Title: No clinical or biochemical evidence for essential fatty acid deficiency in home
patients who depend on long-term olive oil-based parenteral nutrition

Short title: Essential fatty acid status of home parenteral nutrition patients

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1. essential fatty acid
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Abstract

Background: Home parenteral nutrition (HPN) patients depend on lipid emulsions as part of their parenteral nutrition regimen in order to provide essential fatty acids (EFA). Mixed oil sources are used in modern lipid emulsions to decrease the amount of pro-inflammatory EFA, mainly linoleic acid, which is present in large amounts in soybean oil. It is unknown whether patients who fully depend on such mixed lipids have adequate EFA supply and status. In the present study we therefore evaluated whether HPN patients who depend on a mixed lipid emulsion (20% soybean oil, 80% olive oil) show evidence of EFA deficiency, in the form of fatty acid biomarkers, clinical signs and/or decreased innate immune cell functions.

Materials and methods: Fatty acid status was assessed in plasma phosphatidylcholine (PC) and peripheral blood mononuclear cells (PBMCs) from thirty home patients on olive-oil based parenteral nutrition (>3 months, >5 times per week) and thirty age- and sex-matched healthy controls. Innate immune cell functions and phenotype were evaluated by assessing expression of surface membrane molecules, and reactive oxygen species and cytokine production.

Results: None of the HPN patients or controls showed clinical evidence for EFA deficiency in the form of skin rash. Biochemical evidence for EFA deficiency, in the form of an increased Holman index (>0.2) was not found in any of the HPN patients or controls. The Holman index in plasma PC (median (25th – 75th percentile)) was significantly higher (p <0.01) in HPN patients (0.019 (0.015 – 0.028)) compared with controls (0.015 (0.011 – 0.017)). Differences between fatty acid profile of plasma PC and PBMCs were found. No differences were found in innate immune cell functions or phenotype between groups, except for a 3.6-fold higher TNF-alpha production (median (25th – 75th percentile)) in HPN patients (3640 pg/ml (1170 – 4670 pg/ml) compared to controls (1020 pg/ml (770 – 1610 pg/ml)).

Conclusion: We found no clinical or biochemical evidence that HPN patients who fully and long-term depend on olive oil based lipids have an increased risk for EFA deficiency.
Clinical Relevancy Statement

Essential fatty acids (EFA) cannot be made endogenously, which makes intravenous lipid emulsions the only source of these fatty acids for patients who are fully dependent on parenteral nutrition. We studied whether patients reliant upon home parenteral nutrition (HPN) have clinical or biochemical signs of EFA deficiency when a mixture of soybean and olive oils is used as the intravenous lipid emulsion, and compared the fatty acid composition of these patients to healthy controls. The results of this study aid our understanding of the nutritional value of HPN for the most vulnerable patient group, those fully dependent on EFA intake from their parenteral nutrition.
Introduction

Lipid emulsions are essential components of total parenteral nutrition (TPN) formulations as a source of non-glucose calories and of fatty acids, including the essential fatty acids (EFA) alpha-linolenic acid (\(18:3n-3\)) and linoleic acid (LA; \(18:2n-6\)). The first clinically available lipid emulsions were prepared from soybean oil (SO), which is rich in LA, an n-6 polyunsaturated fatty acid (PUFA).

Due to the supposed adverse immune and inflammatory effects of mediators produced from the LA derivative arachidonic acid (20:4n-6), emulsions were developed where part of the SO was replaced by other lipids. One such emulsion is based on 20% SO and 80% olive oil (OO). The latter oil is rich in the immune-neutral n-9 monounsaturated fatty acid oleic acid (\(18:1n-9\)). Compared with pure SO-based lipids, this OO emulsion contains three times less LA, which will comprise about 6.5 percent of energy intake when the emulsion is used as a component of TPN. The minimum dietary requirements for adults to avoid EFA deficiency symptoms are estimated to be 0.5 percent energy from alpha-linolenic acid and 2.5 percent energy from LA.

