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Original research article

Omega-3 supplementation changes the physical properties of leukocytes but not erythrocytes in healthy individuals: An exploratory trial

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ABSTRACT

n3-PUFA impact health in several ways, including cardiovascular protection and anti-inflammatory effects, but the underlying mechanisms are not fully understood. In this exploratory study involving 31 healthy subjects, we aimed to investigate the effects of 12 weeks of fish-oil supplementation (1500 mg EPA+DHA/day) on the physical properties of multiple blood cell types. We used deformability cytometry (DC) for all cell types and Laser-assisted Optical Rotational Red Cell Analysis (Lorrca) to assess red blood cell (RBC) deformability. We also investigated the correlation between changes in the physical properties of blood cells and changes in the Omega-3 Index (O3I), defined as the relative content of EPA+DHA in RBCs. Following supplementation, the mean±SD O3I increased from 5.3 %±1.5 % to 8.3 %±1.4 % ($p < 0.001$). No significant changes in RBC properties were found by both techniques. However, by DC we observed a consistent pattern of physical changes in lymphocytes, neutrophils and monocytes. Among these were significant increases in metrics correlated with the cells' deformability resulting in less stiff cells. The results suggest that leukocytes become softer and have an increased ability to deform under induced short-term physical stress such as hydrodynamic force in the circulation. These changes could impact immune function since softer leukocytes can potentially circulate more easily and could facilitate a more rapid response to systemic inflammation or infection. In conclusion, fish-oil supplementation modulates some physical properties of leukocyte-subfractions, potentially enhancing their biological function. Further studies are warranted to explore the impact of n3-PUFA on blood cell biology, particularly in disease states associated with leukocyte dysregulation.

Abbreviations

ADA	Adrenic acid
ALA	Alpha linolenic acid
ARA	Arachidonic acid
Area	Area under the curve
BMI	Body Mass Index
CHD	Coronary heart disease
CI	Confidence intervals
DC	Deformability cytometry
DGLA	di-homo-gamma-linoleic acid
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
EDTA	Ethylenediaminetetraacetic acid
EI	Elongation index
eO3I	Estimated Omega-3 Index

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EPA	Eicosapentaenoic acid
FO	Fish oil
FORCE	Fatty Acids and Outcomes Research Consortium
gLNA	Gamma linoleic acid
HUFA	Highly unsaturated fatty acids
IL	Interleukin
ICAM-1	Intercellular adhesion molecule-1
LA	linonleic acid
Lorrca	Laser-assisted Optical Rotational Red Cell Analysis
n3	omega-3
NLR	Neutrophil/lymphocyte ratio
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean corpuscular volume
msec	milli second
MUFA	Monounsaturated fatty acids
O3I	Omega-3 Index
Osm	Osmolality
Ohyper	Hyper Osmolality
Omin	Osmolality where deformability is minimal
PLT	Platelets
PUFA	Polyunsaturated fatty acids
RBC	Red blood cell
rTG	*Re*-esterified triglyceride form
SFA	Saturated fatty acids
SCD	Sickle cell disease
SS	Shear Stress
TF	Trans fatty acids
TNF	Tumor necrosis factor
WBC	White blood cells

1. Introduction

The importance of biochemical properties in relation to cell physiology is widely recognised, but the importance of physical properties such as cell shape and mechanics as indicators of cell function have received relatively little attention [1–3]. Cell deformability is crucial for several cellular processes, including cell migration, cell division and interactions with the environment. We here use deformability in terms of "bulk deformability" which refers to the capability of a whole cell to deform under a defined stress [4]. This term ought to be perceived as separate from membrane flexibility. Bulk cell deformability is important in physiological processes such as the passage of cells through capillaries and the ability of immune cells to move from the blood to tissue compartments in response to infections. Cell deformability is determined largely by the cytoskeleton, which in turn is regulated by many different processes. As such, a change in cell deformability is a sensitive indicator of a change in cell function. In the present context, it can also be influenced by the composition of the plasma membrane.

The long-chain polyunsaturated omega-3 fatty acids (n3 PUFA) eicosapentaenoic acid (EPA, 20:5n3) and docosahexaenoic acid (DHA, 22:6n3), and their bioactive metabolites (oxylipins and other lipid mediators) have multiple functions in the heart, blood vessels, brain, lungs, immune and endocrine systems that contribute to long term health maintenance [5–7]. The biochemical/biophysical mechanisms of action of n3 PUFA, including their influence on cell membrane properties, are not fully understood but could play a role in the health benefits of n3 PUFAs. In cell membranes, EPA and DHA are esterified in phospholipids and affect their biophysical properties by interacting with surrounding phospholipids, which in turn alter lipid order and raft formation, membrane fluidity and protein function as well as signal transduction [8–13], which might preserve functional membrane phenotypes. n3 PUFA are incorporated into the membranes of virtually every cell in the body, including blood cells. The levels of EPA and DHA in RBC membranes is used as a biomarker for the n3 body status called the Omega-3 Index (O3I). The O3I is the sum of EPA + DHA in relation to the total FA content in RBCs, and it is considered a valid biomarker of long-chain n3 PUFA intake [14] and reflects the EPA + DHA content of other tissues as well [15].

Experiments with model membranes [16] as well as animal [17] and clinical studies [18–22] investigating the effect of n3 PUFA on the deformability of RBCs using techniques such as ektacytometry have generated inconsistent results. However, the question of whether an increase in the O3I leads to a change in deformability in terms of elastic properties of RBCs has not yet been answered. An increase in deformability would, in theory, allow RBCs to pass through capillaries more readily, possibly delivering oxygen and removing carbon dioxide more efficiently. RBC deformability has been recognized as a sensitive indicator of RBC functionality [23].

Osmotic gradient ektacytometry via Laser-assisted Optical Rotational Cell Analyzer (Lorrca) is a well-established technique to investigate RBC deformability as a function of total membrane surface area, surface-to-volume ratio and hydration status [24]. However, it is unsuitable for investigating the physical properties of leukocytes. Moreover, the Lorrca technique does not give information on the elastic properties of red or white blood cells. Deformability cytometry (DC) allows high-throughput assessment of multiple physical parameters of several individual cell types [25,26]. Cell images are acquired while the cells get deformed on short time scales (msec) by shear stresses in a microfluidic channel. From these images the cells' projected area, capacity to deform, or elasticity, given as the Young's modulus, can be extracted at a rate of up to 1000 cells/*sec*. Additionally, the cells can be classified from the bright field images using neural nets (see Methods).

