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Original Research

The variations of selected serum cytokines involved in cytokine Storm after Omega-3 daily supplements: A Randomized Clinical Trial in Jordanians with vitamin D deficiency

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Abstract

The aim of this study was to investigate the effects of supplementing unsaturated free fatty acids (n-3FA) on the levels of specific cytokines associated with cytokine storm in the blood of 72 Jordanian individuals (both men and women) who had insufficient vitamin D. The study was conducted using a randomized controlled design (RCT). Individuals eligible for the study were randomly assigned to either the n-3FA supplemented (intervention) group or the non-supplemented (control) group. The intervention group received 1,000 mg of wild salmon and fish oil complex, which is equivalent to 300 mg of n-3FA, for a duration of eight weeks, while the control group did not. Blood tests to assess tumor necrosis factor-α (TNF-α), lipid profile, and fasting blood sugar were conducted at baseline and after ten weeks (with a two-week washout period). Additionally, interleukin-6 (IL-6), interleukin-1β (IL-1β), and interleukin-10 (IL-10) levels were measured. Our research indicates that taking n-3FA supplements significantly raised IL-1 β, IL-6, and IL-10 levels compared to their initial levels. However, TNF-α levels did not show any significant changes. The interesting results of this randomized controlled trial could be due to a possible harmful effect of n-3FA supplementation during cytokine storms (CS), specifically on IL-6. Therefore, additional clinical studies are needed in the target patients during cytokine storm to determine if this supplement could affect the treatment with IL-6 antagonists..

Keywords: Omega-3; unsaturated fatty acid; cytokine storm; IL-1β; IL-6; IL-10; TNF-α

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INTRODUCTION

Coronavirus 2 (SARS-CoV-2) is responsible for the severe acute respiratory syndrome (SARS-CoV-19) pandemic viral pneumonia. It has been noted that cytokine storm (CS) and its severe repercussions from infection significantly accompany high blood levels of certain cytokines found during COVID-19.[1] Accordingly, CS has been listed as one of the main COVID-19 hallmarks.[2] Besides interleukin-6 (IL-6), many other cytokines, such as interleukin- 1β, IL-10, and TNF-α changes, were monitored during CS.[3]

Due to their high effectiveness, some supplements such as omega-3 (n-3FA) and vitamin D (VD) are recommended to be accompanied by therapeutic protocols that aim to block IL-6 receptors and suppress cytokine storm.[4,5]

The association between immune responses, including SARS-2 and (n-3FA), has been reported in some previous clinical trials.[6,7] A review of seven RCTs has concluded that routine use of enteral supplementation of n-3FA**,** both EPA and DHA, may reduce the release of inflammatory cytokines.[8]DHA pre-supplementation dramatically reduced IL-6 release.[9] These n-3FAs directly or indirectly inhibit nuclear transcription

factor activity and decrease the generation of pro-inflammatory cytokines and enzymes, such as TNF-α and interleukin IL-1β.[10]

A high-dose (1.5 g/day EPA and 1.0 g/day DHA) n-3FA supplementation can lower plasma levels of both IL-6 and IL-1β, according to an RCT. This RCT was conducted to evaluate the positive impact of EPA and DHA supplementation in the treatment of SARS-2 infection. N-3FA supplements have subsequently been proposed as a supportive treatment and a technique for preventing SARS-2 infection lately.[9]

However, other studies, particularly in those with VDD, have not supported the n-3FA's recommended impact on immune response, including IL-1β, IL-6, and TNF-α levels.[11]

Conversely, VD has been shown to modulate immune responses and may moderate the CS induced by the innate immune system.[12] Conversely, Bader et al.[13] have shown a significant positive association between serum levels of 25OHD and IL-6 in Jordanian people with VDD.[4] Al-Shaer et al.[14] observed that n-3FA supplementation alone without a co-supplementation with VD3 significantly reduced 25OHD levels in Jordanian people with VDD.[15]

In this manner, negative correlations between mean levels of 25OHD and the incidence of Covid-19 were recorded.[16-18] Although n-3FA supplements are recommended as both a supportive therapy and a prevention strategy for SARS-2 infection,[9] some reports have not endorsed the prescribed effect of the n-3FA on the immune response, including IL-1β, IL-6, IL-10, and TNF-α levels and particularly in people with VDD.[11] Therefore, the potential adverse effects of n-3FA supplementation on 25OHD levels may be opposite to the goal of VD as both a supportive therapy and a prevention strategy. Therefore, the potential adverse effects of n-3FA supplementation on 25OHD levels may oppose the goal of VD as both a prevention strategy and supportive therapy, specifically in people with low 25OHD levels.[11]

Dietary n-3FA at a dose of 1 g per day is common in Jordan[5,14] and among people who are susceptible to cardiovascular disease (CVD).[19] But high or/and extensive doses of n-3FA have been shown to have provocative potential consequences on both pro- and anti-inflammatory immune responses.[20]

However, there are diverse mechanisms by which n-3FA might be involved in immune response alterations,[21] and still unclear. Overall, published RTCs, designed to assess the effects of n-3FA supplements on the cytokines involved in the immune response during cytokine storm have not provided a clear conclusion. Therefore, this RCT was designed to measure serum levels of IL-1β, IL-6, IL10, and TNF-α as a part of the immune response during cytokines storm before and after eight weeks of treatment with a dose of n-3FA 300 mg per day in people with VDD.

RESEARCH METHODOLOGY

Study design and Participant

The Applied Science Private University (ASU) Institutional Review Board gave its approval to the present RCT, which is listed at *clinicaltrials.gov* with the identifier NCT04483271.

The trial was undertaken between September 2020 and January 2021 following the Helsinki Declaration. Participants were Jordanian from ASU society (academic staff and administrative employees) with a baseline mean age of 38.5 ±12.3 years (ranging from 27 to 52).

Based on a determination of VDD (25-hydroxyvitamin D (25OHD) < 30 ng/ml) validated by medical advisors at Ibn Al-Haytham clinical labs, eligible individuals were enrolled in the experiment.

