with an increased risk of hospitalization, but not significantly with the risk of death [131]. In addition,

the levels of O3I found, were similar to those proposed as a cut-off for risk of death from cardiovascular disease [150] . In line with these observations, only a few supplementation studies with ω-3 FA have reported an amelioration in clinical symptoms in covid-19 adult patients [100,151] . The strength of our finding is the significant difference between quartiles with respect to DoH. This aspect should also be further investigated in the paediatric population, in order to identify those patients most at risk and provide early intervention, with a larger sample size. In conclusion, in our small group of MIS-C patients, higher levels of O3I were associated with fewer days of hospitalization, whereas the admission to PICU did not seem to be affected.

5.5 Manuscript 4

“Blood Fatty acids profile and inflammatory markers in MIS-C children supplemented with DHA”.

Introduction

Children infected with SARS-CoV-2 may develop multisystem inflammatory syndrome (MIS-C) 4-6 weeks after exposure [152]. Less than 1% of children with proven COVID-19 exposure also acquired MIS-C [153]. The demographic characteristics of the patients showed a predominance of males and an age range of between 7 and 10 years [154,155].

Multiple organ failure is a consequence of the disease, manifested by gastrointestinal, cardiovascular, haematological, mucocutaneous, neurological, and respiratory symptoms [156]. The intensive care unit (ICU) care is required for some patients with more severe disease [157]. According to several studies, ICU care is required in 50–80% of children with MIS–C [158].

The primary function of a cytokine storm and the impact of adaptive immunity following SARS-CoV-2 infection are described for the most widely accepted explanation of the pathogenic mechanism of MIS-C [159,160]. Additionally, some intrinsic susceptibility factors have been described, and evidence of molecular mimicry for MIS-C pathogenesis has also been provided [161]. During the acute phase of MIS-C, altered glucose metabolism (high Homeostasis Model Assessment for Insulin Resistance (HOMA-IR) and Tyg Index values) and a reduction in body mass index (BMI)z-score have been reported in children [162,163]. Regarding blood fatty acid status, lower levels ω-6 LA and AA were found, probably as a result of massive release from phospholipid stores, followed by metabolic conversion into pro-inflammatory lipid mediators [133].

A new genus of metabolites derived from ω-3PUFA has been identified as possessing potent anti-inflammatory and pro-resolvin properties, providing evidence that lipid mediators may also play a key role in the physiological resolution of the inflammatory process [128]. ω-3 FAs have been reported to modulate immunity and activate inflammatory resolution processes. DHA and eicosapentaenoic acid (EPA) metabolites produced by several lipoxygenases have been established as specialized pro-resolving mediators (SPMs), which are a family of compounds involved in tissue regeneration that also includes resolvins, protectins, maresins, and maresin conjugate [164]. By

activating macrophages with an anti-inflammatory phenotype (M2) and promoting phagocytosis in a non-phlogistic manner, SPMs produced by the metabolism of ω-3PUFAs reduce the synthesis of pro-inflammatory mediators and neutrophil recruitment [133]. The ω-3EPA and DHA content of red blood cells is expressed as a proportion of the total weight of FAs in red blood cell membranes, and this is known as the ω-3 Index (O3I) [129]. In adult patients higher circulating ω-3 FAs, have been associated with a better prognosis for COVID-19 [130].

Harris et al. investigated the levels of blood DHA in a large, prospective, population-based cohort of 110,584 individuals to compare the risk of COVID-19 outcomes, including testing positive for SARS-CoV-2, hospitalization, and death, in relation to the baseline plasma DHA levels. Their findings indicate that individuals with high levels of DHA experienced a 26% reduced risk of hospitalization, positive test outcomes, and mortality compared to those with low levels [131]. Additionally, a scoping review of adult patients found that ω-3 supplementation enhances renal and respiratory function, reduces the probability of positive test results for SARS-CoV-2 infection and symptoms, and improves survival rates [132]. A cross-sectional study by Zapata et al. confirmed previous findings, revealing that patients with severe COVID-19 had a reduced O3I, associated with a low consumption of fish and ω-3 supplements. The higher the O3I, the lower the risk of requiring mechanical ventilation and mortality; this association continued to be significant after accounting for age, sex, and other covariates [101]. During the acute phase of MISC in children, LA, AA and DHA were lower by an average of 38, 35 and 38%, respectively [133], when compared to previously reported values for children[97,140,143,147]. Vitamin D supplementation is necessary during this phase, but no other interventions are required in children with MIS-C [165].

Aim

We aimed to evaluate the short-term beneficial effects on inflammatory markers after DHA supplementation in children who suffering from MIS-C.

