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
2. parenteral nutrition
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25 **Abstract**

26

27 **Background**: Home parenteral nutrition (HPN) patients depend on lipid emulsions as part of their
28 parenteral nutrition regimen in order to provide essential fatty acids (EFA). Mixed oil sources are
29 used in modern lipid emulsions to decrease the amount of pro-inflammatory EFA, mainly linoleic
30 acid, which is present in large amounts in soybean oil. It is unknown whether patients who fully
31 depend on such mixed lipids have adequate EFA supply and status. In the present study we
32 therefore evaluated whether HPN patients who depend on a mixed lipid emulsion (20% soybean oil,
33 80% olive oil) show evidence of EFA deficiency, in the form of fatty acid biomarkers, clinical signs
34 and/or decreased innate immune cell functions.

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

40 **Results**: None of the HPN patients or controls showed clinical evidence for EFA deficiency in the
41 form of skin rash. Biochemical evidence for EFA deficiency, in the form of an increased Holman
42 index (>0.2) was not found in any of the HPN patients or controls. The Holman index in plasma PC
43 (median (25th – 75th percentile)) was significantly higher ($p < 0.01$) in HPN patients (0.019 (0.015 –
44 0.028)) compared with controls (0.015 (0.011 – 0.017)). Differences between fatty acid profile of
45 plasma PC and PBMCs were found. No differences were found in innate immune cell functions or
46 phenotype between groups, except for a 3.6-fold higher TNF-alpha production (median (25th – 75th
47 percentile)) in HPN patients (3640 pg/ml (1170 – 4670 pg/ml) compared to controls (1020 pg/ml (770
48 – 1610 pg/ml)).

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

51

52 **Clinical Relevancy Statement**

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

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64 **Introduction**

65 Lipid emulsions are essential components of total parenteral nutrition (TPN) formulations as a
66 source of non-glucose calories and of fatty acids, including the essential fatty acids (EFA) alpha-
67 linolenic acid (18:3n-3) and linoleic acid (LA; 18:2n-6). The first clinically available lipid emulsions
68 were prepared from soybean oil (SO), which is rich in LA, an n-6 polyunsaturated fatty acid (PUFA).
69 Due to the supposed adverse immune and inflammatory effects of mediators produced from the LA
70 derivative arachidonic acid (20:4n-6), emulsions were developed where part of the SO was replaced
71 by other lipids.[1] One such emulsion is based on 20% SO and 80% olive oil (OO). The latter oil is rich
72 in the immune-neutral n-9 monounsaturated fatty acid oleic acid (18:1n-9). Compared with pure SO-
73 based lipids, this OO emulsion contains three times less LA, which will comprise about 6.5 percent of
74 energy intake when the emulsion is used as a component of TPN.[2] The minimum dietary
75 requirements for adults to avoid EFA deficiency symptoms are estimated to be 0.5 percent energy
76 from alpha-linolenic acid and 2.5 percent energy from LA.[3]

77

78 Low EFA intake eventually leads to EFA deficiency, in which case the synthesis of (mono-)
79 unsaturated FA such as oleic acid and palmitoleic acid (16:1n-7) increases. This increased synthesis
80 leads to the production of mead acid (20:3n-9), a n-9 PUFA derived from oleic acid. A mead
81 acid/arachidonic acid ratio (the so-called Holman index) above 0.2 is most commonly used to
82 diagnose EFA deficiency. [4,5] EFA deficiency has been associated with water losses from the skin due
83 to increased permeability, susceptibility to infections, lowered resistance to irradiation injury and
84 impaired wound healing, hematologic disturbances, fat infiltration of the liver, impaired
85 chylomicron synthesis, and aggravated fat absorption. [1,6,7] In addition, changes in (essential) fatty
86 acid status have shown an impact on various aspects of immune function. [8-10]

87

88 Evaluation of the EFA status of some HPN patients has revealed alterations in fatty acid profiles in
89 line with a diagnosis of EFA deficiency.[7,11], but most patients on lipid containing parenteral nutrition

90 do not have a Holman index above 0.2. [7,11-14] A previous double-blind randomized study compared
91 OO-based PN with a pure SO-based lipid emulsion and did not find any evidence for EFA
92 deficiencies after short-term treatment (5 times/week, during 3 months) with either lipid emulsion.
93 [14] We investigated whether patients who fully and long-term depend on OO-based HPN containing
94 low LA concentrations also have adequate EFA intake. To this end, the plasma and cellular fatty acid
95 profile and the presence of scaly skin lesions as a clinical symptom of EFA deficiency were evaluated.
96 Besides the clinical effect, we were also interested in the effect of the EFA status at the cellular level.
97 Accordingly, we compared the function and phenotype of cells of the innate immune system of HPN
98 patients receiving OO-based lipid emulsion with healthy controls.
99