Before enrolling in this RCT, each participant was allowed to give their written approval. Among the reasons for being excluded from the present research were chronic medical diseases, including cancer, osteoporosis, endocrine disorders, and previous experiences of allergic reactions to omega supplements. Those who met the trial's eligibility requirements had their VDD diagnosis validated by physicians at the Ibn Al-Haytham clinical laboratory before admission.

Intervention

Anthropometric and clinical parameters have been evaluated at the beginning and after n-3FA administration. Participants had a 2-week washout before and after n-3FA treatment to prevent any potential effects of its cumulative dosage after the 8-week interventional phase.

Then, follow-up measures for every subject were taken. A separate statistician created an automated randomization procedure. It consisted of two groups made up of one hundred and two (102) qualified individuals' total. Group 1 got 1,000 mg of wild salmon and fish oil complex per day for eight weeks, which is comparable to 300 mg of n-3FA (180 mg of eicosapentaenoic acid (EPA) and 120 mg of docosahexaenoic acid (DHA), Jamieson Laboratories, Canada N8W 585). Group 2 participants received no n-3FA or other omega supplements and served as the control group, as indicated in the consortium chart (Figure 1). Periodic texts sent to each participant's phone were used to track how well they followed the therapy plan.

Anthropometric measurements and Clinical Parameter Assays

Anthropometric measures such as body mass index (BMI), body weight (BW), hip (H) circumference, waist/hip ratio (WHR), height (Ht), and waist (W) circumference were taken at the beginning and conclusion of the experiment.

At Ibn Al-Haytham Hospital's Clinical Laboratories Department in Jordan, serum samples for clinical parameter assays were gathered and placed in labeled Eppendorf tubes. The total serum 25OHD was determined by the chemiluminescence immunoassay LIAISON 25OHD test (DiaSorin, Saluggia, Italy). The test quantifies serum 25OHD, which also measures 25OHD2 and 25OHD3. It could not get below 4 ng/mL. Leptin EIA-5302, manufactured by DRG Diagnostics in Marburg, Germany, was used to detect blood leptin levels. The test has a sensitivity of 0.1 ng/mL.

An enzyme immunoassay kit (PTH Intact EIA-3645, DRG

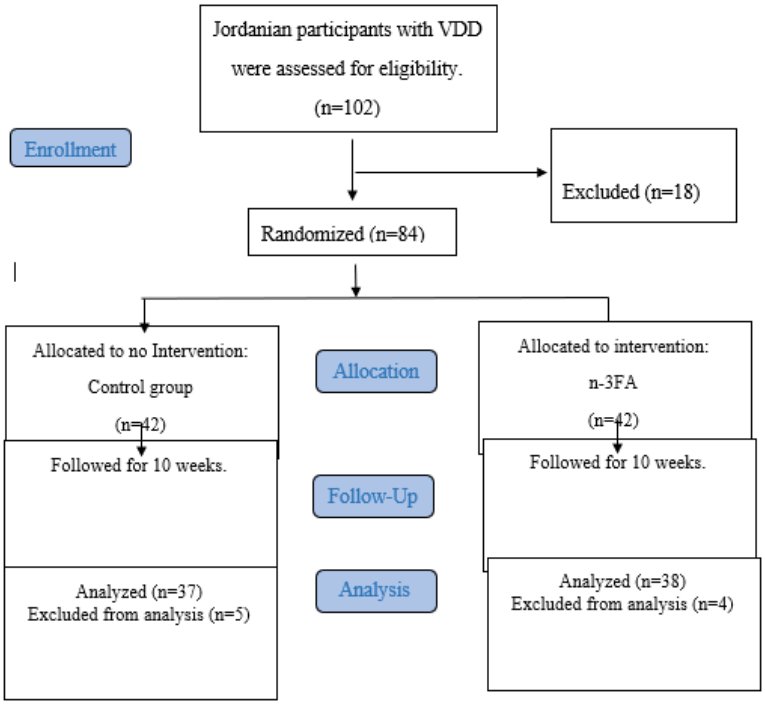

Figure 1. CONSORT flow diagram for the study, indicating the number of subjects screened, recruited, and randomly assigned to the different intervention groups.

Diagnostics, Marburg, Germany) was used to measure serum PTH levels; 1.57 pg/mL was the sensitivity.

Calcium and phosphorus (PO4) levels in serum were determined using the calcium-ARSENAZO test (M11570i-15) and the phosphorus phosphomolybdate/Uv kit (M11508i-18, BioSystems, Barcelona, Spain). Utilising the Human ELISA KIT (ab178013, Abcam, Newark, NJ, USA), the level of IL-6 in the serum was determined.

Serum IL-10 was tested using a Human ELISA Kit (ab185986, Abcam). A human ELISA KIT (ab214025, Abcam) was used to measure serum IL-1β. The Human ELISA KIT (ab181421, Abcam) test also evaluated blood TNF-α.

Using enzymatic kits from HumaStar 200 - Human Diagnostics, Germany, serum levels of fasting blood glucose (FBG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), triglycerides (TG), and high-density lipoprotein cholesterol (HDL-C) were measured.

Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST) levels were determined using BioSystems kits (M21533i-03 and M11531i-23, respectively). Using specialised BioSystems kits (M12502i-19 and M11536i-17), serum creatinine and urea levels were evaluated.

Statistical Analysis

The statistical analysis was conducted using the latest version of SPSS 26 for Windows. To find any notable differences between each study group before and following the administration of the n-3FA supplementation, a paired t-test was carried out.

The significance of any variance between all items of the two different groups (control, n-3FA) was assessed by two independent sample t-tests.

The correlation analysis method compared TNF-α, IL-1β, IL-6, and IL-10 plasma levels and their ratios (TNF-α/IL-10, IL-1β / IL-10, and IL-6/IL-10).

The impact of n-3FA intake on the variables (TNF-α/IL-10, IL-1β/IL-10, IL-6/IL-10, ratio (TNF-α/IL-10), and IL-6/IL-10) was examined using simple linear regression. At the follow-up level for the (D) group, a multiple linear regression analysis identified the predictors of the items (TNF-α, IL-1β, IL-6, IL-10, ratio (TNF-α/IL-10), IL-1β, and IL-6/IL-10).