Materials and methods

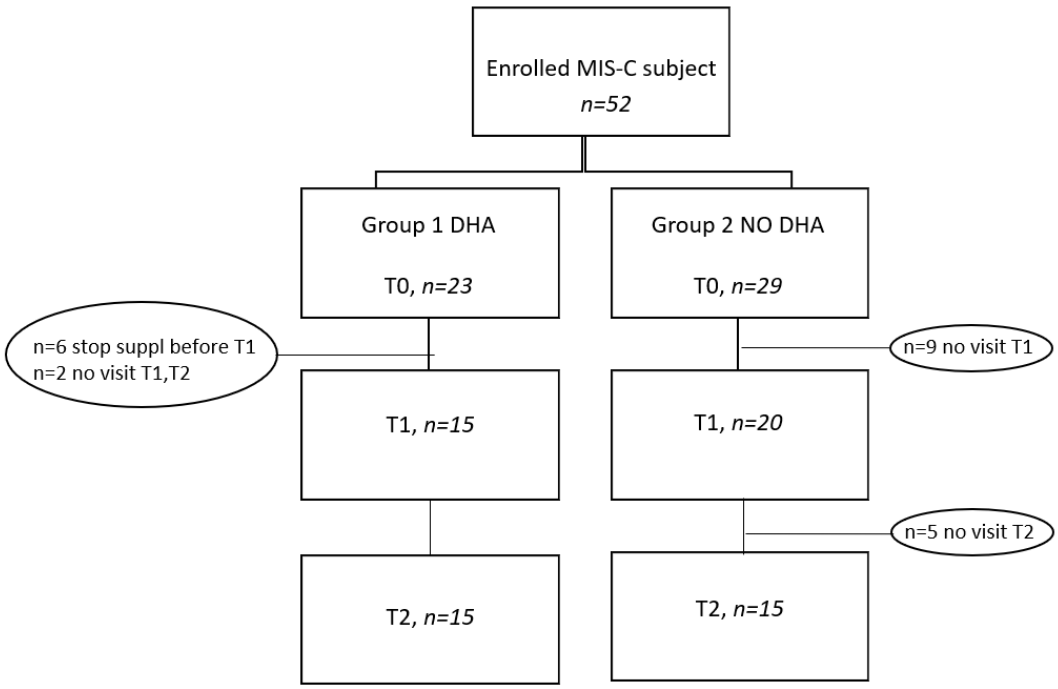
Subjects

A group of 52 children and adolescents (2-18 years old) with MIS-C, as defined according to the CDC classification [10], were enrolled at the Department of Paediatrics at the Vittore Buzzi Children's Hospital in Milan, Italy, between 1 December 2020 and 31 March 2022. For all patients, a clinical and biochemical assessment was recorded on admission. Anthropometric measurements were collected at admission and before hospital discharge.

After 5–7 days from admission (T0), a drop of blood was collected on Guthrie test paper from each patient and stored in a refrigerator until analysis as described below. At this time, the drug therapy of children was also recorded. Consecutively, 23 patients at the time of hospital discharge received instructions for DHA supplementation (1ml/day equals to 250 mg of DHA) (**see Suppl. Table 1**) to be carried out for three consecutive months. The control group of 29 children did not receive any supplementation advice . Recommendations on healthy eating habits, following the Mediterranean diet, were explained and given to each child and caregivers at discharge. At the follow-up visit three months after discharge (T1), blood samples were also collected as described.

Six months after discharge (T2), the patients were examined in a multidisciplinary setting and a further blood sample was carried out and stored as described above. In the supplemented group (Group 1), six children who had taken DHA for less than 3 months, and two children who had not attended the visits at 3 and 6 months, were excluded from the final analysis. In the other group (Group 2) nine children had not attended the T1 visit, and five who had not attended T2 were excluded from the final analysis. The final analysis was thus performed on 15 patients in each group, as shown in Figure 16.

Figure 16. Flow chart of enrolled patients



The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Review Board of the hospital (protocol number 2021/ST/004). Children's caregivers gave their written consent for inclusion after being informed about the nature of the study.

Anthropometric and Blood Measurements

Physical examination included anthropometric measurements of weight and height and BMI. Weight and height were measured using a mechanical column scale with an altimeter (Seca 711 and Seca 220), arm and waist circumferences were measured with a tape measure (Seca 201), and tricipital skinfolds were measured using a calliper (Holtain 610). BMI (kg/m2) and BMI z-scores were calculated according to CDC growth chart reference values[166].

The diagnostic procedure for confirming the MIS-C diagnosis involved a complete blood count and measurements of the C-reactive protein (CRP), procalcitonin, ferritin, cardiac troponin T (cTnT), N-terminal pro-brain natriuretic peptide (NT-proBNP), coagulative

parameters, creatine kinase, electrolytes, and interleukin-6 (IL-6). These measurements were compared to our clinical laboratory's normal range values. Additionally, the metabolic profile including total and high-density lipoprotein cholesterol (HDL), fasting plasma glucose (FPG), insulin and triglycerides (TG) was acquired from a blood sample obtained in a fasting state between 8:30 and 9:00 a.m. Insulin was measured using the electrochemiluminescence immunoassay (ELCIA) method. The triglyceride–glucose (TyG) index as a surrogate for insulin resistance was calculated as [ln(fasting triglycerides (mg/dL) fasting plasma glucose (mg/dL)/2)] [167,168]; the cutoff point for pathological IR was set at 7.88 [169,170]. The blood analysis was repeated at the six- month visit (T2), except for those parameters closely related to MIS-C diagnosis.

Fatty Acid Analysis

The FA profile was evaluated in a drop of blood collected on Guthrie paper embedded with butylated hydroxy toluene (BHT) as an antioxidant. After direct transmethylation, FA methyl esters were analyzed by gas chromatography using a GC-2100 (Shimadzu Italia S.r.l., Milan, Italy) equipped with a 15 m capillary column (DBB Agilent), PTV injector and FID detection [135,171] . Relative percentages were used to report 23 FAs; total saturated FA (SAT), monounsaturated FA (MUFA), and PUFA were also reported. In addition, the O3I was calculated in accordance with Stark et al [148].

Statistical analysis

The statistical analyses were performed using IBM SPSS statistics v. 28.01. The analyses and differences were considered significant for $p<0.05$.

Results

At the start of the study 52 subjects (children and adolescents) with MIS-C were enrolled. For each group, 15 patients attended all the follow- up visits (T1 and T2). **Table 8** shows the general characteristics of the sample.

Table 8. Study participants' characteristics at baseline.

	Average ± SD
Number of recruited subjects	52
M/F	37/15
Age (y)	8.78±3.95
Height (cm)	134±25
Weight (Kg)	34.72±15.67
BMI z-score WHO	0.53±1.21
Tricipital Skinfold (p.le)	58.88±20.51

Table 9 shows the FA profile in Group 1 at the various times. At T1 there was a significant increase in DHA, DPA and EPA with respect to T0. At the same time, ω-6 FAs (i.e. LA, AA) had increased, whereas oleic acid (18:1) had decreased considerably. At T2, LA was significatively higher than T0 and T1, and oleic acid was lower than T0 and higher than T1. In the case of AA (increase), DHA, DPA and EPA (decrease), the levels were not significantly different from T1.

The FA profile in Group 2 revealed a similar trend over time (**Table 10**). In fact, there was an increase in LA, AA, DPA and DHA from T0 to T2, and a decrease of oleic and palmitic acids. The FA levels in Group 1 versus Group 2 (**Table 11**) at T0 were very similar, with the exception of γ-linolenic acid (GLA 18:3 ω6), EPA and DPA (lower and higher levels in Group 1 vs Group 2) with significant differences. At T1, after DHA supplementation, the differences between groups were more evident for saturated and monounsaturated FAs, and for both ω-6 and ω-3 FA. At T2 significant differences between groups were retained only for stearic acid (18:0), and for EPA and DHA (**Table 11**).