Low EFA intake eventually leads to EFA deficiency, in which case the synthesis of (mono-)unsaturated FA such as oleic acid and palmitoleic acid (\(16:1n-7\)) increases. This increased synthesis leads to the production of mead acid (\(20:3n-9\)), a n-9 PUFA derived from oleic acid. A mead acid/arachidonic acid ratio (the so-called Holman index) above 0.2 is most commonly used to diagnose EFA deficiency. EFA deficiency has been associated with water losses from the skin due to increased permeability, susceptibility to infections, lowered resistance to irradiation injury and impaired wound healing, hematologic disturbances, fat infiltration of the liver, impaired chylomicron synthesis, and aggravated fat absorption. In addition, changes in (essential) fatty acid status have shown an impact on various aspects of immune function.

Evaluation of the EFA status of some HPN patients has revealed alterations in fatty acid profiles in line with a diagnosis of EFA deficiency, but most patients on lipid containing parenteral nutrition
do not have a Holman index above 0.2.\textsuperscript{7,11,14} A previous double-blind randomized study compared OO-based PN with a pure SO-based lipid emulsion and did not find any evidence for EFA deficiencies after short-term treatment (5 times/week, during 3 months) with either lipid emulsion.\textsuperscript{14} We investigated whether patients who fully and long-term depend on OO-based HPN containing low LA concentrations also have adequate EFA intake. To this end, the plasma and cellular fatty acid profile and the presence of scaly skin lesions as a clinical symptom of EFA deficiency were evaluated. Besides the clinical effect, we were also interested in the effect of the EFA status at the cellular level. Accordingly, we compared the function and phenotype of cells of the innate immune system of HPN patients receiving OO-based lipid emulsion with healthy controls.
Methods

Subjects

Thirty adult (≥ 18 years) HPN patients without active underlying immune-mediated disease, who had been using a parenteral nutrition formulation containing 80% OO and 20% SO at least five times per week for at least 3 months and thirty sex- and age-matched healthy controls were included in the study. Subjects with metabolic disorders, active allergic, inflammatory or otherwise immune-mediated diseases, those who consumed more than two portions of fatty fish per week, smoked more than five cigarettes per day, or who used immune suppressive medication, vitamins or fish oil supplements, were excluded from enrollment. Four patients had been hospitalized for a few days due to infectious complications at the moment of inclusion: presuming that these events did not alter FA profile but did have an impact on immune function, immunologic assays were not performed in these patients. The Ethical Review Board of the Radboud University Medical Center approved the study. All procedures were performed after obtaining written informed consent from the patients and controls. The study was registered at ClinicalTrial.gov (NCT01986153).

Laboratory variables

Blood cell counts, including automated leucocyte differentiation, and C-reactive protein were determined on an automated analyzer (AdviaTM 120; Siemens Medical Solutions, The Hague, The Netherlands).

Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were purified from venous whole blood in 10 ml Monoject tubes containing 170 IU of lithium heparin (Beliver Industrial Estate, Plymouth PL6 7BP, UK) as described previously. Briefly, the blood, 1:1 diluted with phosphate buffered saline (PBS, B. Braun Melsungen AG, Melsungen, Germay), was layered on Ficoll-Paque™ Plus (GE Healthcare Life Sciences, Uppsala, SE) and centrifuged (700xg, 20 min, RT). The PBMC-containing interphase was
collected, washed twice with PBS and suspended in RPMI medium to the desired final cell concentration.