The Kolmogorov-Smirnov test evaluated the normality of distribution for laboratory measurements. The data showed a conventional distribution curve.

RESULTS

Baseline anthropometric characteristics of the Participants

Seventy-two (72, 70.58 %) out of One hundred two (102) participants adhered to the protocol and completed the ten weeks whole trial and an eight-week intervention regimen time, as shown in Figure 1. Twenty-seven (27) individuals left the study early because of non-compliance (10), failing to meet the criteria for eligibility (8), and quitting the treatment group (n = 4) and the control group (n=5).

As demonstrated in Table 1, the mean initial BMI for all individuals was *28.53±5.99* kg/m^2, indicating that the study group being studied was overweight. Around half (44%) of the group studied was female, as seen in Table 1. The initial chosen

potential anthropometric and lifestyle parameters are listed in Table 1. And initial levels of selected cytokines and clinical parameters are also provided in Table 2.

Table 1. Statistical description of anthropometric parameters at baseline (n = 72)

Parameter	Mean (SD)
Age (years)	38.23 ± 9.58
Body weight (kg)	82.29 ± 18.63
Hight (cm)	197.28 ± 8.03
Body mass Index (kg/m$^{2)}$	28.53 ± 5.99
Waist (cm)	96.52 ± 16.04

Table 2. Baseline Levels of selected cytokines and clinical parameters in the entire clinical trial population (n = 72)

Parameter	Mean ± SD	Normal range
25OHD	22.49±6.46	30-70 ng/ml
IL-1β (ng/ml)	31.16±4.89	N/A
IL-6 (ng/ml)	3.49±2.25	N/A
IL-10 (ng/ml)	4.15±2.78	N/A
TNF-α (ng/ml)	2.06±0.56	N/A
TNF-α/IL-1β0	1770.42±1083.89	N/A
IL-1β/IL-6	197.53±175.80	N/A
IL-6/IL-1β0	204.77±129.73	N/A
FBG (mg/dl)	84.18±18.53	70-110
TG (mg/dl)	134.38±81.63	Up to 150
TC (mg/dl)	138.22±65.09	Up to 200
HDL (mg/dl)	35.12±12.96	> 60
LDL (mg/dl)	96.18±32.40	Up to 100

ABBREVIATIONS: N/A: not applicable; TNF-α, Tumor Necrosis Factor; IL-1β: interleukin 1beta; IL-6: Interleukin 6; IL-10 Interlukin10; FBG, fasting blood glucose, TG: Triglyceride; TC: total cholesterol; HDL: high-density protein; LDL: low-density protein; SD: standard deviation.

Baseline Clinical Characteristics

Table 2 lists the mean initial values for the ratios of the serum concentrations of IL-1β, IL-6, IL-10, and TNF-α. Fasting blood glucose (FBG) and serum lipid profile measurements were all within normal limits at baseline for all individuals.

Changes in 25OHD and PTH levels

The follow-up means of the 25OHD levels among the participants of the n-3FA group were significantly different, according to paired samples T-test (24.52±4.91 ng/ml vs. 15.75±6.39 ng/ml, P^A= 0.001). Independent sample T-tests were used to establish any noteworthy discrepancies in PTH levels and 25OHD between the control and n-3FA groups. In the follow-up period, no significant variations in serum 25OHD and serum PTH levels between the control and n-3FA groups were seen (18.61±7.75 ng/ml vs 15.75±6.39 ng/ml and 33.35±10.64 ng/ml vs 30.05±11.57 ng/ml, P^C = 0.244 and P^C= 0.226, respectively, as shown in Table 3).

Table 3. Changes in the serum levels of 25OHD and PTH after 10 weeks follow up

Group				
Variable		C	n-3FA	
		Mean ± SD	Mean ± SD	*P*-value
25OHD	Baseline	20.15±7.28	24.52±4.91	P^B = 0.054
	Follow up	18.61±7.75	15.75±6.39	P^C = 0.244
	Change	-1.54	-8.77	
	P^A	0.054	< 0.001	
PTH	Baseline	34.68± 8.22	29.22±7.43	P^B = 0.057
	Follow up	33.35±10.64	30.05±11.57	P^C = 0.226
	Change	-1.33	0.84	
	P^A	0.506	0.656	

Abbreviations: Note $P^{A:}$ *P*-value; follow-up changes in each group (paired t-test); P^B: *P*-value for baseline comparison between groups (Independent-test) P^C: *P*-value for follow-up comparison (Independent-test), C: control group; n-3FA: n-3FA supplementation group; 25OHD: vitamin D level.

Changes in cytokines levels

The mean levels of IL-1β, IL-6, and IL-10 were significantly different after the study completion, as shown in Table 4, according to a paired t-test. In the n-3FA group, the mean IL-1β level decreased extensively with a reduction of about -3.37ng/ml (7.29±1.87 ng/ml vs 3.92±2.79 ng/ml, P^A= 0.001).

The study utilized a statistically independent t-test to establish whether there was a notable variation in the mean levels of IL-1β between the n-3FA group and the control group. The results showed that the n-3FA group had higher levels of IL-1β (7.29 ± 1.87 ng/ml) than the control group (5.86 ± 3.87 ng/ml), with a significant *p*-value of less than 0.001.

In the group that consumed n-3FA, there was a notable contrast between the average IL-6 levels before and after (22.67±12.24 ng/ml compared to 3.57±2.93 ng/ml, p<0.001).

Table 3 shows that there were no notable differences in the average IL-6 levels between the n-3FA and control groups at the beginning and end of the study, as confirmed by the independent t-test. However, the mean IL-10 levels were significantly higher in the n-3FA group at the 10-week follow-up, with a difference of around 0.76 ng/ml (2.70±1.69 ng/ml compared to 1.95±0.63 ng/ml in the control group, with a P^A-value of 0.019).

In the group that consumed n-3FA, IL-10 levels were found to be significantly higher compared to the control group, with a *P*-value of PC 0.017. The IL-10 levels were 2.70±1.69 ng/ml in the control group and 3.73±1.80 ng/ml in the n-3FA group. Additionally, the recent RCT showed a minimal increase in the average TNF-α during follow-up (P^A = 0.989).