Table 9. Whole blood fatty acid profile in Group 1 (n=15) (supplemented with DHA).

Fatty Acids	% w/w±SD at T0 in Group 1	% w/w±SD at T1 in Group 1	% w/w±SD at T2 in Group 1
16:0	27.92±1.66[a]	26.53±2.08[b,c]	25.29±1.70[c]

18:0	10.15±1.09[a]	14.07±1.16[b]	12.46±1.34[c]
20:0	0.41±0.10[a]	0.57±0.08[b]	0.44±0.11[a,c]
22:0	1.04±0.22[a]	1.86±0.30[b]	1.59±0.24[c]
24:0	1.72±0.55[a]	3.21±0.70[b]	2.64±0.59[c]
16:1	3.14±0.83[a]	1.28±0.37[bc]	1.20±0.54[c]
18:1 n-9	27.07±3.39[a]	16.13±2.15[b]	18.58±3.20[c]
18:1 n-7	1.65±0.28[a]	1.66±0.37[a]	1.39±0.32[b,c]
20:1	0.20±0.13	0.16±0.04	0.18±0.04
22:1	0.08±0.03[a]	0.13±0.08[b]	0.04±0.06[c]
24:1	2.11±0.58[a]	2.97±0.60[b]	2.56±0.47[c]
20:3 n-9	0.17±0.10	0.14±0.04	0.13±0.03
18:2 ω-6	12.57±2.22[a]	14.56±1.58[b]	17.71±2.82[c]
18:3 ω-6	0.68±0.41[a]	0.25±0.18[b,c]	0.19±0.08[c]
20:3 ω-6	1.13±0.30[a]	1.43±0.38[b,c]	1.34±0.20[c]
20:4 ω-6	6.60±1.21[a]	9.05±1.79[b,c]	9.29±2.20[c]
22:4 ω-6	0.84±0.26[a]	1.13±0.42[b,c]	1.06±0.32[c]
22:5 ω-6	0.36±0.08[a]	0.79±0.32[b]	0.33±0.09[a,c]
18:3 ω-3	0.20±0.09	0.29±0.16	0.21±0.08
20:5 ω-3	0.32±0.09[a]	0.46±0.10[b,c]	0.41±0.08[c]
22:5 ω-3	0.48±0.10[a]	0.65±0.16[b,c]	0.60±0.20[a,c]
22:6 ω-3	1.19±0.25[a]	2.67±0.78[b,c]	2.37±0.87[c]
SAT	41.23±2.42[a]	46.23±3.13[b]	42.42±2.36[a,c]
MONO	34.24±3.61[a]	22.33±2.05[b,c]	23.94±3.38[c]
POLY	24.52±2.75[a]	31.43±3.09[b]	33.64±2.33[c]
I.I.	108.56±6.99[a]	124.07±10.84[b,c]	127.31±9.08[c]
ω-6	22.17±2.71[a]	27.21±2.76[b]	29.93±2.10[c]
ω-3	2.18±0.35[a]	4.08±0.80[b,c]	3.59±1.13[c]
ω-6/ω-3	10.42±2.05[a]	6.88±1.31[b]	9.12±2.79[c]
DHA/AA	0.18±0.3[a]	0.31±0.10[b]	0.25±0.06[c]
EPA/AA	0.05±0.02[a,b]	0.05±0.01[a]	0.05±0.01[b,c]

Data are expressed as mean ± standard deviations (SDs) of FAs of the relative percentage (weight/weight) of all the FAs considered - see Methods. Statistical analysis performed: paired Student's t test. Values that do not share the same suffix (abc) are significantly different for p-value < 0.05.

Table 10. Whole blood fatty acid profile in Group 2(n=15) (no DHA supplementation).

Fatty Acids	% w/w±SD at T0 in Group 2	% w/w±SD at T1 in Group 2	% w/w±SD at T2 in Group 2
16:0	29,43±2,45[a]	24,56±2,12[b,c]	24.39±0.9[c]
18:0	10.62±1.57[a]	12.74±1.34[b]	11.84±0.65[c]
20:0	0.39±0.12[a]	0.54±0.09[b]	0.46±0.05[a,c]
22:0	1.06±0.21[a]	1.62±0.20[b,c]	1.52±0.19[c]
24:0	1.67±0.44[a]	2.74±0.47[b,c]	2.66±0.37[c]
16:1	2.77±1.21[a]	1.09±0.25[b,c]	1.26±0.42[c]
18:1 n-9	27.67±4.27[a]	21.64±3,97[b,c]	20.15±1.81[c]
18:1 n-7	1.63±0.32[a]	1.44±0.24[a]	1.31±0.21[b,c]
20:1	0.19±0.08	0.18±0.08	0.16±0.04
22:1	0.07±0.05	0.11±0.15	0.11±0.15
24:1	2.00±0.44[a]	2.75±0.45[b,c]	2.74±0.33[c]
20:3 n-9	0.15±0.09	0.12±0.04	0.16±0.06
18:2 ω-6	12.70±2.27[a]	17.92±2.66[b,c]	19.29±1.94[c]
18:3 ω-6	0.33±0.31	0.29±0.14	0.24±0.09
20:3 ω-6	0.94±0.32[a]	1.09±0.18[a]	1.30±0.24[b,c]
20:4 ω-6	5.31±1.84[a]	7.35±1.38[b,c]	8.30±1.55[c]
22:4 ω-6	0.65±0.29[a]	0.91±0.26[b,c]	1.04±0.26[c]
22:5 ω-6	0.33±0.16	0.34±0.16	0.31±0.08
18:3 ω-3	0.17±0.06[a]	0.36±0.16[b]	0.20±0.06[a,c]
20:5 ω-3	0.41±0.11[a]	0.28±0.10[b,c]	0.33±0.09[c]
22:5 ω-3	0.41±0.22[a]	0.48±0.10[a,c]	0.57±0.13[b,c]
22:6 ω-3	1.09±0.51[a]	1.44±0.47[a,c]	1.70±0.43[b,c]
SAT	43.17±3.34	42.20±3.22	40.88±1.46
MONO	34.35±4.64[a]	27.20±3.71[b,c]	25.68±2.16[c]

POLY	22.48±4.60[a]	30.60±4.03[b]	33.44±2.24[c]
I.I.	100.61±12.45[a]	115.86±10.40[b]	123.57±6.84[c]
ω-6	20.26±4.12[a]	27.91±3.74[b]	30.48±2.19[c]
ω-3	2.07±0.75[a]	2.57±0.57[a,c]	2.80±0.56[b,c]
ω-6/ω-3	10.42±2.72	11.24±2.11	11.27±2.27
DHA/AA	0.20±0.05	0.19±0.05	0.21±0.04
EPA/AA	0.09±0.05[a]	0.04±0.01[b,c]	0.04±0.01[c]

Data are expressed as mean ± standard deviation (SD) of FAs of the relative percentage (weight/weight) of all the FAs considered - see Methods. Statistical analysis performed: paired Student's t test. Values that do not share the same suffix (abc) are significantly different for p-value < 0.05.