In leukocytes, changes in physical properties can influence physiological processes such as the movement of cells through blood vessels [27,28]. Recent studies have described changes in the physical properties of immune cells that accompany several disorders such as malaria, viral respiratory infections, and hematological oncological diseases such as acute lymphoblastic and myeloid leukaemia and malignant pleural effusions [29–31]. How important these physical changes are in the pathophysiology of these (and possibly other) diseases is under investigation.

The aim of this exploratory proof-of-concept study was to investigate the influence of fish oil supplementation (rich in n3 PUFAs) on the physical properties of RBCs and leukocytes using Lorrca and DC. Based on the postulated effects of n3 PUFA on RBC deformability in the literature, we aimed at proving these findings by reproducing an increase in RBC deformability after fish oil (FO) supplementation. The study was conducted in a group of healthy young adults with a low fish consumption in whom we expected a relatively low baseline O3I. FO supplementation should increase the O3I as EPA and DHA become incorporated into blood cell membranes. We hypothesized that there would be an increase in blood cell deformability metrics with supplementation, and that it would correlate with an increase in the O3I.

2. Material and methods

2.1. Study design

The present study was conducted as a single-center, open-label, intervention trial. It was carried out at the Institute of Food Science and Human Nutrition, Leibniz University Hannover, Germany. The study design is described in Fig. 1. Briefly, the study consisted of a screening and a 12-week intervention phase with FO supplementation. An examination was conducted at the beginning (t0) and at the end of the intervention (t12). Ethical approval (date: 2021–10–01, reference number: Bo/45/2020) was provided by the Ethics Commission of the Medical Chamber of Lower Saxony (Hannover, Germany). In accordance with the guidelines of the Declaration of Helsinki, written informed consent was obtained from all participants prior to their participation in the study. This trial is registered in the German Clinical Trials Register (DRKS00025685).

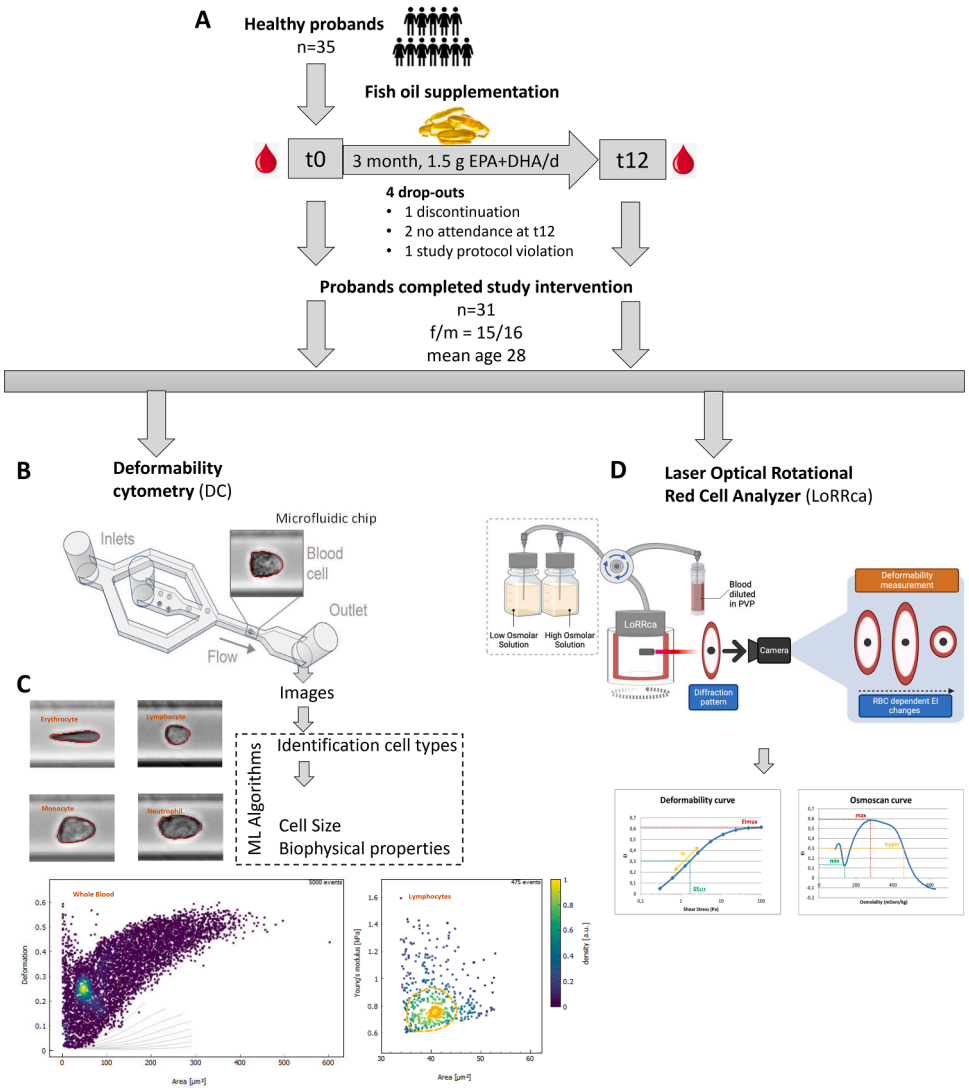

Fig. 1. A) Study design; B,C) deformability cytometry (DC) methodology and D) Laser Optical Rotational Red Cell Analyzer (Lorrca) ektacytometry; the two techniques used to measure the physical properties of circulating blood cells.
A) Schematic representation of the study design including the study cohort and intervention. B) Schematic representation of the deformability cytometry methodology. Whole, diluted blood is injected in the microfluidic chip where single-blood cells are passing through a microchannel (20 × 20 µm^2). C) Representative bright field images of blood cells are taken. Subsequently blood cell types are identified and analyzed for cell size (area) as well as morpho-rheological properties using machine learning (ML) algorithms. Representative scatter plots illustrate the identification of various physical features including area-ratio, aspect, deformation, Young 's modulus, and inert-ratio of individual cells from which the cell deformation can be concluded. Each dot represents a single measured event. D) Schematic setup of the ektacytometer (Lorrca) with osmotic gradient ektacytometry module. The ektacytometer uses a couette geometry with a static bob and rotating cylinder (cup) to create a simple shear flow. A thin layer of RBC suspension is sheared between the two concentric cylinders. The rotation of the outer cylinder causes deformation (elongation) of the RBCs. Light from the laser beam is scattered by the presence of RBCs and this diffraction pattern is detected and analysed. By means of pumps an osmotic gradient is formed and the change in deformability in response to changing osmotic conditions is recorded. The Figure was created with Biorender (https://www.biorender.com).