Table 4. Changes in the serum levels of cytokines

Cytokine	Change	Group		*p*-value
		Control Mean (SD)	n-3FAs Mean (SD)	
IL-1β				
	Baseline	2.98±1.25	3.92±2.79	P^B=0.081
	Follow up	5.86±3.87	7.29±1.87	P^C=0.048
	Change	2.88	3.37	
	P^A	.065	<.001	
IL-6				
	Baseline	4.82± 2.47	3.57±2.93	P^B=0.063
	Follow up	5.12±4.93	22.67±12.24	P^C>0.001
	Change	0.29	19.10	
	P^A	.752	<.001	
IL-10				
	Baseline	2.19±0.43	1.95±0.63	*P^B=0.067*
	Follow up	3.73 ±1.80	2.70±1.69	*P^C=0.017*
	Change	1.54	0.76	
	P^A	.061	.019	
TNF-α				
	Baseline	30.75±5.22	31.52±4.63	*P^B=0.516*
	Follow up	37.7 ±12.73	32.54±5.91	*P^C=0.010*
	Change	6.95	1.02	
	P^A	.053	.989	

Note *P^A: P*-value; follow-up changes in each group (paired t-test); PB: *P*-value for baseline comparison between groups (Independent-t test) PC: *P*-value for follow-up comparison (Independent-test).
Abbreviations: C, control group; n-3FA, n-3FA supplementation group; TNF-α, Tumor Necrosis Factor; IL-1β: interleukin-1 beta; IL-6: Interleukin 6; IL-10 Interlukin

Changes in the serum levels of cytokines ratios

Based on the data in Table 5, it was found that the follow-up IL-6/IL-10 ratio in n-3FA was markedly higher than the baseline values (1022.94±762.89 ng/ml vs 191.50±148.77 ng/ml, with a *p*-value of less than .001).

The results of an independent t-test showed that the group taking n-3FA had a significantly higher mean IL-6/IL-10 ratio than the control group, with values of 1022.94±762.89 and 174.40±141.84 respectively, and a P^C value of >0.001. There were no significant differences between the mean IL-1β /IL-10 or TNF-α/IL-10 values across both groups (349.28±193.41 ng/ml vs 244.19±221.93 ng/ml, P^A = 0.062) or (1522.60±797.49 ng/ml vs 2016.19±1358.52 ng/ml, P^A = 0.077).

The group that received n-3FA supplements had a noticeable alteration in their serum levels of IL-1β, IL-6, and IL-10 between the initial test and the follow-up (with a *P*-value of 0.05). However, the baseline and follow-up levels of IL-1β, IL-6, and IL-10 in the C group did not indicate any significant changes (with a *P*-value greater than 0.05). As per Table 5, there were no significant differences in the blood TNF-α levels between the n-3FA and C groups between the initial test and the follow-up (with a *P*-value greater than 0.05).

Stepwise Regression Analysis

Tables 5 and 6 hold valuable information regarding the impact of certain variables (IDVs) on cytokine levels and ratios in the blood, particularly in relation to CS. Our multivariate stepwise regression analysis has revealed significant findings in this area. In addition, we have also studied the effects of taking 1,000 mg of n-3FA daily for eight weeks. Our investigation has shown that IL-10 and waist circumference have a direct impact on IL-6 levels with an R value of 0.678, R2 value of 0.459 and a *p*-value of 0.001. These findings are conclusive and should not be overlooked.

Table 5. Changes in the serum levels of pro to anti-inflammatory cytokines ratios

Cytokine	Change	Group		*p*-value
		Control Mean (SD)	n-3FAs Mean (SD)	
IL-6/IL-10	Baseline	*220.13± 103.73*	*191.50±148.77*	*P^B=0.365*
	Follow up	*174.40±141.84*	*1022.94±762.89*	*P^C>0.001*
	Change	*-45.72*	*831.44*	
	P^A	*1.28*	*<.0.001*	
IL-1β/IL-10	Baseline	*143.59±70.69*	*244.19±221.93*	P^B=0.233
	Follow up	*206.43±205.92*	*349.28±193.41*	*P^C=0.004*
	Change	*62.84*	*105.09*	
	P^A	*-1.79*	*.062*	
TNF-α/IL-10	Baseline	1486.24±527.55	2016.19±1358.5	P^B=0.052
	Follow up	1293.55 ±812.24	1522.60±797.49	P^C=0.242
	Change	-192.69	-493.60	
	P^A	1.58	.077	

Note *P^A: P*-value; follow-up changes in each group (paired t-test); PB: *P*-value for baseline comparison between groups (Independent-t test) PC: *P*-value for follow-up comparison (Independent-test).
Abbreviations: C, control group; n-3FA, n-3FA supplemented group; IL-6/IL-10, Tumor necrosis factor to interleukin- 6 ratio; IL-1β/IL-10, interlukin-1beta to interlukin-10 ratio, TNF-α/IL-10, tumor necrosis factor to interlukin-10 ratio.

Changes in TNF-α levels are affected by Gender, LDL, WHR, and the IL-1β /IL-10 ratio. This is indicated by a correlation of R = 0.758, R^2 = 0.536, and a *p*-value of less than 0.001. Meanwhile, Age, gender, and waist circumference all play a significant role in changes in IL-10 level values in the n-3FA interventional group. The correlation is also R = 0.758, R2 = 0.536, and the p-value is less than 0.001. Lastly, only the height of the body component is chosen by the stepwise regression model among all IVDs that have a relation to changes in serum IL-1β levels. This is shown Table 5 with a correlation of R = 0.448, R^2 = 0.201, and a *p*-value of 0.005.

During the investigation, it was found that various factors known as IDVs had a significant impact on the levels of serum IL-6/IL-10 ratio. In the group that was given n-3FA, it was observed that the follow-up levels of IL-6/IL-10 were closely related to follow-up levels of WHR, TNF-α, and FBG (r=0.762). Predictors such as gender, WHR, TNF-α, and GBG accounted for approximately 60% of the variation. The IDVs that played a crucial role in mediating the effect of n-3FA supplementation on serum levels of IL-1β/IL-10 ratio and IL-1β levels were hip circumference, TNF-α, Age, WHR, gender, and BMI.