Table 11. Statistical analysis of fatty acid levels between Group 1 (suppl DHA) vs Group 2 (no DHA) at T0, T1 and T2.

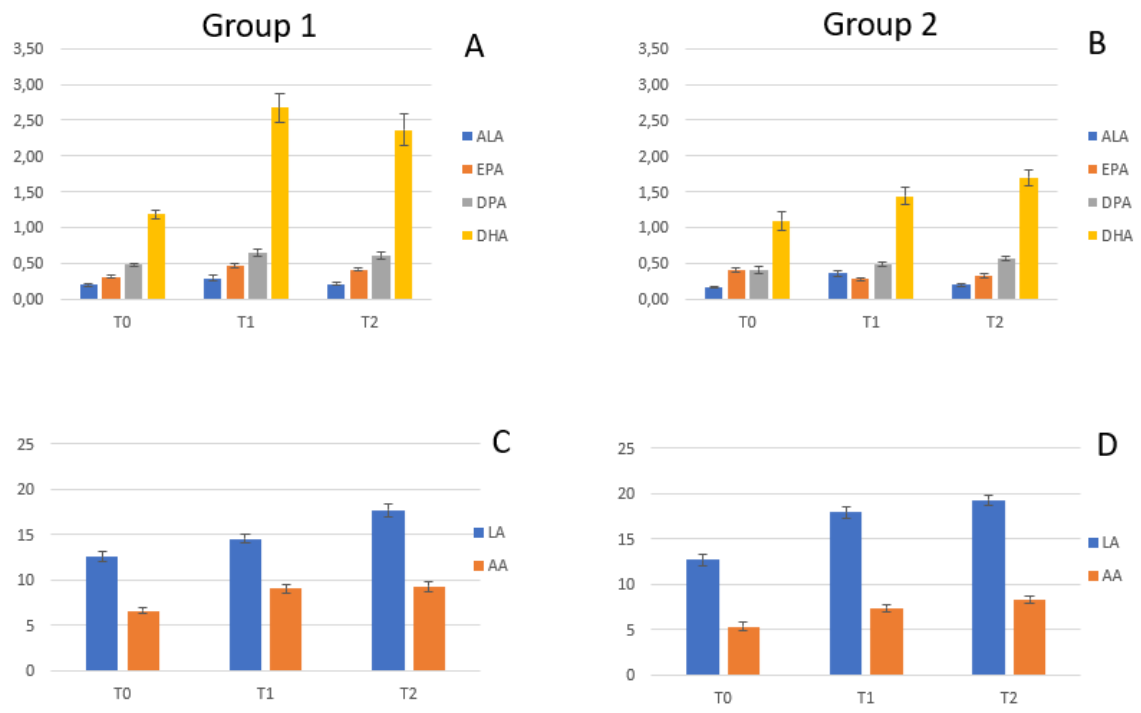
Fatty Acids	Group 1 vs Group 2 T0	Group 1 vs Group 2 T1	Group 1 vs Group 2 T2
16:0	0.081	**0.013**	0.061
18:0	0.461	**0.003**	**0.023**
20:0	0.775	0.233	0.806
22:0	0.806	**0.026**	0.305
24:0	0.713	**0.041**	0.838
16:1	0.217	0.202	0.744
18:1 n-9	0.87	**<0.001**	0.05
18:1 n-7	0.624	0.074	0.412
20:1	0.713	0.902	0.174
22:1	0.325	0.233	0.412
24:1	0.838	0.325	0.148
20:3 n-9	0.653	0.217	0.098
18:2 ω-6	0.902	**<0.001**	0.089
18:3 ω-6	**0.015**	0.174	0.126
20:3 ω-6	0.098	**0.003**	0.595
20:4 ω-6	0.067	**0.013**	0.25
22:4 ω-6	0.098	0.233	0.967

22:5 ω-6	0.081	**<0.001**	0.744
18:3 ω-3	0.461	0.067	1
20:5 ω-3	**0.019**	**<0.001**	**0.023**
22:5 ω-3	**0.037**	**0.003**	0.713
22:6 ω-3	0.345	**<0.001**	**0.023**
SAT	0.174	**0.002**	0.098
MONO	0.967	**<0.001**	0.056
POLY	0.412	0.461	0.87
I.I.	0.098	0.089	0.285
ω-6	0.325	**0.002**	0.436
ω-3	0.233	**<0.001**	**0.037**
ω-6/ω-3	0.902	0.461	**0.041**
DHA/AA	0.161	0.089	**0.041**
EPA/AA	**0.008**	**0.015**	0.461

Statistical analysis performed: U Mann Whitney test for non-parametric values that are significantly different for p-values <0.05.

Figure 17 shows the trend of the amounts of FAs measured at the different time points in the two groups. ω-3 FAs increased in both groups, but the increase in Group 1 (supplemented with DHA) was higher: + ◎ 124% versus + ◎ 32% of DHA respectively at T1, and + ◎ 99% versus +◎ 56% of DHA, respectively, at T2 (panel A, panel B). In addition, Group 1 DHA showed (panel A) a typical bell-shaped trend, due to supplementation followed by wash-out. FAs in the ω-6 series showed a similar trend in both groups; LA and AA increased from T0 to T2; the increase was more pronounced for Group 2 (panel C, panel D).

Figure 17. Fatty acid levels in Group 1 and Group 2 at T0, T1 and T2.