2.2. Study participants

The participants were recruited via notices at the Leibniz University of Hannover, between October 2021 and January 2022. The main inclusion criteria were: Age range ≥ 20 to ≤ 50 years and willingness to take FO capsules for 12 weeks. The exclusion criteria were: severe chronic diseases, treatment with lipid-lowering agents or other drugs affecting lipid metabolism (e.g., statins, fibrates, bile acid exchange resins, phytosterols, etc.), intake of n3 PUFA supplements, regular consumption of fatty fish > 250 g (1 portion)/week, diagnosed blood coagulation disorders and intake of anticoagulant drugs (except aspirin), known allergy or intolerance to components of the test products. Both inclusion and exclusion criteria were determined using a screening questionnaire and screening examination.

2.3. Composition of supplements

The FO supplement (OMEGA-3 PureMax™, Croda International PLC, England) contained 300 mg EPA and 200 mg DHA per capsule. With a daily intake of 3 capsules, the total EPA+DHA intake was 1500 mg and 1620 mg of total n3 PUFA. In this product, n3 PUFAs are present in the re-esterified triglyceride form (rTG). The capsules were to be ingested with meals (breakfast, lunch, or dinner) with sufficient liquid (at least 200 ml). Subjects were considered compliant only if they consumed at least 80 % of the required capsules. At the end of the intervention period, the non-consumed capsules were returned and used for the calculation of compliance.

2.4. Intervention phase and blood sampling

During the intervention phase (February to May 2022), participants were invited to the baseline (t0) and final visit (t12). Before the start of the examination, questionnaires were used to ask about the participants physical condition and existing illnesses, including possible infections. Study participants were asked not to change their dietary habits (especially fish and seafood consumption) and physical activity during the intervention period. Furthermore, subjects were queried regarding their medications and/or supplement use and asked to make no changes throughout the study.

Blood samples were taken by a licensed physician after an overnight fast ($\geq$ 12 h) between 06.00 and 10.00 h (for each participant at the same time of each examination visit, if possible). The samples were obtained by venipuncture of an arm vein using multiflyneedles (Sarstedt, Nümbrecht, Germany) into serum and EDTA monovettes (Sarstedt, Nümbrecht, Germany). Whole EDTA blood was immediately used to prepare dried RBC samples for O3I analysis (see below). Serum and EDTA blood samples were sent to an external accredited and certified laboratory (Laborärztliche Arbeitsgemeinschaft für Diagnostik und Rationalisierung e.V. Hannover, Germany) for the measurement of blood cell metrics (red blood cell counts, hemoglobin, hematocrit, mean corpuscular hemoglobin [MCH], mean corpuscular hemoglobin concentration [MCHC], mean corpuscular volume [MCV]), white blood cells (WBC), platelets (PLT). Complete blood count analysis (including a differential white blood cell count) was performed using fluorescence flow cytometry (Fluorocell PLT, XN-9000, Norderstedt, Germany). Neutrophil/lymphocyte ratio (NLR) was calculated.

2.5. Analysis of fatty acids in red blood cell membranes and the Omega-3 index

Fatty acid (FA) levels in RBC membranes including the O3I were analyzed using dry blood spots (Harris and Polreis, 2016) and gas chromatography (DeFina et al., 2016) in a certified laboratory (OmegaQuant Analytics, Sioux Falls, SD, USA). Briefly, EDTA blood tubes were spun for 15 min at 2000 rpm to isolate the RBC fraction. After removing the plasma and buffycoat, 100 uL of packed RBCs were mixed with 100 μL of a solution (3 mg EDTA per mL of normal saline) to increase the adhesion of the RBCs on the filter cards. 50 μL of the diluted RBCs were spotted on OmegaQuant antioxidant-treated filter cards which were then sent to the laboratory for FA analysis.

2.6. Deformability cytometry (DC)

DC was performed as described elsewhere [26,29] and is illustrated in Fig. 1. In brief, blood samples are diluted in a viscosity adjusted measurement buffer (25 mPa s) at a 1 to 20 ratio, respectively, and then pumped through a microfluidic chip mounted to an inverted microscope. The cells deform while passing through a microfluidic channel constriction of 20 μm by 20 μm dimension to different extents depending on their physical properties at a total flow rate of 0.6 μL per second. Multiple parameters are recorded at the end of the constriction calculated from the cells' brightfield images at an imaging rate of 3600 fps over a 10 min measurement time. In-house developed classification algorithms are used to separate the images into cell types (RBCs, thrombocytes, and leukocyte subtypes - neutrophils, monocytes, eosinophils, basophils, and lymphocytes). Based on those images several physical parameters are calculated (for a detailed description see Supplementary Table 1 and https://shapeout2.readthedocs.io/en/stable/). The measured deformation, area and the temperature at the measurement region are used to calculate the Young's modulus [32]. We used numerical simulations to disentangle the recorded steady-state deformation and the projected area to get the cells' elastic property represented as Young's modulus [33]. The current models depend on initially spherical objects. As RBCs have a discocyte shape a calculation of the cells Young's modulus is not feasible.

Further information on the data, classification algorithms, training procedure, and model performance are described in "Supplementary Information".

2.7. Laser optical rotational red cell analyzer (Lorrca) ektacytometry

Deformability of RBCs was measured by means of ektacytometry. Measurements are performed with the Lorrca (Laser Optical Rotational Red Cell Analyzer, RR Mechatronics, Zwaag, The Netherlands). In this ektacytometer, RBCs in an isotonic viscous solution (Elon-Iso) are exposed to shear stress at 37 °C. RBCs are forced to elongate into an elliptical shape. A diffraction pattern is generated by a laser beam and measured by a camera. From the captured ellipse the vertical axis (A) and the horizontal axis (B) are used to calculate the elongation index (EI) by the formula (A-B)/($A + B$). The EI reflects the RBC deformability of the RBC population in the ektacytometer.

2.7.1. Osmotic gradient ektacytometry

In osmotic gradient ektacytometry RBCs are brought in a continuous changing increasing osmolar condition. This test is performed using the Osmoscan module on the ektacytometer. For the Osmoscan, whole blood is standardized to a fixed RBC count of 1000E6/5 mL Elon-ISO (RR Mechatronics). Subsequently RBCs are exposed to an osmolality gradient from approximately 60 – 600 mOsm/kg, under a continuous shear stress of 30 Pa. Based upon a formed Osmoscan curve, elongation and osmolality features of RBCs are determined. Detailed description of the parameters is given in Supplementary Table 2 and in reference [34].