Fatty acid composition assessment of PBMCs and plasma phosphatidylcholine

Total lipid was extracted from thawed plasma (0.5 mL, collected in EDTA and stored at -80 °C) and PBMCs (1 x 10⁷ /mL RPMI medium supplemented with 500 units/ml penicillin/500 µg/ml streptomycin and stored at -80 °C) with 5 mL of chloroform:methanol (2:1) containing the antioxidant butylated hydroxytoluene (50 mg/ L). Solid phase extraction was used to isolate phosphatidylcholine (PC) from the total plasma lipid extract. Next, fatty acid methyl esters (FAMEs) from plasma PC and from PBMC total lipid were formed by incubation in methanolic H₂SO₄ for 2 h at 50 °C. FAMEs were separated using gas chromatography on a Hewlett Packard 6890 gas chromatograph fitted with a BPX70 fused silica capillary column (0.25 lm · 30 m · 0.22 mm); helium was used as the carrier gas. Following injection, the temperature was rapidly raised to 115 °C for 2 min. Then, the temperature was raised to 200 °C at the rate of 10 °C/ min, where it was held for 18 min. Finally, the temperature was raised to 245 °C at a rate of 60 °C/ min where it was held for 8 min. FAMEs were detected by flame ionization detection. FAMEs were identified by comparison with run times of authentic standards. CHEMSTATION software was used to calculate peak areas and the percentage contribution of each peak to the total.

Functional and phenotypic analysis of leukocytes

Leucocyte functions were determined by evaluating the expression of surface activation markers, the oxygen radical production and the cytokine production.

Cytokine production: TNF-alpha and IL-10

Isolated PBMCs (1x10⁶ cells/mL) were cultured in RPMI. Cells were stimulated with phytohaemagglutinin (PHA; 10 µg/mL) at 37 °C and 5% CO₂. Aliquots of the culture supernatant
were removed after 48 h of incubation and stored at -80 °C until use for determination of IL-10 and TNF-alpha concentrations. Cytokine concentrations were determined in the supernatants using a specific ELISA kit for human TNF-alpha and IL-10 (R&D Systems Europe, Abingdon, UK) according to instructions of the manufacturer.

Surface activation markers

Immunofluorescent staining followed by flow cytometric analysis was used to determine markers for activation, expressed on the membrane surface of neutrophils and monocytes, as described previously. Monocytes and neutrophils were gated based on their CD14 and CD45 expression. Characterization of activation markers was performed using antibodies (purchased from Beckman Coulter (Miami, FL, USA)) directed against an adhesion molecule of the β2 integrin family (CD11b), a degranulation marker for specific granulae (CD66b) and L-selectin (CD62L). Immune-fluorescent staining was performed according to the "lyse and wash" method. Flow cytometry analyses were carried out on a Beckman Coulter Cytomics FC500 (Miami, FL, USA).

Oxygen radical production

Spontaneous and stimulus-induced oxygen radical production in whole blood was evaluated using Luminol-enhanced chemiluminescence and determined in an automated LB96V Microlumat Plus Luminometer (EG & G Berthold, Bald Wilberg, Germany), as described in detail previously. Briefly, 200 microliters of 1:100 HBSS diluted blood were added to a 96 well microplate, either without stimulus, or in the presence of a receptor-independent (phorbol 12-myristate 13-acetate, PMA) or receptor-dependent (serum-treated zymosan particles, STZ) stimulus. Luminol was added to each well to start the chemiluminescence reaction. Each measurement was carried out in at least four replicates. Chemiluminescence was determined every 145 seconds at 37 °C for one hour. Luminescence was expressed as relative light units per second (RLU/sec). Data were analyzed with
Winglow software (EG & Berthold). After subtraction of background signal, the signal intensity in whole blood samples was corrected for the neutrophil population count.