The fatty acid values at the three times in the two groups were also compared with reference values found in the literature (**Supplementary Table 2**). In Group 1, the DHA values remained higher than the reference values, even after the 3-month wash-out, and likewise for EPA. LA values were also higher in Group 1 than in Group 2. Clinical parameters were measured at T0 and at T2 (**Table 12**). At T0, all the parameters were higher than the reference values due to the inflammatory status. At T2, the parameters returned to the normal range, except for the TyG index which was still high in both groups.

Table 12. Blood values in Group 1 and Group 2 at T0 and T2.

Biochemistry	Group 1 T0	Group 1 T2	Group 2 T0	Group 2 at T2	Reference values
CRP (mg/L)	199.8±105.6	0.8±1.1	175.5±88.3	0.9±0.5	≤10 mg/L
Ferritin (µg/L)	1537.1±1881.3	30.7±12.7	593.7±504.2	25.5±14.4	<300 µg/L
ESR (mm/h)	51±27.2	5.6±5.4	59.9±32.6	9.1±6.4	≤30 mm
IL-6 (ng/L)	33±67.6	0.5±0.5	6±83.6	0.4± 81.8	7 (ng/L)

Albumin (g/dL)	2.6±0.4	4.5±0.3	2.8±0.5	7.1±10.5	35–50 g/L
D-Dimer (μg/L)	3763,5±3535.7	439.8±294.9	5736.7±6062.4	425.5±402.6	<500 μg/L
Fibrinogen (g/L)	6.5±0.8	4.1±0.7	5.6±1.5	3.9±0.6	<4 g/L
Glucose (mg/dL)	111.53±37.11	87.47±7.93	118.36±30.81	86.53±5.13	70–110 mg/dL
Triglycerides (mg/dL)	227±96.42	89.93±51.68	207.29±103.47	74.67±24.09	<75 mg/dL 0–9 yo <90 mg/dL 10–19 yo
Tryg index	9.3±0.52	8.16±0.51	9.28±0.49	8.03±0.33	<7.88

Data are expressed as mean ± standard deviation (SD) of blood values.

Analyses were performed to search for possible correlations between inflammatory-related parameters such as CRP, ferritin, IL-6, TyG index, ESR, D-dimer, fibrinogen, and the most important FA, i.e. LA, AA, ALA, EPA, DPA and DHA (see Supplementary Tables 3, 4, 5, 6). At T0, in Group 1 the only correlation found was a negative one between DPA and TyG index, whereas there were no correlations in Group 2. At T2, in Group 1 positive correlations were found between EPA and ESR and D-dimer, and positive correlations between DHA, O3I and ferritin. In Group 2, there were negative correlations between EPA and ESR, and between DHA and CRP **(Table 13).**

Table 13. Significant correlations between blood values and fatty acid profile of Group 1 and Group 2 at T0 and T2. (All correlation values are shown in Supplementary Tables 3, 4, 5 and 6).

Group 1

T0		**LA**	**AA**	**ALA**	**EPA**	**DPA**	**DHA**	**O3I**
Tryg-Index	r	0.06	-0.36	0.21	0.22	**-.59***	-0.33	-0.33

	p	0.82	0.18	0.43	0.42	**0.02**	0.22	0.22
	N	*15*	*15*	*15*	*15*	*15*	*15*	*15*

T2		**LA**	**AA**	**ALA**	**EPA**	**DPA**	**DHA**	**O3I**
ESR	r	-0.23	0.02	0.23	**.53***	0.22	0.09	0.13
	p	0.42	0.94	0.41	**0.04**	0.43	0.73	0.63
	N	*15*	*15*	*15*	*15*	*15*	*15*	*15*
Ferritin	r	-0.35	0.43	0.16	0.33	0.42	**.52***	**.52***
	p	0.19	0.11	0.56	0.23	0.11	**0.04**	**0.04**
	N	*15*	*15*	*15*	*15*	*15*	*15*	*15*
D-dimer	r	0.00	0.27	-0.19	**.53***	0.24	0.14	0.16
	p	0.99	0.32	0.49	**0.04**	0.37	0.60	0.57
	N	*15*	*15*	*15*	*15*	*15*	*15*	*15*

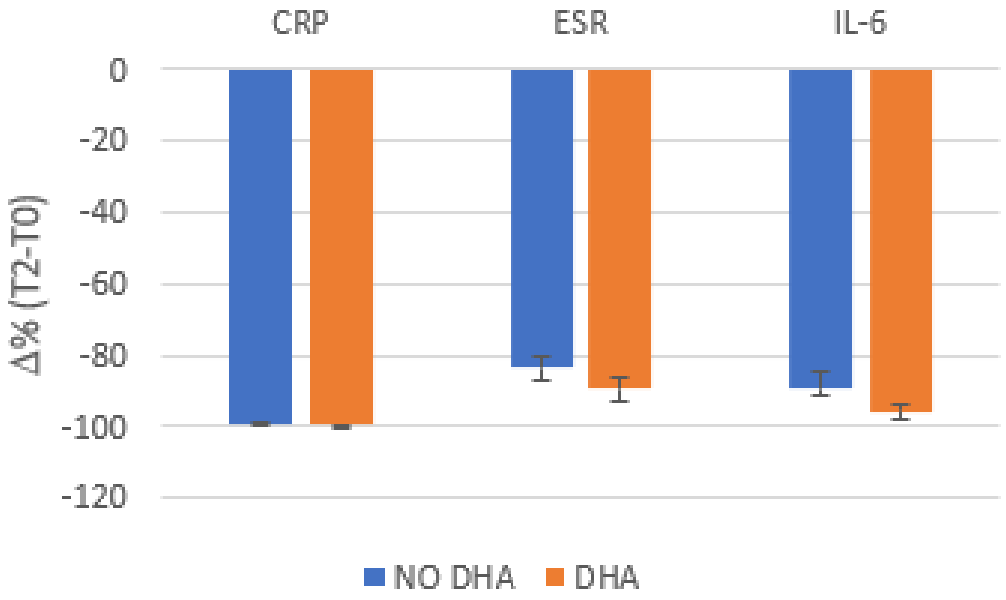
Group 2

T2		**LA**	**AA**	**ALA**	**EPA**	**DPA**	**DHA**	**O3I**
CPR	r	**.52***	0.14	-0.06	-0.19	-0.13	**-.60***	-0.16
	p	**0.05**	0.61	0.83	0.49	0.63	**0.01**	0.55
	N	*15*	*15*	*15*	*15*	*15*	*15*	*15*
ESR	r	-0.06	-0.20	0.05	-0.24	**-.60***	-0.44	0.24
	p	0.83	0.46	0.85	0.39	**0.02**	0.09	0.38
	N	15	15	15	15	15	15	15

N= number of sample size. r = correlation. p = p value

Lastly. **Figure 18** shows the reduction levels, expressed as ⊚% (T2-T0), of the inflammation markers measured in the two groups. CRP decreased equally in both groups, while ESR and IL-6 decreased more in Group 1.