2.7.2. Deformability ektacytometry

The deformability test was performed using the Deformability module on the ektacytometer [24]. Briefly, whole blood is standardized to a fixed RBC count of 200E6/5 mL Elon-ISO (RR Mechatronics). RBCs are exposed to an increasing shear stress. Shear stress is increased from 0.3 – 50 Pa in nine steps. The deformability module quantifies the stress dependent deformability of the RBCs. Parameters are elucidated in Supplementary Table 2.

2.8. Statistical analyses

Statistical analyses were performed using R. For all DC metrics of the different cells, the medians and SDs were calculated based on each individual's single-cell data.

The effect of FO supplementation on blood count markers as well as DC and Lorrca parameter was assessed using repeated measures ANOVA. The analysis was conducted within the entire cohort and also within subgroups based on sex, baseline O3I, and delta O3I change after fish oil supplementation. In addition, the DC parameters (t0, t12 and delta change) were compared with blood count markers (mean cell count, percentage of leukocyte subpopulations) and the O3I.

Data were analyzed by per-protocol meaning that only data from participants who completed the study (31/35) were included (see Results). Statistical significance was set at an exploratory threshold of alpha=0.05. With $n = 31$ subjects, we have 80 % power to detect medium sized effects (Cohen's d = Change/SD = 0.5) when using a significance level of 0.05.

3. Results

3.1. Characteristics of the study population and study course

35 of the participants met the eligibility criteria and were included in the study collective (Fig. 1). There were 4 drop outs during the study (Fig. 1). One subject dropped out during the study, two did not attend the final examination and one violated the study protocol. Accordingly, data from 31 individuals were statistically evaluated. No side effects

were reported. The average compliance was 93 % of capsules consumed. At baseline, the subjects were all healthy, and none reported illness or infection. Supplementary Table 3 depicts baseline demographic and clinical characteristics of the study population.

The participants were mostly non-smokers who ate a mixed diet (Supplementary Table 3). Their mean age was 28, and with a mean BMI of 23.4 kg/m^2, the population can be classified as normal weight.

3.2. Fatty acid levels in red blood cells and Omega-3 index

Most subjects (n = 21) had a O3I in the range of 4.0 % and 6.5 % (Supplementary Fig. 1). Nevertheless, given the presence of subjects with both higher and lower O3I values, the inter-individual variability appeared substantial (Table 1). After the 12-week FO intervention, there were numerous significant changes in the relative FA levels of the RBCs. The increase in the relative proportion of total PUFA in the FA pattern in RBCs was minor. Likewise, the changes in FA with > 18 carbon atoms (linonleic acid, gamma linoleic acid and alpha linolenic acid) are small. First and foremost, a shift in highly unsaturated fatty acids (HUFA) between n6 HUFA (decreased) and n3 HUFA (increased) can be observed. Although all n3 PUFA increased, the largest increases being seen in the percentages of EPA and DHA (both +1.5). Median O3I rose from 5.3 % to 8.6 %. Median Delta-O3I$_{t0\text{-}t12}$ was 2.9 %. Individual change in O3I in response to fish oil supplementation is shown in Supplementary Fig. 1. As n3 HUFA increased, there was a concomitant decrease in the relative levels of n6 PUFAs, especially with arachidonic acid (ARA, −1.7 and adrenic acid (ADA, −1.0). As a result of these shifts in the FA pattern, the proportions of total PUFAs and MUFAs in RBCs increased slightly, while the proportion of total SFA slightly decreased.

3.3. Blood count

No change in mean RBC count and related indices were observed after FO intervention, except for a marginal increase in MCH and MCV (Table 2). Likewise, no change was observed in the mean platelet and total leukocyte counts. There was a decrease in the percentage of neutrophils (−4.0 %) and an increase in the percentage of lymphocytes (+3.2 %), however the NLR was not significantly altered. An analysis stratified by sex yielded similar results (data not shown).

3.4. Deformability cytometry (DC)

The effects of 12 weeks of FO supplementation on DC metrics of RBCs and leukocytes, specifically neutrophils, monocytes and lymphocytes are shown in Fig. 2. No significant changes in any of the physical measures were observed in the RBCs (Fig. 2A), except minimal effects on SD area-ratio and SD deformation. This result was not influenced by the baseline O3I level (data not shown). No changes were found in the size (area) of monocytes, leukocytes, and neutrophils (only minimal changes in the area SD for monocytes).

We observed a consistent pattern of changes in the shape markers, aspect ratio (aspect), deformation and inertia-ratio (inert-ratio), of leukocyte subpopulations with higher median values. All these parameters are correlated with the cells' capability to deform under the standard measurement conditions in DC. However, the cell size given as projected area did not change. Thus, the cells are less stiff after FO supplementation which is reflected in a lower Young's modulus (Fig. 2B-D). In the case of monocytes, the median of the area-ratio was additionally reduced indicating a reduction in surface roughness, a marker for cell activation. With a few exceptions, no changes in the SDs were

Table 1
Fatty acid levels of red blood cells (given as a percentage of total fatty acids) and Omega-3 Index (O3I) at the beginning (t0) and the end (t12) of the study.