Statistical analyses

Values are expressed as median with interquartile range unless stated otherwise. Differences between numeric variables of patients and controls were analyzed using the nonparametric Mann-Whitney U test. To evaluate whether immune status was correlated with fatty acid profile of the PBMCs, all immune parameters were tested for the presence of a correlation with the value of the Holman index in PBMCs by using Pearson’s correlation test. In order to correct for multiple testing, a p-value < 0.01 was considered statistically significant. All statistical analyses were performed using SPSS software (version 20.0; IBM SPSS, Inc., Chicago, IL, USA).
Results

Characteristics of HPN patients and healthy controls

The majority of patients (n=19/30) and controls (n=21/30) was female. The median age (25<sup>th</sup> – 75<sup>th</sup> percentile) of patients and controls was 57 (51 – 64) and 58 (46 – 61) years, respectively. The BMI (median (25<sup>th</sup>- 75<sup>th</sup> percentile) of patients (22.3 (19.4 – 23.8) kg/m<sup>2</sup>) was significantly (p=0.009) lower than that of controls (23.8 (22.0 – 25.8) kg/m<sup>2</sup>). The primary indication for intestinal failure was short bowel syndrome (SBS, in 15/30) while nine patients suffered from a gastrointestinal motility disorder and six patients had various problems, including systemic sclerosis, chronic intestinal pseudoobstruction, or Crohn's disease. Patients received parenteral nutrition five (n=8), six (n=8) or seven (n=14) times per week. The amount of fat (median (25<sup>th</sup> - 75<sup>th</sup> percentile) given to the HPN patient per kilogram bodyweight per day was 0.97 gram (0.79 – 1.23 gram). Most patients had been dependent on HPN for more than one year (median (25<sup>th</sup>- 75<sup>th</sup> percentile) 1151 (438 – 2241) days).

Increased n-9 and lower n-6 FA in plasma PC of HPN patients

Plasma PC FA profiles of patients and controls are presented in Table 1. Patients and controls differed in n-6 and n-9 FA: the relative amounts of LA and total n-6 FA were significantly lower in patients, while the relative amounts of oleic acid and total n-9 FA were significantly higher. Small but statistically significant differences in cis-vaccenic acid, total n-3 and total n-7 fatty acids were found between groups. Minor, but statistically significant differences were found between patients and controls in myristic-, behenic-, α-linolenic-, eicosatetraenoic-, γ-linolenic-, eicosadienoic-, palmitoleic- and mead acid in plasma PC.

Increased Holman index in HPN patients

The Holman index (i.e. the mead acid/arachidonic acid ratio) in plasma PC that is regarded as a measure for EFA status and is suggestive for deficiency when increased above 0.2 is presented for all
subjects in Figure 1. A Holman index above 0.2 was not found in any of the HPN patients or controls.

The median (25th – 75th percentile) Holman index in plasma PC was significantly higher (p < 0.01) in patients (0.019 (0.015 – 0.028)) compared with controls (0.015 (0.011 – 0.017)).

Lower n-9 and higher n-6 FA in PBMCs of HPN patients

The differences between groups in PBMC FA profiles were different from those of plasma PC (Table 1). PBMCs of patients and controls differed in n-6 and n-9 FA profiles. The relative amount of total n-6 FA was significantly higher in patients, whereas that of oleic acid and total n-9 FA was lower in patients. Minor, yet statistically significant differences were found between patients and controls in palmitic, stearic, behenic, eicosatetraenoic, eicosapentaenoic, docosapentaenoic, eicosadienoic, cis-vaccenic, total n-3 and total n-7 FA in PBMCs. The median (25th – 75th percentile) mead acid/arachidonic acid ratio in PBMCs was not statistically different between patients (0.029 (0.011 – 0.076)) and controls (0.019 (0.013 – 0.040).

Increase in TNF-alpha production by PBMCs from HPN patients

Innate immune function was assessed by evaluating the expression of surface membrane activation markers, and the stimulus-induced production of ROS and cytokines by leukocytes (Table 2). A 3.6-fold increase in TNF-alpha production was found for PBMCs from patients compared to those from controls, while IL-10 production was not different between groups. A 2.4-fold decrease in IL-10/TNF-alpha ratio was found for PBMCs from patients compared to those from controls. No statistically significant differences between patients and controls were found in the expression of the activation marker L-selectin or of adhesion and degranulation markers of granulocytes and monocytes. Receptor-independent (PMA) and receptor-dependent (STZ) induced ROS production were not statistically different between groups.