Figure 18. Delta (Δ) % reduction (T0-T2) of inflammatory markers in Group 1 (DHA) and Group 2 (NO DHA).



Discussion

Current evidence supports the use of ω-3 FA supplementation in the prevention and treatment of a wide range of human diseases, including coronary artery disease, diabetes, hypertension, arthritis, and other inflammatory and autoimmune conditions[172,173]. ω-3 FAs appear to play an important therapeutic role in the treatment of several diseases. By triggering the production of SPMs, ω-3 FAs actively control the inflammatory process. These mediators reduce the severity of the inflammatory process and promote its active resolution by suppressing the overproduction of pro-inflammatory lipid-derived compounds and cytokines [174].

Inflammation has two main phases: initiation and resolution. During the initiation phase, ω-6 ARA enters the cells through the phospholipid membrane and produces a series of molecules called eicosanoids, which are involved in the inflammatory process[175]. At the same time, the ω-3 EPA, DHA and DPA also enter the cells, and act as substrates for specialised pro-resolving mediators (resolvins, protectins and maresins), thereby modulating inflammation[128]. In adults, lower levels of DHA and O31 have been associated with a higher risk of adverse COVID-19 outcomes[131].

In this study, we analysed the FA profile and inflammation-related blood values in a cohort of children with MIS-C. One group was supplemented with DHA for three months. Our aim was to investigate correlations between FA levels and blood values during the acute phase, and after six months, and to examine the short-term benefits of DHA supplementation.

Interestingly, our results showed significant differences between Group 1 and Group 2 for DHA levels at T2. EPA levels were significantly higher in Group 1 than in Group 2 at all three times. DPA increased significantly in the supplemented Group 1 compared to Group 2 at both T0 and T1. At the same time, O3I and DHA/AA were significantly higher in Group 1 at T2. A significantly higher ω-6:ω-3 ratio (typical of a Western diet) was found in Group 2 compared to Group 1 at T2.

The study highlights the continued presence of ω-3 levels in Group 1, which is the group that received supplementation. Both groups showed complete resolution of inflammation at T2, which can be attributed to the physiological process that naturally follows the acute phase of the disease. Nevertheless, the correlations between blood levels and FAs show a positive trend, possibly due to our small sample size.

Another limitation of our study was the inability to measure SPMs, which represent the inflammatory status. Nonetheless, we recommend early DHA supplementation in children with inflammatory diseases such as MIS-C to improve the inflammatory response and facilitate a better prognosis. Ongoing supplementation over time is also advisable to support full recovery of daily activity and normal function in children. Studies in adults with severe COVID-19 show the benefits of early use of ω-3 PUFA supplementation [106,176,177]. Therefore, further research should evaluate the benefits for critically ill paediatric patients of an early use of ω-3 PUFA in the hospital setting and during follow-up.

5.6 Discussion and conclusion

Glucose and lipid metabolism profile was found to be altered during hospitalisation in children diagnosed with MIS-C, suggesting multi-organ inflammatory involvement. In severely ill patients it is common to have alterations in glucose metabolism [62,63]. In the adult population an interaction between COVID-19 and glucose-insulin metabolic disturbances has been proven [178,179], and in the paediatric population a relationship between SARS-CoV-2 infection and type 1 diabetes mellitus has been described[180].

The results we observed are no different from what is expected in the adaptive metabolic response to inflammation. However, it is possible to hypothesise a bidirectional relationship between COVID-19 and glucose impairment. Glycaemic fluctuations could be caused by the inflammatory cascade of the SARS-CoV-2 attack on the pancreas and potentially impaired β-cell function. Both hepatic and peripheral insulin action were supported by the significant prevalence of abnormal values in the HOMA-IR and TyG indices in our cohort of children. Despite hyperinsulinemia, the effect of counterregulatory hormones on IR in skeletal muscles may be mediated via an increase in circulating free fatty acid levels.

In addition, in order to observe the FA levels for each patient we collected a blood sample (using the Guthrie test) during inflammatory status, as lipid mediators also play a role in the physiological evolution of inflammation and its resolution. We found lower relative amounts of ω-6 PUFA (LA and AA) in MIS-C subjects than those reported in the literature for healthy children. These values remain lower after dividing the subjects according to age (<9 years and >9 years), probably due to a massive release of phospholipids followed by metabolic conversion into pro-inflammatory lipid mediators. The levels of ω-3 PUFAs ALA and EPA were broadly in line with those found in the literature [94, 95, 96, 97, 98], whereas the levels of DHA were lower, likely due to increased SPM formation, which, however, does not seem to be sufficient to ameliorate the hyperinflammatory status.

Only one direct correlation was found between relative amounts of ALA and CRP concentration, while no statistically significant correlation was found between markers of

inflammation and relative amounts of relevant FAs (i.e. LA, AA, EPA and DHA). Although ALA is a precursor to ω-3 PUFAs, its conversion in humans is limited and more pronounced in females than males [181] . Therefore, we can speculate that ALA may also have led to a reduction in pre-resolution SPM formation and an increase in the number of inflammatory biomarkers. In our cohort, O3I was not found to be associated with any markers of inflammation (IL-6, CRP or ESR), but it should be noted that in adult patients with an O3I of 5.7% or higher, the risk of death was lower than in patients with an O3I below this value [131].

PUFA ω-6 and ω-3 values observed in MIS-C children may reflect the severity of the inflammatory status, with higher values in milder cases, and vice versa. The FA profile of MIS-C children is altered during hospital stay, and the levels of O3I are significantly lower than those of healthy children. However, higher levels of O3I are associated with a lower number of DoH , whereas the admission to PICU does not seem to be affected.