	Fatty acids	Median [95 % CI] t0	Median [95 % CI] t12	Median [95 % CI] △ t12-t0	p-value[a] t0-t12
SFA	C14:0	0.25 [0.22,0.28]	0.29 [0.27,0.35]	+0.04 [+0.10,+0.01]	**0.015**
	C16:0	24.9 [25.0,25.0]	23.4 [23.2,23.7]	−1.51 [−1.31,−1.98]	**<0.001**
	C18:0	16.9 [16.8,17.4]	17.0 [16.8,17.2]	−0.01 [+0.18,−0.41]	0.428
	C20:0	0.22 [0.20,0.24]	0.21 [0.20,0.25]	−0.1 [+0.03,−0.02]	0.752
	C22:0	0.58 [0.56,0.60]	0.60 [0.54,0.63]	+0.05 [+0.05,−0.04]	0.885
	C24:0	0.39 [0.35,0.40]	0.52 [0.48,0.55]	+0.12 [+0.25,+0.15]	**<0.001**
MUFA	C16:1n7t	0.20 [0.18,0.23]	0.15 [0.14,0.19]	−0.03 [0.00,−0.08]	**0.035**
	C16:1n7	0.19 [0.16,0.22]	0.32 [0.28,0.32]	+0.12 [+0.14,+0.08]	**<0.001**
	C18:1t	0.51 [0.47,0.60]	0.51 [0.44,0.57]	−0.03 [+0.05,−0,11]	0.501
	C18:1n9	15.4 [15.0,15.6]	15.6 [15.4,15.9]	+0.37 [+0.54,+0.01]	**0.045**
	C20:1n9	0.32 [0.28,0.33]	0.27 [0.25,0.30]	−0.04 [0.00,−0.07]	**0.042**
	C24:1n9	0.18 [0.16,0.22]	0.52 [0.49,0.58]	+0.33 [+0.39,+0.28]	**<0.001**
n6 PUFA	C18:2n6, LA	11.2 [10.9,11.9]	11.3 [11.2,12.2]	+0.44 [+0.65,−0.05]	0.085
	C18:3n6, gLNA	0.07 [0.06,0.09]	0.06 [0.05,0.08]	−0.01 [+0.07,−0.28]	0.248
	C20:2n6	0.25 [0.23,0.28]	0.24 [0.21,0.27]	−0.02 [+0.02,−0.05]	0.464
	C20:3n6, DGLA	1.67 [1.60,1.83]	1.37 [1.27,1.45]	−0.33 [−0.26,−0.45]	**<0.001**
	C20:4n6, ARA	14.2 [13.8,14.4]	12.5 [12.3,13.0]	−1.65 [−1.09,−1.84]	**<0.001**
	C22:4n6, ADA	3.0 [2.91,3.26]	2.0 [1.91,2.15]	−1.02 [−0.92,−1.18]	**<0.001**
n3 PUFA	C22:5n6, DPAn6	0.48 [0.44,0.55]	0.35 [0.30,0.37]	−0.14 [−0.11,−0.21]	**<0.001**
	C18:3n3, ALA	0.17 [0.15,0.19]	0.25 [0.23,0.28]	+0.09 [+0.11,+0.06]	**<0.001**
	C20:5n3, EPA	0.74 [0.70,1.03]	2.38 [2.22,2.65]	+1.52 [+1.76,+1.38]	**<0.001**
	C22:5n3, DPAn3	2.65 [2.50,2.769]	3.21 [3.17,3.27]	+0.58 {+2.80,+2.55]	**<0.001**
	C22:6n3, DHA	4.47 [4.01,4.89]	5.92 [5.70,6.27]	+1.53 [+1.80,+1.26]	**<0.001**
Sum formula	SFA	43.1 [43.0,44.1]	42.0 [41.8,42.3]	−1.16 [−0.86,−2.11]	**<0.001**
	MUFA	16.6 [16.2,16.9]	17.4 [17.0,17.5]	+0.65 [+1.0,+0.36]	**<0.001**
	PUFA	39.5 [38.8,39.8]	40.4 [39.8,40.7]	+0.79 [+1.59,+0.35]	**0.003**
	n6 PUFA	31.2 [30.6,31.7]	28.4 [27.9,28.9]	−3.09 [−2.11,−3.39]	**<0.001**
	n3 PUFA	8.08 [7.51,8.73]	12.0 [11.4,12.3]	+3.56 [+4.20,+3.25]	**<0.001**
	n6:n3 ratio	3.80 [3.65,4.39]	2.38 [2.30,2.56]	−1.28 [−1.28,−1.90]	**<0.001**
	TFA	1.12 [1.06,1.30]	0.94 [0.88,1.08]	−0.22 [−0.05,−0.35]	**0.012**
O3I	EPA+DHA	5.30 [4.77,5.87]	8.58 [7.98,8.87]	+2.93 [+3.49,+2.71]	**<0.001**

LA= linonleic acid, gLNA= gamma linoleic acid, DGLA= di-homo-gamma-linoleic acid, ARA= arachidonic acid, ADA= adrenic acid, ALA= alpha linolenic acid, EPA= eicosapentaenoic acid, DPA= docosapentaenoic acid, DHA= docosahexaenoic acid, SFA= saturated fatty acids, MUFA= monounsaturated fatty acids, PUFA= polyunsaturated fatty acids, TFA= trans fatty acids, O3I= Omega-3 Index.

[a] Statistical changes were calculated by ANOVA for repeated measures, significant changes are marked in bold.

Table 2
Hematologic metrics at the beginning (t0) and the end (t12) of the study.

	Mean t0	SD	Mean t12	SD	Mean △ t12-t0	SD	p-value[a] t0-t12
Red blood cells (10^{12}/L)							
female:	4.65	0.26	4.62	0.18	−0.04	0.21	0.511
male:	4.95	0.24	4.88	0.31	−0.08	0.21	0.174
Hemoglobin (g/dL)							
female:	13.4	0.84	13.4	0.75	−0.05	0.59	0.763
male:	14.8	0.49	14.7	0.72	−0.08	0.57	0.606
Hematocrit (%)							
female:	0.40	0.02	0.40	0.02	0	0.02	0.891
male:	0.43	0.01	0.43	0.02	0	0.02	0.491
MCH (pg/cell)	29.4	1.46	29.6	1.46	+0.20	0.44	**0.016**
MCHC (g/dl)	34.2	1.04	34.2	1.02	−0.03	0.52	0.784
MCV (10^{-15} l)	86.0	3.78	86.7	3.60	+0.65	1.16	**0.004**
Platelets (10^9/l)	267	57.5	275	62.1	+8.07	31.4	0.162
Leukocytes (10^9/l)	5.92	1.39	5.92	1.31	0	1.13	1
Neutrophils (%)	50.9	7.97	47.0	9.06	−3.96	8.01	**0.010**
Monocytes (%)	9.79	2.05	9.88	2.16	+0.09	1.56	0.741
Eosinophils (%)	2.59	1.85	2.98	2.15	+0.39	1.31	0.108
Basophils (%)	0.83	0.35	0.86	0.33	+0.03	0.25	0.574
Lymphocytes (%)	35.9	7.52	39.1	8.78	+3.24	6.69	**0.011**
NLR	1.55	0.68	1.40	1.07	−0.15	0.92	0.361

MCH= mean corpuscular hemoglobin, MCHC= mean corpuscular hemoglobin concentration, MCV= mean corpuscular volume.
[a] Statistical changes were calculated by ANOVA for repeated measures, significant changes are marked in bold.

observed. A sex-stratified analysis consistently revealed alterations in DC markers that closely resembled those observed across the entire study population (data not shown). This is also clear from a visual inspection of the distribution between females and males in Fig. 2.

The complete statistical analysis can be found in "Supplementary Table 4". In addition to the median and SD values, Supplementary Table 4 also contains the mean values, which give almost similar results as the median values.