The study found that six specific parameters, which had a strong correlation (r=0.891), were responsible for more than 80% of the variation in the IL-1/IL-10 ratio during the follow-up period. Even after accounting for confounding variables, the researchers identified certain factors, including hip circumference, age, WHR, gender, and BM, as significant independent predictors for the TNF-α/IL-10 ratio. These predictors, shown in Table 6, accounted for approximately 80% of the variation in serum TNF-α/IL-10 ratio levels throughout the study in the group that received n-3FA supplementation. The study listed all the variables chosen by stepwise regression at trial follow-up for the n-3FA study group.

DISCUSSION

The study aimed to explore the impact of taking n-3FA supplements (1000 mg daily for eight weeks) on the levels of specific cytokines (IL-1β, TNF-α, IL-6, and IL-10) in adults with VDD. The study's primary outcome revealed a significant increase (*P*<0.001) in the levels of pro-inflammatory cytokines IL-6 and IL-1β, as well as the anti-inflammatory cytokine IL-10 (*P*=0.019) after ten weeks of follow-up due to substantial n-3FA supplementation.

A significant rise in IL-6 levels, which is a major signal of cytokine storm,[13] was observed when taking 1 g n-3FA (120 mg EPA + 180 mg DHA) daily. This dosage could activate both pro- and anti-inflammatory immune responses, as previous studies have shown.[20,22,23]

Research by Rutting S et al.[24] has shown that PUFAs can raise IL-6 levels in primary pulmonary fibroblasts. Similarly, Kiecolt-Glaser et al.[25] found that n-3 FA supplementation increased IL-6 blood levels in young men. Additionally, Grimble et al.[26] reported that children who were given 300 mg/day of n-3 PUFA for 12 weeks experienced an increase in pro-inflammatory cytokines (IL-6, IL-1β, TNF-α), and the anti-inflammatory cytokines (IL-10).

In a study conducted in 2002,[22] rats with experimental acute pancreatitis who were treated with n-3FA showed an increase in their blood IL-10 levels. Similarly, in a study conducted in 2016.[27] Diabetic males who consumed 3 g/day of n-3FA for eight weeks experienced a significant increase in the ratio of IL-6 to IL-10,[28] which is comparable to our findings.

Despite several additional RCTs,[21,29,30] there is insufficient evidence to support the potential immunosuppressive impact of n-3FA supplementation.

Table 6. Significant correlations of cytokine levels with trial variables at follow-up in n-3FA study group

Dependent variable	**Univariate effect estimate**	**Coefficient**				
		B	**F**	**r**	**r²**	***P*-value**
IL-6						
	IL10	3.872	13.902	0.533	0.284	< 0.001
	Waist	-0.270	14.425	0.678	0.459	< 0.001
TNF-α						
	Gender	*-6.142*	*11.876*	*0.503*	*.253*	< 0.001
	LDL	0.093	15.091	0.686	.470	< 0.001
	WHR	0.313	15.349	0.763	.583	< 0.001
	IL-1β/IL-10	0.149	19.322	0.798	.637	< 0.001
IL-10						
	Age	0.098	15.120	0.549	0.282	< 0.001
	Waist	-0.046	17.391	0.711	0.477	< 0.001
	Gender	-1.220	14.843	0.758	0.536	< 0.001
IL-1β						
	Height	0.117	8.805	0.448	0.201	0.005
	25OHD	0.100	7.626	0.556	0.310	0.002

Abbreviations: TNF-α, Tumor Necrosis Factor; IL-1β: interleukin-1 beta; IL-6: Interleukin 6; IL-10 Interlukin10; (TNF/IL-10): Ratio TNF to IL10; WHR: waist to Hip ratio; BMI: body mass index; FBG: fasting blood glucose; LDL: low density lipoprotein.

Table 7. Significant correlations of cytokine levels ratios with trial variables at follow-up in n-3FA study group

Dependent variable	Univariate effect estimate	Coefficient				
		B	F	r	r^2	*P*-value
IL-6/IL-10						
	Gender	909.152	17.447	0.577	0.333	<0.001
	WHR	37.071	13.479	0.665	0.442	<0.001
	TNF-α	-50.710	11.502	0.715	0.511	<0.001
	FBG	8.709	11.050	0.762	0.580	<0.001
IL-1β/IL-10						
	Hip	6.289	7.655	0.424	0.179	0.009
	TNF-α	-15.079	10.0830	0.610	0.372	<0.001
	Age	-6.428	9.149	0.674	0.454	<0.001
	WHR	7.864	10.558	0.754	0.569	<0.001
	Gender	190.108	11.726	0.809	0.654	<0.001
	BMI	-32.634	19.362	0.891	0.795	<0.001
TNF-α/IL-10						
	Hip	33.178	14.560	0.542	0.294	0.001
	Age	-29.742	11.854	0.641	0.411	<0.001
	WHR	28.770	11.302	0.712	0.507	<0.001
	Gender	1234.125	22.186	0.857	0.735	<0.001
	BMI	-88.011	24.688	0.894	0.799	<0.001
	25OHD	-30.973	25.930	0.916	0.838	<0.001

Abbreviations: TNF-α, Tumor Necrosis Factor; IL-1β: interleukin 1β; IL-6: Interleukin 6; IL-10 Interlukin10; (TNF-α/IL-10): Ratio TNF-α to IL710;(IL-1β/IL-10): Ratio IL-1β to IL-10; (IL-6/IL-10): Ratio IL-6 to IL-10; WHR: waist to Hip ratio; BMI: body mass index; FBG: fasting blood glucose

Research has shown that severe COVID-19 effects caused by CS can lead to a considerable increase in blood levels of IL-6, IL-1, and TNF-α.[31] To reduce the risk of COVID-19-related negative effects, several papers have suggested the intake of n-3 fatty acids.[32-34] For example, Asher et al.[35] found that increased intake of omega-3 fatty acids could reduce the probability of dying from COVID-19 by 75%. The research team[35] emphasized that it is not known what hospital treatment was given, and other circumstances may have impacted the outcome, making further research necessary.