According to recent findings, ω-3 PUFAs play a critical therapeutic function in treating various diseases[172,173]. They actively regulate the inflammatory process by inducing the formation of SPMs [174]. To enable the best possible inflammatory response, it is crucial to maintain an omega 3–6 balance [128] . A number of clinical studies conducted during the SARS-CoV2 pandemic have indicated the potential benefits of supplementing with ω-3 PUFAs to control and improve a patient's inflammatory status [101,104,149]. Some trials revealed an improvement in clinical symptoms in Covid-19 adult patients after ω-3 PUFAs supplementation [100,151].

We observed consistently higher levels in the group that had received supplementation, but at the same time no significant correlations between fatty acid levels and markers of inflammation in the total population included in our study. However, it may be interesting to further investigate the use of ω-3-supplementation in the critically ill paediatric population, both in the acute phase and afterwards, by detecting SPMs as markers of inflammation.

Chapter 6

Future perspectives

Our results on the paediatric population with MIS-C would seem to indicate that although the clinical course is positive, hospitalisation is prolonged. This is a key aspect as the risk of malnutrition can set in insidiously and create a loss of muscle mass. Screening and monitoring are useful in an acute hyperinflammatory disease such as in this case.

The role of ω-3 PUFA appears to be pivotal. Indeed, due to the high inflammatory response, endogenous utilisation is high. Therefore, an appropriate supplementation of ω-3 would seem to be supportive to ameliorate the inflammatory status in acute phase and later . In addition, children who have had this pathology experienced a particular historical moment that accentuated various aspects related to sedentariness, altered eating habits, and the difficulty of resuming normal daily activities as well as spontaneous movements and sports. Although the Sars-cov-2 pandemic in the paediatric population did not have the same impact as it did in the adult population, it was nevertheless significant. We recommend that children and teenagers should be screened for the risk of malnutrition in long hospitalization setting and that targeted advice regarding diet and use of specific supplements can improve short- and long-range inflammatory outcomes.

As a future prospectives, in the paediatric setting it would be interesting to evaluate the efficacy of ω-3 supplementation in the acute phase by means of SPM dosage. In fact, in critical adults (ARDS, sepsis, COVID-19, and organ injury) its efficacy has been proven by several systematic reviews and meta-analyses. Future research needs to focus on the optimal dosages of ω-3 PUFAs, the optimal routes of administration, and which target paediatric population would benefit the most.

Chapter 7

Copyright Statement

All non-original figures used in the manuscript contain the reference source in the caption.

The author confirms that she collected all the anthropometric and blood data and samples for fatty acid analysis of the patients included in the study. In addition, she participated in writing and revising the papers, interpreting the data, and producing the graphs, pictures and figures.

Chapter 8

Supplementary Tables

Suppl. Table 1: composition of DHA supplement

	100 ml
Energy kcal	845 kcal
Fat	94 g
-Saturated fat	69 g
-Monounsaturated fat	0 g
-Polyunsaturated fat	25 g
Carbohydrates	0 g
-Sugar	0 g
Protein	0 g
Salt	< 0.1 g
DHA (from algae oil)	25.000 mg
Vitamin D3	40.000 UI (1000 µg)

Suppl. Table 2 Whole blood fatty acids profile in Group 1 and Group 2 at T0, T1 and T2 compared to references range from literature.

Group/Author	**Group 1 at T0, T1 and T2**			**Group 2 at T0, T1 and T2**			**Risé[93]**	**Crippa[94]**	**Bonafini[95]**	**Van der Wurff[96]**	**Ryan[97]**
Age (year)	2-18 y			2-18 y			<9	7-14	7-9	13-15	4
% LA	12,57 ±2,22	14.56±1.58	17.71±2.82	12.70±2.27	17.92±2.66	19.29±1.94	17.6±1.92	22.54±2.45	19.9±2.32		

% AA	6.60±1.21	9.05±1.79	9.29±2.20	5.31±1.84	7.35±1.38	8.30±1.55	8.33±1.04	10.10±0.92	12.21±1.67	11.01±11.33	7.50±1.89
% ALA	0.20±0.09	0.29±0.16	0.21±0.08	0.17±0.06	0.36±0.16	0.20±0.06	0.15±0.05		0.16±0.08		
% EPA	0.32±0.09	0.46±0.10	0.41±0.08	0.41±0.11	0.28±0.10	0.33±0.09	0.23±0.08	1.13±0.45	0.30±0.17	0.34±0.42	0.30±0.39
% DHA	1.19±0.25	2.67±0.78	2.37±0.87	1.09±0.51	1.44±0.47	1.70±0.43	1.40±0.37	1.93±0.53	2.92±0.76	2.49±2.63	1.00±0.34

Suppl. Table 3. Correlation analysis between blood values and fatty acids Group 1 at T0

Group 1

Blood values a T0		**LA**	**AA**	**ALA**	**EPA**	**DPA**	**DHA**	**O3I**
CPR	r	-0.229	-0.013	0.433	-0.214	-0.188	0.218	0.282
	p	0.412	0.965	0.107	0.443	0.503	0.435	0.308
	N	*15*	*15*	*15*	*15*	*15*	*15*	*15*
ESR	r	0.074	-0.260	-0.526	-0.077	-0.218	-0.488	-0.540
	p	0.820	0.415	0.079	0.812	0.497	0.108	0.070
	N	*12*	*12*	*12*	*12*	*12*	*12*	*12*
Il-6	r	-0.382	-0.430	0.091	0.212	-0.406	-0.055	0.006
	p	0.276	0.214	0.803	0.556	0.244	0.881	0.987
	N	*10*	*10*	*10*	*10*	*10*	*10*	*10*
Ferritin	r	-0.236	-0.168	0.057	0.196	-0.268	0.082	0.079
	p	0.398	0.550	0.840	0.483	0.334	0.771	0.781
	N	*15*	*15*	*15*	*15*	*15*	*15*	*15*
D-Dimer	r	-0.211	-0.086	-0.225	0.111	0.129	0.007	-0.025
	p	0.451	0.761	0.420	0.694	0.648	0.980	0.930
	N	*15*	*15*	*15*	*15*	*15*	*15*	*15*
Fibrinogen	r	-0.129	0.000	-0.165	-0.020	-0.298	0.113	0.205
	p	0.647	1.000	0.556	0.943	0.280	0.689	0.463
	N	*15*	*15*	*15*	*15*	*15*	*15*	*15*
Tryg-Index	r	0.063	-0.363	0.218	0.225	**-.595***	-0.332	-0.336
	p	0.825	0.184	0.435	0.420	**0.019**	0.226	0.221
	N	*15*	*15*	*15*	*15*	*15*	*15*	*15*