There was no correlation between changes in physical properties of leukocytes with changes in (RBC) O3I (neither in the total population, nor in subgroups with baseline O3I values ⟨ 5.5 % or O3I responses ⟩ 2.5 % delta changes; see "Supplementary Table 5").

3.5. Lorrca data

Only a few, minor changes were detected in the various osmotic and deformability metrics by Lorrca analysis (Table 3). This result was not affected by the baseline level of the O3I (data not shown).

4. Discussion

As anticipated, 12 weeks of FO supplementation resulted in an increase in the mean O3I from 5.3 % to 8.4 %, which is the change expected based on prior studies [35]. While we did not observe any changes in physical parameters of the RBCs neither using DC nor Lorrca, we found a change in several DC parameters in monocytes, neutrophils and lymphocytes, namely an increased ability to deform under short term hydrodynamic force.

While it is widely assumed that n3 PUFAs "increase cell membrane flexibility" [36–38], the evidence for this is not consistent. Some early clinical trials investigating the physical behavior of RBCs reported that n3 PUFA supplementation increased RBC deformability and reduced whole blood viscosity [19–21], but others did not. Oostenbrug et al. investigated the effects of exercise and FO supplementation, with or without vitamin E, on RBC deformability in 24 trained cyclists using Lorrca and found no improvement in RBC deformability [22]. In two small clinical trials in healthy subjects, Hagve et al. observed a reduced osmotic fragility of RBCs, but no changes in RBC membrane fluidity in response to FO supplementation [18,39]. An in vitro study also failed to show an effect of n3 PUFA on bilayer fluidity in a gel-liquid crystalline phase transition of dipalmitoyl phosphatidylcholine [16].

This is the first investigation of the influence of n3 PUFA supplementation on the physical properties of RBCs using DC. Compared to older techniques investigating visco-elastic behavior of cells on long timescales, DC measures the elastic behavior of RBCs at high shear-rates (>10,000 s^{-1}, [40] and at short timescales (msec). This is particularly important in healthy individuals with non-atherosclerotic vessels where intravascular shear occurs on rather short time scales. The mere replacement of n3 PUFAs with n6 PUFAs in RBC membranes in healthy young adults does not affect the bulk deformation of RBCs, as measured in DC. Given that ~400 lipid species have been identified in RBC membranes alone [41], it would seem unlikely that increasing the levels of just two fatty acids (but not altering total PUFA content) would alter such complex processes as viscous or elastic properties. However, the "fine-tuning" of lipid bilayers in cell membranes plays a crucial role in cell function [42], and even low-abundant lipids can be important for membrane function. Therefore, changes in composition, even in small amounts, could theoretically affect bilayer properties and ultimately cell deformability. Another interpretation could be that the potential impact on RBC deformability is concealed by the varied RBC membrane distribution of EPA+DHA between subjects. However, despite significant variability in O3I among subjects, we observed no impact of the O3I on the physical characteristics of RBCs.

The deformability of RBCs is influenced by factors beyond just their membranes. The cytoskeletal structure, cellular hydration and metabolic conditions also play an important role in determining RBC deformability. It may be that n3 PUFA supplementation in people with blood cell disorders causes a change in RBC properties. For example, studies in mice with sickle cell disease (SCD) showed that treatment with n-3 PUFA normalised the low deformability of sickle cells as measured by ektacytometry and atomic force microscopy, whereas no changes in the properties of healthy RBCs in healthy wild-type mice were observed [17]. Clinical trials have shown improved symptoms in SCD patients following n3 PUFA supplementation [43], but whether this is the result of changes in membrane properties or other factors (e.g., inflammation and hemolysis [43] is unclear. Future studies facilitating DC should be conducted on patients with RBC membrane disorders (i.e., SCD or thalassaemia) to investigate the influence of n3 PUFA on the physical properties of RBCs and the influence of those properties on the disease.

Our results suggest that n3 PUFA supplementation makes neutrophils, monocytes and lymphocytes softer and more susceptible to deformation under short term physical stress in healthy adults. Whether

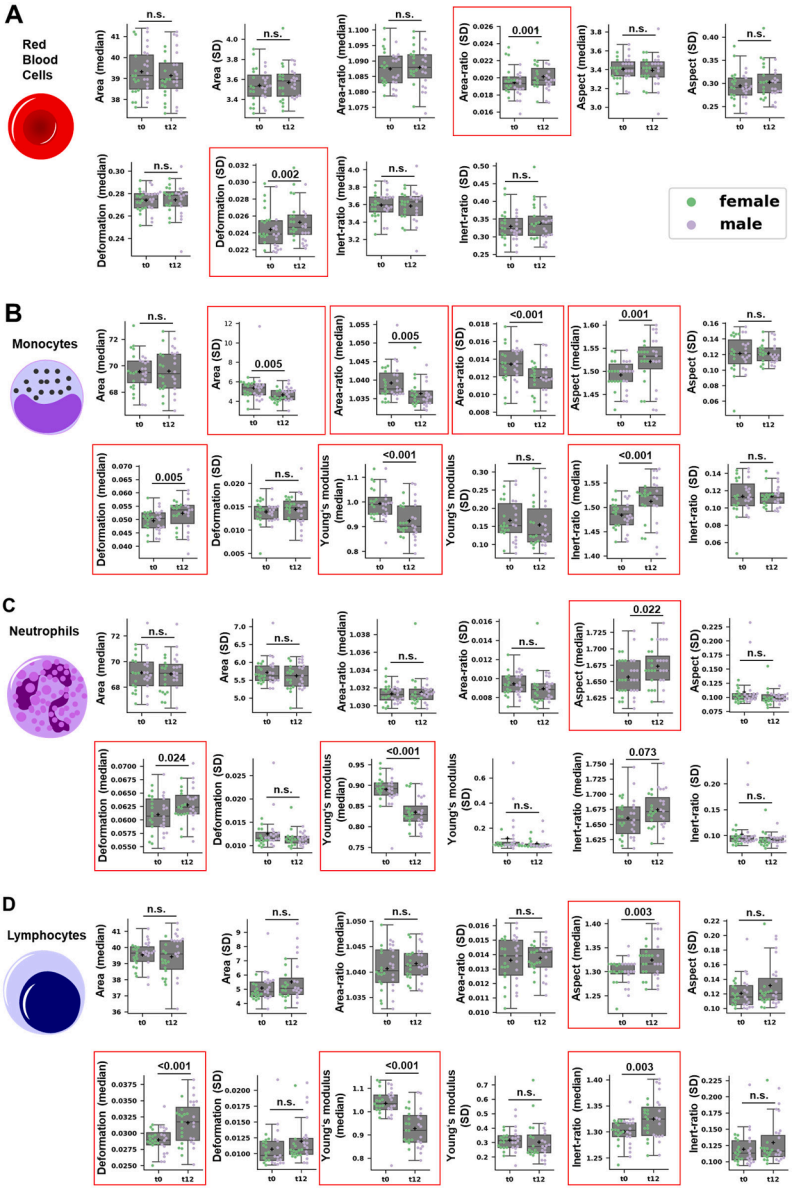

Fig. 2. Effects of 12 weeks of fish oil supplementation on selected physical properties of A) red blood cells, B) monocytes, C) neutrophils and D) lymphocytes obtained using deformability cytometry.