No correlation between leukocyte functions and EFA status in PBMCs
To evaluate whether immune status was correlated with EFA status, cytokine production, ROS production and expression of surface activation markers were tested for the presence of a correlation with the mead acid/arachidonic acid ratio in PBMCs. None of the immune parameters was significantly correlated with the mead acid/arachidonic acid ratio in PBMCs.
In the present study we found no clinical or biochemical evidence for EFA deficiency in patients who long-term and fully depend on OO-based parenteral nutrition. None of the patients or controls had a Holman index above 0.2, meaning none of them met the criteria for the diagnosis of EFA deficiency.

Scaly skin lesions, the most prominent feature of EFA deficiency, were not seen in any of the patients or controls. Accordingly, functional immunological parameters were not different between groups, with the exception of evidence for increased inflammatory potential (TNF-alpha production) in the patients.

We found significantly lower relative plasma PC concentrations of LA and alpha-linolenic acid in HPN patients than controls. About 90 percent of the total amount of EFA in plasma PC consisted of LA in both groups. We found average relative concentrations of LA in the plasma PC of 14.7 percent in the patients and 24.7 percent in the controls, which is in line with previous reports, ranging from 11 to 24 percent in patients and from 22 to 30 percent in controls. The lower LA in plasma PC of HPN patients was compensated for by a higher average relative concentration of oleic acid (17.5 vs 10%). The different relative proportions of oleic acid and LA in plasma PC of patients and controls probably reflect the differences in relative supply of those two fatty acids in the TPN regimen (patients) compared with the diet (controls). Alpha-linolenic acid represents only a small proportion of the total amount of EFA present in plasma, as has previously been described for both HPN patients and healthy controls.

EFA deficiency is traditionally defined as a mead acid/arachidonic acid ratio (Holman index) above 0.2 in plasma, since a low EFA intake will eventually lead to increased mead acid synthesis. Although a significant difference between patients and controls was found with a maximum Holman index in patients of 0.051 and in controls of 0.030, none of the patients or controls met the criterion for EFA
deficiency. A Holman index above 0.2 is sporadically described, but most HPN patients like our patients have a Holman index lower than 0.2.

To establish the clinical relevance of the fatty acid profile, we evaluated patients for the presence of the most prominent clinical sign of EFA deficiency, scaly skin lesions, on the day that blood was withdrawn for analysis. We did not find such evidence in any of the patients or controls. Interestingly, clinical signs of EFA deficiency have been described in HPN patients with a Holman index lower than 0.2. For instance, Jeppesen reported a median (25th–75th percentile) Holman index for HPN patients without skin problems of 0.10 (0.04–0.028), while for HPN patients with skin problems a Holman index of 0.05 (0.02–0.20) was found. However, these skin problems were self-reported, and might not be related to the EFA status of the patients.

Besides the effects of EFA status on clinical symptoms, we were also interested in the effect of the EFA status at the cellular level, since it is known that EFA deficiency impairs cellular aspects of the immune response. A previous study showed no major differences in FA profile between neutrophils and monocytes so the PBMC lipids were considered to be representative for immune cells. We therefore analyzed fatty acid profiles of PBMCs and performed functional tests to evaluate immune function. The FA profile of PBMCs was different from that in plasma PC: while the concentration of n-6 FA was lower and that of n-9 FA higher in plasma PC of patients compared to controls, the opposite was found in PBMCs. Differences between plasma and PBMC FA profiles have been described before, and may be explained by the possibility that cells exert a significant level of control over their plasma membrane composition, and a second explanation may be that cells can metabolize PUFAs and thereby modify the FA composition of their plasma membrane.