Suppl. Table 4. Correlation analysis between blood values and fatty acids Group 2 at T0

Group 2

Blood values a T0		**LA**	**AA**	**ALA**	**EPA**	**DPA**	**DHA**	**O3I**
CPR	r	-0.157	0.186	-0.004	0.238	-0.007	0.191	0.286
	p	0.576	0.508	0.990	0.393	0.980	0.495	0.302
	N	*15*	*15*	*15*	*15*	*15*	*15*	*15*
ESR	r	0.108	-0.020	0.175	-0.134	-0.207	0.022	-0.121
	p	0.714	0.946	0.550	0.647	0.478	0.940	0.681
	N	*14*	*14*	*14*	*14*	*14*	*14*	*14*
Il-6	r	-0.148	0.482	0.093	-0.259	0.262	0.296	0.296
	p	0.751	0.274	0.842	0.574	0.571	0.518	0.518
	N	*7*	*7*	*7*	*7*	*7*	*7*	*7*
Ferritin	r	0.044	-0.214	0.352	-0.319	-0.003	-0.121	-0.236
	p	0.887	0.482	0.238	0.288	0.993	0.694	0.437
	N	*13*	*13*	*13*	*13*	*13*	*13*	*13*
D-Dimer	r	0.157	0.221	0.207	0.061	0.038	0.302	0.254
	p	0.576	0.428	0.460	0.830	0.894	0.274	0.362
	N	*15*	*15*	*15*	*15*	*15*	*15*	*15*
Fibrinogen	r	-0.050	-0.165	0.249	-0.011	-0.115	-0.335	-0.275
	p	0.858	0.557	0.370	0.968	0.683	0.222	0.321
	N	*15*	*15*	*15*	*15*	*15*	*15*	*15*
Tryg-Index	r	-0.275	-0.398	0.044	0.051	-0.181	-0.508	-0.301
	p	0.342	0.159	0.880	0.863	0.536	0.064	0.296
	N	*14*	*14*	*14*	*14*	*14*	*14*	*14*

Suppl. Table 5. Correlation analysis between blood values and fatty acids Group 1 at T2

Group 1

Blood values a T2		**LA**	**AA**	**ALA**	**EPA**	**DPA**	**DHA**	**O3I**
CPR	r	-0.049	-0.054	-0.053	0.323	-0.018	-0.197	-0.161
	p	0.863	0.849	0.852	0.240	0.949	0.482	0.566
	N	*15*	*15*	*15*	*15*	*15*	*15*	*15*
ESR	r	-0.227	0.019	0.227	**.526***	0.220	0.095	0.134
	p	0.416	0.945	0.416	**0.044**	0.430	0.736	0.634
	N	*15*	*15*	*15*	*15*	*15*	*15*	*15*
Il-6	r	0.433	0.000	0.187	-0.124	-0.310	-0.124	-0.124
	p	0.107	1.000	0.506	0.660	0.262	0.660	0.660
	N	*15*	*15*	*15*	*15*	*15*	*15*	*15*
Ferritin	r	-0.353	0.427	0.164	0.327	0.425	**.516***	**.524***
	p	0.197	0.112	0.560	0.235	0.114	**0.049**	**0.045**
	N	*15*	*15*	*15*	*15*	*15*	*15*	*15*
D-Dimer	r	0.004	0.271	-0.190	**.532***	0.247	0.146	0.161
	p	0.990	0.328	0.497	**0.041**	0.376	0.603	0.567
	N	*15*	*15*	*15*	*15*	*15*	*15*	*15*
Fibrinogen	r	-0.329	0.339	0.474	0.342	0.375	0.232	0.284
	p	0.232	0.216	0.074	0.212	0.168	0.405	0.305
	N	*15*	*15*	*15*	*15*	*15*	*15*	*15*
Tryg-Index	r	-0.231	-0.262	0.100	-0.148	-0.116	-0.020	-0.026
	p	0.427	0.366	0.735	0.614	0.692	0.946	0.929
	N	*14*	*14*	*14*	*14*	*14*	*14*	*14*

Suppl. Table 6. Correlation analysis between blood values and fatty acids Group 2 at T2

Group 2

Blood values a T2		**LA**	**AA**	**ALA**	**EPA**	**DPA**	**DHA**	**O3I**
CPR	r	**.516***	0.141	-0.059	-0.192	-0.133	**-.603***	-0.167
	p	**0.049**	0.615	0.835	0.493	0.636	**0.017**	0.551
	N	*15*	*15*	*15*	*15*	*15*	*15*	*15*
ESR	r	-0.058	-0.205	0.052	-0.240	**-.600***	-0.447	0.243
	p	0.839	0.464	0.853	0.390	**0.018**	0.094	0.384
	N	15	15	15	15	15	15	15
Ferritin	r	0.156	-0.217	0.286	0.472	-0.016	-0.178	0.181
	p	0.579	0.437	0.301	0.076	0.954	0.527	0.518
	N	15	15	15	15	15	15	15
D-Dimer	r	-0.174	-0.035	0.218	-0.483	-0.440	-0.301	0.405
	p	0.552	0.905	0.455	0.080	0.115	0.295	0.151
	N	14	14	14	14	14	14	14
Fibrinogen	r	0.145	0.116	-0.091	0.001	-0.099	-0.317	-0.048
	p	0.620	0.694	0.758	0.997	0.736	0.270	0.869
	N	14	14	14	14	14	14	14
Tryg-Index	r	0.191	-0.134	-0.098	0.043	0.004	-0.298	-0.102
	p	0.495	0.634	0.729	0.879	0.990	0.280	0.718
	N	15	15	15	15	15	15	15

IL-6 correlation with FA was not possible to conduct due to low number of available values.

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