The widespread use of n-3FA supplements[36] has resulted in conflicting findings[37] regarding their immunomodulatory effects. It is imperative to identify the parameters responsible for these discrepancies, while considering confounding variables. Only then can we hope to settle the debate surrounding the impact of these supplements on the immune system.

It has been found through a meta-analysis of previous n-3 fatty acid supplementation studies[38,39] that the benefits of marine n-3 fatty acids in reducing systemic inflammation are more pronounced in individuals who suffer from chronic inflammation and have utilized the supplement for an extended period.

The research conducted by Sindhu et al.[40] Remla et al.[41] El-Mikkawy et al.[42], and Mahase[43] has established a strong association between elevated levels of IL-6 in the bloodstream and age, BMI, and body fat indicators. The current study's sample included these variables, and stepwise regression analysis revealed their significant role in the relationship between 25OHD and IL-6 levels. Moreover, age and body weight independently affected the n-3FA effects, leading to significant increases in IL-6 and IL-10 correlations.

Our research has shown that there are various factors that can affect the impact of n-3FA on cytokines. However, we can confidently conclude that the baseline means levels of 25OHD hold the greatest significance, regardless of the subjects' health status. This finding is without a doubt the most important takeaway from our research.

Based on the study conducted by Costenbader et al.[30] taking 1 g/day of n-3 FA did not lead to significant reductions in IL-6 or levels in both men and women aged above 55 and below 50 years after a year of therapy. Patients with polycystic ovarian syndrome also reported similar findings (Rahmani E., 2017). Furthermore, research by Zhu Z. et al.[44] and Al-Shaer et al.[14] found that COVID-19 patients and individuals with VD deficiency respectively had higher BMI values.

Schmidt et al.[45] conducted research that found higher levels of IL-6 and TNF-α in the blood of obese individuals compared to lean ones. It is worth noting that most of the participants in the current study were overweight and deficient in vitamin D at the beginning.

According to numerous studies concerning vitamin D levels, there exists a negative correlation between lower levels of vitamin D and an increased chance of getting COVID-19[17,46,47]. This correlation may be attributed to the fact that a deficiency in vitamin D has been linked to a more intense cytokine storm.[48]

A meta-analysis of 21 studies found that 25(OH)D3 has an opposing effect on pro-inflammatory cytokines such as IL-6.[49] Our own research has shown that n-3FA significantly reduces vitamin D deficiency, albeit with a concomitant increase in IL-6 levels at the conclusion of the trial.

Vitamin D is known to trigger the production of excessive pro-inflammatory cytokines such as IL-6, TNF-α, and IL-1β. This leads to the cytokine storm and its associated complications. This fact has been suggested by Contreras-Bolívar et al.[50]

Research conducted by Hao et al.[51] conclusively proves that individuals who consume n-3FA supplements exhibit a significant increase in IL-10 levels. Furthermore, Satoh-Asahara's study[20] demonstrated that high purity EPA, a type of n-3FA, elevates IL-10 levels in monocytes present in the peripheral blood of obese individuals with dyslipidemia. It is worth noting that alongside gender, LDL is one of the most influential predictors that mediate the impact of n-3FA on IL-10 levels.

Overall, the widespread effects of n-3FA supplements are complicated findings of the current trial, but they help interpret unexpected observed changes, particularly a significant increase in IL-6 levels. Furthermore, because healthcare providers and clinical trials widely use dietary VD3 supplements have given mixed results, as previously mentioned, public health must establish mechanisms that underlie their essential requirements. It is possible that the results of previous studies did not align with expectations due to the specific amounts and lengths of time that n-3FA supplements were administered. However, A larger sample size should theoretically lead to more representative results, but more funding resources could have achieved that goal. Overall, we hypothesize that daily supplementation of n-3FA may elevate serum cytokines associated with cytokine storm, in particularly IL-6, but larger RCTs are needed to prove these findings.

CONCLUSION

Our research findings demonstrate that the administration of n-3FA supplements significantly increased IL-1 β, IL-6, and IL-10 levels compared to their baseline levels. TNF-α levels did not exhibit significant alterations.

The intriguing findings of this randomized controlled trial may be attributed to a potential adverse impact of n-3FA supplementation on IL-6 levels during cytokine storms. Further clinical studies are required to investigate the potential effects of this supplement on the treatment of IL-6 antagonists in patients experiencing cytokine storm.

References

1. Mangalmurti N, Hunter CA. Cytokine Storms: Understanding COVID-19. Immunity. 2020;53(1):19-25. https://doi.org/10.1016/j.immuni.2020.06.017
2. Kempuraj D, Selvakumar GP, Ahmed ME, et al. COVID-19, Mast Cells, Cytokine Storm, Psychological Stress, and Neuroinflammation. Neuroscientist. 2020;26(5-6):402-414. https://doi.org/10.1177/1073858420941476
3. Costela-Ruiz VJ, Illescas-Montes R, Puerta-Puerta JM, et al. SARS-CoV-2 infection: The role of cytokines in COVID-19 disease. Cytokine Growth Factor Rev. 2020;54:62-75. https://doi.org/10.1016/j.cytogfr.2020.06.001
4. Bader DA, Abed A, Mohammad BA, et al. The Effect of Weekly 50,000 IU Vitamin D3 Supplements on the Serum Levels of Selected Cytokines Involved in Cytokine Storm: A Randomized Clinical Trial in Adults with Vitamin D Deficiency. Nutrients. 2023;15(5):1188. https://doi.org/10.3390/nu15051188
5. Daboul SM, Abusamak M, Mohammad BA, et al. The effect of omega-3 supplements on the serum levels of ACE/ACE2 ratio as a potential key in cardiovascular disease: A randomized clinical trial in participants with vitamin D deficiency. Pharmacy Practice. 2023;21(1):1-9. https://doi.org/10.18549/pharmpract.2023.1.2761
6. BourBour F, Mirzaei Dahka S, Gholamalizadeh M, et al. Nutrients in prevention, treatment, and management of viral infections; special focus on Coronavirus. Arch Physiol Biochem. 2023;129(1):16-25. https://doi.org/10.1080/13813455.2020.1791188
7. Radzikowska U, Rinaldi AO, Celebi Sozener Z, et al. The Influence of Dietary Fatty Acids on Immune Responses. Nutrients. 2019;11(12):2990. https://doi.org/10.3390/nu11122990
8. Harris WS, Tintle NL, Etherton MR, et al. Erythrocyte long-chain omega-3 fatty acid levels are inversely associated with mortality and with incident cardiovascular disease: The Framingham Heart Study. J Clin Lipidol. 2018;12(3):718-727 e6. https://doi.org/10.1016/j.jacl.2018.02.010
9. Szabo Z, Marosvolgyi T, Szabo E, et al. The Potential Beneficial Effect of EPA and DHA Supplementation Managing Cytokine Storm in Coronavirus Disease. Front Physiol. 2020;11:752. https://doi.org/10.3389/fphys.2020.00752
10. Kang JX, Weylandt KH. Modulation of inflammatory cytokines by omega-3 fatty acids. Subcell Biochem. 2008;49:133-43. https://doi.org/10.1007/978-1-4020-8831-5_5
11. Balic A, Vlasic D, Zuzul K, et al. Omega-3 Versus Omega-6 Polyunsaturated Fatty Acids in the Prevention and Treatment of Inflammatory Skin Diseases. Int J Mol Sci. 2020;21(3):741. https://doi.org/10.3390/ijms21030741
12. Meftahi GH, Jangravi Z, Sahraei H, et al. The possible pathophysiology mechanism of cytokine storm in elderly adults with COVID-19 infection: the contribution of "inflame-aging". Inflamm Res. 2020;69(9):825-839. https://doi.org/10.1007/s00011-