PBMC function was evaluated by measuring the stimulus (PHA)-induced production of pro- and anti-inflammatory cytokines, yielding a 3.6-fold increase in production of the pro-inflammatory cytokine
TNF-alpha in patients compared to controls. Since the IL-10/TNF-alpha ratio was only 2.4 times lower in patients, the difference in TNF-alpha seems to be partly neutralized by the anti-inflammatory IL-10. The increased TNF-alpha production was not related to EFA status, since no correlation was found between TNF-alpha and the mead acid/arachidonic acid ratio in PBMCs. Other immune functions were not correlated to this ratio of fatty acids.

Differences in stimulus-induced cytokine production may have consequences in clinical situations where the immune system needs to be triggered, like during infection. However, we found no evidence of differences between patients and controls with regard to other markers of immune function (stimulus-induced ROS production, expression of surface activation markers), a finding that is in line with previous work in this field.

It was not unexpected that the healthy controls had no evidence of EFA deficiency, since EFA are abundantly present in the western diet. Although some HPN patients have been described with an increased Holman index, most patients with lipid containing parenteral nutrition did not meet the criterion of a Holman index above 0.2 before. The LA caloric intake of about 6.5 percent present in OO-based HPN seems to be adequate for our patients to have a Holman index below 0.2.

Limitations of the present study should be taken into account. First, besides scaly skin lesions, no other, but also less prominent, clinical features of EFA deficiency, like infection susceptibility or wound healing were evaluated in our study population. Secondly, only innate immune functions were evaluated, since these seem to be particularly affected in HPN patients, as is exemplified by the increased risk for pneumonia and wound infections in mildly malnourished surgical patients on PN. Furthermore, the limited power because of small study groups precludes the detection of subtle changes in FA profile and immune function.
In conclusion, we found no clinical or biochemical evidence that HPN patients who fully and long-
term depend on olive oil based lipids have an increased risk for EFA deficiency.
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Statement of authorship

ED Olthof planned and executed the study, analyzed the data and drafted the manuscript.

HM Roelofs executed the study, analyzed the data and revised the manuscript.

HL Fisk executed the study and revised the manuscript.

PC Calder executed the study and revised the manuscript

GJA Wanten designed the study, obtained funding and revised the manuscript.

All authors read and approved the final manuscript.

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Conflict of Interest Statement

ED Olthof, HM Roelofs and HL Fisk declare no conflict of interest. PC Calder has received speaking fees from Baxter, BBraun and Fresenius Kabi. GJA Wanten has received speaking fees from Baxter, BBraun, Fresenius Kabi, and Geistlich and research grants from Baxter, Fresenius Kabi and Geistlich.
References


**Figure legends**

**Figure 1:** Mead acid/arachidonic acid ratio (Holman index) in plasma phosphatidylcholine of HPN patients and healthy controls. Essential fatty acid deficiency is characterized by a Holman index > 0.2. * All values are presented as medians. * A p-value of <0.01 was considered to be statistically significant.
Tables

Table 1: Fatty acid composition of plasma phosphatidylcholine (PC) and PBMCs as percentage by weight of total fatty acids. All data are presented as median with interquartile range. A p-value of <0.01 was considered statistically significant.

a HPN patients are statistically different from healthy controls with a p-value < 0.001.

b HPN patients are statistically different from healthy controls with a p-value < 0.01.
Table 2: Innate immune function of HPN patients and age- and sex-matched healthy controls.

Immune function was evaluated by determining C-reactive protein, the expression of membrane surface activation markers (L-selectin, adhesion, specific and azurophilic degranulation) on granulocytes and monocytes, the production of reactive oxygen species during stimulation with a receptor-independent (phorbol 12-myristate 13-acetate) and receptor-dependent (serum treated zymosan) stimulus and cytokine production (TNF-alpha and IL-10) in PBMCs. All data are presented as median with interquartile range. A p-value of <0.01 was considered to be statistically significant.

* Immune function was not assessed because they were hospitalized (n=4) or because of logistic problems (n=1).

* HPN patients are statistically different from healthy controls with a p-value < 0.001.