Each dot in each graph represents the indicated median (or SD) value for one individual. Hence, in each graph, there are 31 dots at t0 and t12. Lines within the boxes represent the median values for the cohort. The + sign in the boxes represents the mean values. The upper and lower lines in the boxes represent the 25th to 75th percentiles, and the lines outside the boxes represent the SD. Statistical changes were calculated by ANOVA for repeated measures. Red frames indicate findings with significant differences between t0 and t12. n.s. = not significant.

this is also true for other leukocytes (i.e., the 3 % not represented by these three) is not known. We can only speculate about the implications of these findings for the biological function of leukocytes and immune function in health and disease. Physical changes in leukocytes are closely linked to physiological processes such as cell migration within blood vessels [27,28]. Leukocyte migration through the capillaries plays a crucial role in facilitating the movement of these cells to tissue sites of infection, injury or inflammation [28]. Neutrophils encounter tight constrictions in capillaries, some as narrow as 5 μm, which necessitates their deformation for successful passage. Consequently, alterations in the dimensions, contours, and stiffness of leukocytes could profoundly impact their ability to traverse these intricate junctions.

In the case of monocytes, we observed a reduction in the area-ratio, which describes the structuring or roughness of the cell surface. A decrease in the area-ratio means that the surface of the monocytes becomes smoother and more homogeneous (uniform). In previous studies we found the area-ratio of monocytes to be associated with immune cell activation. A lower area-ratio was shown to indicate cell deactivation [29]: Several studies providing EPA and DHA as supplements to healthy human volunteers have reported decreased production of TNF, IL-1β and IL-6 by LPS-stimulated monocytes or mononuclear cells [44].

All types of leukocytes face challenges in navigating different microenvironments during trafficking, including organ capillary beds, epithelial sheets, fibrillar networks and cell-dense lymphatic organs [45,46]. The later challenges include adhesive crawling under high shear stress, pathfinding in complex 3D mazes, and cellular deformation to overcome physical barriers [45]. However, there is an extremely large combinatorial range of trafficking signals and endothelial barrier regulatory molecules that impact leukocyte recruitment, and deformability is

Table 3
Laser Optical Rotational Red Cell Analyzer (Lorrca) data at the beginning (t0) and the end (t12) of the study.

	Mean t0	SD	Mean t12	SD	Mean △ t12-t0	SD	p-value[a] t0-t12
Osmoscan							
EI_{min}	0.14	0.01	0.13	0.01	+0.02	0.14	**0.001**
O_{min} (mOsm/kg)	139	3.69	137	5.94	+1.39	4.45	0.096
EI_{max}	0.59	0	0.59	0	−0.02	0	**0.002**
Osm (EI_{max}) (mOsm/kg)	288	7.96	289	10.4	−0.77	5.62	0.449
O_{hyper} (mOsm/kg)	444	13.7	444	11.6	−0.39	6.04	0.724
Area (AUC)	155	6.45	155	5.93	−0.66	3.53	0.305
Deformability							
$SS_{1/2}$ (Pa)	1.37	0.20	1.36	0.19	+0.01	0.15	0.807
EI_{max}	0.65	0.01	0.66	0.011	−0.01	0.01	**0.001**
m, slope	0.79	0.05	0.77	0.05	+0.02	0.05	**0.012**
EI @ Shear 0.3	0.04	0.01	0.04	0.02	0	0.01	0.480
EI @ Shear 0.57	0.12	0.01	0.12	0.01	0	0.01	0.132
EI @ Shear 1.08	0.22	0.01	0.22	0.01	0	0.01	0.468
EI @ Shear 2.04	0.32	0.01	0.32	0.01	0	0.01	0.606
EI @ Shear 3.87	0.41	0.01	0.41	0.01	0	0.01	0.608
EI @ Shear 7.34	0.49	0.01	0.49	0.01	0	0.01	0.943
EI @ Shear 13.92	0.54	0.01	0.54	0.01	0	0	0.645
EI @ Shear 26.38	0.58	0.01	0.58	0.01	0	0	0.070
EI @ Shear 50.0	0.61	0.01	0.61	0.01	0	0.01	**0.004**

EI_{min}= Minimum Elongation Index; O_{min}= Osmolality where deformability is minimal; EI_{max}= Maximum Elongation Index; Osm (EI_{max})= Osmolality at EImax; O_{hyper}= Hyper Osmolality at 50 % of EI_{max}; Area= Area under the curve (O_{min} – 500mOsm/kg); Pa= Pascal; $SS_{1/2}$= Shear Stress at 50 % of EI_{max}; m, slope= slope of the curve.

[a] Statistical changes were calculated by ANOVA for repeated measures, significant changes are marked in bold.

only one factor among many. Thus, n3 PUFA-induced changes in deformability does not allow for a generalized statement about the effects of these FAs on the biological function of leukocytes [46]. Clearly, membrane PUFA composition is not the only determinant of blood cell membrane flexibility or even overall cell deformability [47].

Similarly, our data do not allow us to draw conclusions about the mechanisms by which EPA and DHA alter the biophysical properties of leukocytes. We did not see a correlation between the increase in O3I and the change in most DC deformability metrics in leukocytes. Of course, the O3I is measured in RBCs, not leukocytes, and so how EPA and DHA levels may have changed in these cell types in this experiment is not clear. However, previous studies have confirmed that the rise in EPA+DHA levels in mononuclear cells, which encompass lymphocytes and monocytes, following supplementation mirrors that observed in RBCs [48]. Future research should aim to investigate fatty acid profiles across various leukocyte classes for a more comprehensive understanding. It is possible that an increase in EPA and DHA in leukocyte membranes and the resulting altered fluidity may only partially contribute to the altered physical properties of leukocytes. The deformability of leukocytes is regulated by a complex interplay between membrane fluidity, cytoskeletal dynamics, adhesion molecules, cell signaling, inflammatory mediators (i.e. cytokines, chemokines and lipid mediators) and mechanical forces (i.e. shear stress), allowing leukocytes to adapt their shape and squeeze through narrow spaces in the body in response to physiological and pathological conditions. As an example, the effect of EPA and DHA on neutrophil function has been studied in several aspects. Neutrophils are the most abundant leukocytes in humans and the first cells recruited to the site of inflammation or infection [49]. EPA and DHA have been shown to be incorporated into phospholipids of neutrophil cell membranes [50,51], where they are likely to alter lipid bilayer function. Changes in membrane fluidity can modulate the assembly and disassembly of cytoskeletal components.