020-01372-8
13. Bader DA, Abed A, Mohammad BA, et al. The Effect of Weekly 50,000 IU Vitamin D(3) Supplements on the Serum Levels of Selected Cytokines Involved in Cytokine Storm: A Randomized Clinical Trial in Adults with Vitamin D Deficiency. Nutrients. 2023;15(5):1188. https://doi.org/10.3390/nu15051188
14. Al-Shaer AH, Abu-Samak MS, Hasoun LZ, et al. Assessing the effect of omega-3 fatty acid combined with vitamin D3 versus vitamin D3 alone on estradiol levels: a randomized, placebo-controlled trial in females with vitamin D deficiency. Clin Pharmacol. 2019;11:25-37. https://doi.org/10.2147/CPAA.S182927
15. Al-Shaer AH, Abu-Samak MS, Hasoun LZ, et al. Assessing the effect of omega-3 fatty acid combined with vitamin D3 versus vitamin D3 alone on estradiol levels: a randomized, placebo-controlled trial in females with vitamin D deficiency. Clinical Pharmacology: Advances and Applications. 2019:25-37. https://doi.org/10.2147/cpaa.s182927
16. Ilie PC, Stefanescu S, Smith L. The role of vitamin D in the prevention of coronavirus disease 2019 infection and mortality. Aging Clin Exp Res. 2020;32(7):1195-1198. https://doi.org/10.1007/s40520-020-01570-8
17. Meltzer DO, Best TJ, Zhang H, et al. Association of Vitamin D Deficiency and Treatment with COVID-19 Incidence. medRxiv. 2020:2020:20095893. https://doi.org/10.1101/2020.05.08.20095893
18. Grant WB, Lahore H, McDonnell SL, et al. Evidence that vitamin D supplementation could reduce risk of influenza and COVID-19 infections and deaths. Nutrients. 2020;12(4):988. https://doi.org/10.3390/nu12040988
19. Takruri HR, Alkurd RA. Intakes of Fats, Cholesterol, Fiber and Micronutrients as Risk Factors for Cardiovascular Disease in Jordan. Jordan Journal of Biological Sciences. 2014;7(2)
20. Satoh-Asahara N, Shimatsu A, Sasaki Y, et al. Highly purified eicosapentaenoic acid increases interleukin-10 levels of peripheral blood monocytes in obese patients with dyslipidemia. Diabetes Care. 2012;35(12):2631-9. https://doi.org/10.2337/dc12-0269
21. Gutierrez S, Svahn SL, Johansson ME. Effects of Omega-3 Fatty Acids on Immune Cells. Int J Mol Sci. 2019;20(20):5028. https://doi.org/10.3390/ijms20205028
22. Foitzik T, Eibl G, Schneider P, et al. Omega-3 fatty acid supplementation increases anti-inflammatory cytokines and attenuates systemic disease sequelae in experimental pancreatitis. JPEN J Parenter Enteral Nutr. 2002;26(6):351-6. https://doi.org/10.1177/0148607102026006351
23. Sierra S, Lara-Villoslada F, Comalada M, et al. Dietary fish oil n-3 fatty acids increase regulatory cytokine production and exert anti-inflammatory effects in two murine models of inflammation. Lipids. 2006;41(12):1115-25. https://doi.org/10.1007/s11745-006-5061-2
24. Rutting S, Papanicolaou M, Xenaki D, et al. Dietary omega-6 polyunsaturated fatty acid arachidonic acid increases inflammation, but inhibits ECM protein expression in COPD. Respir Res. 2018;19(1):211. https://doi.org/10.1186/s12931-018-0919-4
25. Kiecolt-Glaser JK, Belury MA, Andridge R, et al. Omega-3 supplementation lowers inflammation and anxiety in medical students: a randomized controlled trial. Brain Behav Immun. 2011;25(8):1725-34. https://doi.org/10.1016/j.bbi.2011.07.229
26. Grimble RF, Howell, W. M., et al. The ability of fish oil to suppress tumor necrosis factor α production by peripheral blood mononuclear cells in healthy men is associated with polymorphisms in genes that influence tumor necrosis factor α production. The American journal of clinical nutrition. 2002;76(2):454-459. https://doi.org/10.1093/ajcn/76.2.454
27. Karachentsev Y, Gorshunska M, Krasova N, et al. The influence of interleukin-6 gene polymorphism on omega-3 fatty acid effect in type 2 diabetic patients. Diabetes & Metabolism. 2016;42(4):300.
28. Lalia AZ, Lanza IR. Insulin-Sensitizing Effects of Omega-3 Fatty Acids: Lost in Translation? Nutrients. 2016;8(6):329. https://doi.org/10.3390/nu8060329
29. Muldoon MF, Laderian B, Kuan DC, et al. Fish oil supplementation does not lower C-reactive protein or interleukin-6 levels in healthy adults. Journal of internal medicine. 2016;279(1):98-109. https://doi.org/10.1111/joim.12442
30. Costenbader KH, MacFarlane LA, Lee IM, et al. Effects of One Year of Vitamin D and Marine Omega-3 Fatty Acid Supplementation on Biomarkers of Systemic Inflammation in Older US Adults. Clin Chem. 2019;65(12):1508-1521. https://doi.org/10.1373/clinchem.2019.306902
31. Fajgenbaum DC, June CH. Cytokine Storm. N Engl J Med. 2020;383(23):2255-2273. https://doi.org/10.1056/NEJMra2026131
32. Kothapalli KS, Park HG, Brenna JT. Polyunsaturated fatty acid biosynthesis pathway and genetics. Implications for interindividual variability in prothrombotic, inflammatory conditions such as COVID-19☆,☆☆,★,★★. Prostaglandins, Leukotrienes and Essential Fatty Acids. 2020;162:102183. https://doi.org/10.1016/j.plefa.2020.102183
33. Panigrahy D, Gilligan MM, Huang S, et al. Inflammation resolution: a dual-pronged approach to averting cytokine storms in COVID-19? Cancer Metastasis Rev. 2020;39(2):337-340. https://doi.org/10.1007/s10555-020-09889-4
34. Suh W, Urits I, Viswanath O, et al. Three cases of COVID-19 pneumonia that responded to icosapent ethyl supportive treatment. The American Journal of Case Reports. 2020;21:e928422-1. https://doi.org/10.12659/ajcr.928422
35. Asher A, Tintle NL, Myers M, et al. Blood omega-3 fatty acids and death from COVID-19: A pilot study. Prostaglandins Leukot Essent Fatty Acids. 2021;166:102250. https://doi.org/10.1016/j.plefa.2021.102250
36. Bailey RL, Gahche JJ, Miller PE, et al. Why US adults use dietary supplements. JAMA Intern Med. 2013;173(5):355-61. https://doi.org/10.1001/jamainternmed.2013.2299
37. Yates CM, Calder PC, Ed Rainger G. Pharmacology and therapeutics of omega-3 polyunsaturated fatty acids in chronic inflammatory disease. Pharmacol Ther. 2014;141(3):272-82. https://doi.org/10.1016/j.pharmthera.2013.10.010