Studies have shown that long chain n3 PUFA are metabolised by neutrophils to various lipid mediators such as prostaglandins, leukotrienes, thromboxanes, maresins, protectins and resolvins [52,53]. Several of these n3-derived lipid mediators have been shown to inhibit neutrophil migration both in vitro and in vivo [54]. In the present study, we also found a slight decrease in neutrophil counts. Long chain n3 PUFAs can affect the cytoskeleton via their lipid mediators. In vitro studies with human polymorphonuclear leukocytes showed that incubation with resolvin D1 resulted in decreased actin polymerisation [55]. Actin is a key component of the cytoskeleton and the main determinant of cell elastic properties [4]. It is also involved in cell motility, shape change and intracellular transport. Altered actin polymerisation leads to changes in actin filament dynamics and cytoskeletal rearrangements, ultimately altering the biophysical properties of cells. In a recent study in healthy humans, we showed that EPA + DHA supplementation reduced cytoskeleton-associated gene expression in blood cells [56], suggesting an affect on the actin cytoskeleton and changes in cytoskeletal dynamics. Moreover, in vivo and ex vivo analyses revealed that EPA and DHA induced changes in the membrane composition of CD4+ *T* cells, consequently prompting cytoskeletal rearrangements and elucidating the enhanced mobility of these cells [57].

We observed a decrease in lymphocyte counts and a slight (n.s.) decrease in NLR. Although a healthy or target value for NLR has not been yet defined, NLR is considered as a marker for immune system homeostasis, inflammatory status and various diseases (sepsis, pneumonia, COVID-19, cancer, etc.) [58]. A slight increase in lymphocyte count following n3 PUFA supplementation at a dose of EPA+DHA similar to our study has previously been reported in healthy adults [59]. One could speculate that FO supplementation might reduce the expression of the cellular adhesion molecules l-selectin and soluble intercellular adhesion molecule-1 (ICAM-1), which could lead to reduced lymphocyte adhesion to the endothelium, resulting in a higher proportion of freely circulating lymphocytes in the peripheral blood [60,61]. The extent to which n3 PUFA supplementation influenced the expression of adhesion molecules in our study, and whether there is a link with the increased deformability of leukocytes, is not known. Infection as a trigger for increased lymphocyte counts is unlikely since at the t12 visit, all subjects reported that they were healthy and had no symptoms of active infection. Other causes of immune activation with slightly elevated lymphocyte counts (e.g., allergies, autoimmune disorders, thyroid disorders) or a change in medication (e.g., corticosteroids or anticonvulsants) can be ruled out as these subjects were excluded. Whether mental stress was another influencing factor (many of the subjects were students with possible exam stress) cannot be answered at this point, as stress status was not recorded.

5. Strength and limitations

The strength of the study was that the deformability of RBCs was examined with two completely different tools (DC and Lorrca). We also provided a sufficiently high dose of EPA and DHA given for a sufficiently long time to increase the O3I from typical German levels (~5 %) to optimal levels (>8 %). Had we not done so, then the question would remain of whether enough n3 PUFA had been given to elicit changes in cell physiology.

This was an exploratory, proof-of-principle study in which we hoped to generate preliminary data on the effects of n3 PUFAs on blood cell physiochemical properties. The study has a number of limitations. We did not include a placebo group, but this should obviously be done in future such trials. The study was primarily limited by the small number of subjects which resulted in reduced power to detect effects. Even though we aimed to recruit subjects with a low initial EPA+DHA status, we observed considerable variability in the O3I both at the beginning (t0) and after twelve months of fish oil supplementation (t12). Our study was constrained by not targeting subjects within a specific “tight” O3I

range (e.g., 4.5 to 5.5 %). Future studies should aim to recruit subjects with a more consistent O3I at baseline.

6. Conclusion

The results of this exploratory study suggest that n3 PUFA supplementation has no detectable effect on the membrane deformability properties (Lorrca) nor whole cell elastic properties (DC) of RBCs. However, it does seem to influence the morpho-rheological characteristics of leukocytes, enhancing their ability to deform when subjected to physical stress.

Future directions

Further research is essential to understand the clinical implications of these findings. To determine whether the changes in leukocyte morpho-rheological properties have any significant effects on immune function or inflammatory response, future studies should be conducted using suitable participant groups, such as those with low-grade inflammation, and appropriate biomarkers, including assessments of leukocyte function and inflammatory markers. Additionally, these investigations should consider potential variations related to sex, age, and different forms of n3 PUFAs (e.g., bound as rTG, ethyl ester, phospholipid, or wax ester), as well as distinguishing between EPA and DHA. Such inquiries may shed light on how alterations in leukocyte physical properties contribute to the immunomodulatory or anti-inflammatory mechanisms associated with n3 PUFAs.

CRediT authorship contribution statement

Jan Philipp Schuchardt: Writing – original draft, Visualization, Supervision, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **Martin Kräter:** Writing – review & editing, Validation, Methodology, Investigation, Conceptualization. **Maximilian Schlögel:** Formal analysis. **Jochen Guck:** Writing – review & editing, Resources, Methodology, Conceptualization. **Brigitte A. van Oirschot-Hermans:** Formal analysis. **Jennifer Bos:** Writing – review & editing, Formal analysis. **Richard van Wijk:** Writing – review & editing, Resources, Methodology, Conceptualization. **Nathan L Tintle:** Writing – review & editing, Formal analysis. **Jason Westra:** Formal analysis. **Felix Kerlikowsky:** Formal analysis. **Andreas Hahn:** Writing – review & editing, Resources, Conceptualization. **William S. Harris:** Writing – review & editing, Supervision, Methodology, Conceptualization.

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Data availability statement

All DC raw data is available on the deformability cytometry open repository (https://dcor.mpl.mpg.de/) by searching for “omega3 blood cell deformability study”.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.plefa.2024.102636.

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