38. Li K, Huang T, Zheng J, et al. Effect of marine-derived n-3 polyunsaturated fatty acids on C-reactive protein, interleukin 6 and tumor necrosis factor alpha: a meta-analysis. PLoS One. 2014;9(2):e88103. https://doi.org/10.1371/journal.pone.0088103
39. Rangel-Huerta OD, Aguilera CM, Mesa MD, et al. Omega-3 long-chain polyunsaturated fatty acids supplementation on inflammatory biomakers: a systematic review of randomised clinical trials. Br J Nutr. 2012;107 Suppl 2(S2):S159-70. https://doi.org/10.1017/S0007114512001559
40. Sindhu S, Thomas R, Shihab P, et al. Obesity is a positive modulator of IL-6R and IL-6 expression in the subcutaneous adipose tissue: significance for metabolic inflammation. PloS one. 2015;10(7):e0133494. https://doi.org/10.1371/journal.pone.0133494
41. Remla N, Hadjidj Z, Ghezzaz K, et al. Increased Gustatory Response Score in Obesity and Association Levels with IL-6 and Leptin. J Nutr Metab. 2016;2016:7924052. https://doi.org/10.1155/2016/7924052
42. El-Mikkawy DM, EL-Sadek MA, EL-Badawy MA, et al. Circulating level of interleukin-6 in relation to body mass indices and lipid profile in Egyptian adults with overweight and obesity. Egyptian Rheumatology and Rehabilitation. 2020;47(1):1-7.
43. Mahase E. Covid-19: Why are age and obesity risk factors for serious disease? : British Medical Journal Publishing Group; 2020.
44. Zhu Z, Cai T, Fan L, et al. The potential role of serum angiotensin-converting enzyme in coronavirus disease 2019. BMC Infect Dis. 2020;20(1):883. https://doi.org/10.1186/s12879-020-05619-x
45. Schmidt FM, Weschenfelder J, Sander C, et al. Inflammatory cytokines in general and central obesity and modulating effects of physical activity. PLoS One. 2015;10(3):e0121971. https://doi.org/10.1371/journal.pone.0121971
46. Cozier YC, Castro-Webb N, Hochberg N. S, et al. Lower serum 25 (OH) D levels associated with higher risk of COVID-19 infection in US Black women. PLoS One. 2021;16(7):e0255132. https://doi.org/10.1371/journal.pone.0255132
47. D'Avolio A, Avataneo V, Manca A, et al. 25-Hydroxyvitamin D Concentrations Are Lower in Patients with Positive PCR for SARS-CoV-2. Nutrients. 2020;12(5):1359. https://doi.org/10.3390/nu12051359
48. Baktash V, Hosack T, Patel N, et al. Vitamin D status and outcomes for hospitalised older patients with COVID-19. Postgrad Med J. 2021;97(1149):442-447. https://doi.org/10.1136/postgradmedj-2020-138712
49. Pereira M, Dantas Damascena A, Galvao Azevedo LM, et al. Vitamin D deficiency aggravates COVID-19: systematic review and meta-analysis. Crit Rev Food Sci Nutr. 2022;62(5):1308-1316. https://doi.org/10.1080/10408398.2020.1841090
50. Contreras-Bolívar V, García-Fontana B, García-Fontana C, et al. Vitamin D and COVID-19: where are we now?. Postgraduate Medicine. 2023;135(3):195-207. https://doi.org/10.1038/s41577-022-00765-6
51. Hao W, Wong OY, Liu X, et al. omega-3 fatty acids suppress inflammatory cytokine production by macrophages and hepatocytes. J Pediatr Surg. 2010;45(12):2412-8. https://doi.org/10.1016/j.jpedsurg.2010.08.044