100 **Methods**

101 Subjects

102 Thirty adult (> 18 years) HPN patients without active underlying immune-mediated disease, who
103 had been using a parenteral nutrition formulation containing 80% OO and 20% SO at least five
104 times per week for at least 3 months and thirty sex- and age-matched healthy controls were
105 included in the study. Subjects with metabolic disorders, active allergic, inflammatory or otherwise
106 immune-mediated diseases, those who consumed more than two portions of fatty fish per week,
107 smoked more than five cigarettes per day, or who used immune suppressive medication, vitamins or
108 fish oil supplements, were excluded from enrollment. Four patients had been hospitalized for a few
109 days due to infectious complications at the moment of inclusion: presuming that these events did
110 not alter FA profile but did have an impact on immune function, immunologic assays were not
111 performed in these patients. The Ethical Review Board of the Radboud University Medical Center
112 approved the study. All procedures were performed after obtaining written informed consent from
113 the patients and controls. The study was registered at ClinicalTrial.gov (NCT01986153).
114
115 Laboratory variables

116 Blood cell counts, including automated leucocyte differentiation, and C-reactive protein were
117 determined on an automated analyzer (AdviaTM[120]; Siemens Medical Solutions, The Hague, The
118 Netherlands).
119
120 Isolation of peripheral blood mononuclear cells

121 Peripheral blood mononuclear cells (PBMCs) were purified from venous whole blood in 10 ml
122 Monoject tubes containing 170 IU of lithium heparin (Beliver Industrial Estate, Plymouth PL6 7BP,
123 UK) as described previously.[15] Briefly, the blood, 1:1 diluted with phosphate buffered saline (PBS, B.
124 Braun Melsungen AG, Melsungen, Germay), was layered on Ficoll-Paque™ Plus (GE Healthcare Life
125 Sciences, Uppsala, SE) and centrifuged (700xg, 20 min, RT). The PBMC-containing interphase was

126 collected, washed twice with PBS and suspended in RPMI medium to the desired final cell
127 concentration.
128
129 Fatty acid composition assessment of PBMCs and plasma phosphatidylcholine
130 Total lipid was extracted from thawed plasma (0.5 mL, collected in EDTA and stored at -80 °C) and
131 PBMCs (1 x 10^7 /mL RPMI medium supplemented with 500 units/ml penicillin/500 µg/ml
132 streptomycin and stored at -80 °C) with 5 mL of chloroform:methanol (2:1) containing the
133 antioxidant butylated hydroxytoluene (50 mg/ L). Solid phase extraction was used to isolate
134 phosphatidylcholine (PC) from the total plasma lipid extract. Next, fatty acid methyl esters (FAMEs)
135 from plasma PC and from PBMC total lipid were formed by incubation in methanolic H_2SO_4 for 2 h at
136 50 °C. FAMEs were separated using gas chromatography on a Hewlett Packard 6890 gas
137 chromatograph fitted with a BPX70 fused silica capillary column (0.25 lm · 30 m · 0.22 mm); helium
138 was used as the carrier gas. Following injection, the temperature was rapidly raised to 115 °C for 2
139 min. Then, the temperature was raised to 200 °C at the rate of 10 °C/ min, where it was held for 18
140 min. Finally, the temperature was raised to 245 °C at a rate of 60 °C/ min where it was held for 8 min.
141 FAMEs were detected by flame ionization detection. FAMEs were identified by comparison with run
142 times of authentic standards. CHEMSTATION software was used to calculate peak areas and the
143 percentage contribution of each peak to the total.
144
145 Functional and phenotypic analysis of leukocytes
146 Leucocyte functions were determined by evaluating the expression of surface activation markers,
147 the oxygen radical production and the cytokine production.
148
149 Cytokine production: TNF-alpha and IL-10
150 Isolated PBMCs (1x10^6 cells/mL) were cultured in RPMI. Cells were stimulated with
151 phytohaemagglutinin (PHA; 10 µg/mL) at 37 °C and 5% CO_2. Aliquots of the culture supernatant

152 were removed after 48 h of incubation and stored at -80 °C until use for determination of IL-10 and
153 TNF-alpha concentrations. Cytokine concentrations were determined in the supernatants using a
154 specific ELISA kit for human TNF-alpha and IL-10 (R&D Systems Europe, Abingdon, UK) according
155 to instructions of the manufacturer.
156

157 Surface activation markers

158 Immunofluorescent staining followed by flow cytometric analysis was used to determine markers
159 for activation, expressed on the membrane surface of neutrophils and monocytes, as described
160 previously.[15] Monocytes and neutrophils were gated based on their CD14 and CD45 expression.
161 Characterization of activation markers was performed using antibodies (purchased from Beckman
162 Coulter (Miami, FL, USA)) directed against an adhesion molecule of the β2 integrin family (CD11b), a
163 degranulation marker for specific granulae (CD66b) and L-selectin (CD62L). Immune-fluorescent
164 staining was performed according to the "lyse and wash" method. Flow cytometry analyses were
165 carried out on a Beckman Coulter Cytomics FC500 (Miami, FL, USA).
166

167 Oxygen radical production

168 Spontaneous and stimulus-induced oxygen radical production in whole blood was evaluated using
169 Luminol-enhanced chemiluminescence and determined in an automated LB96V Microlumat Plus
170 Luminometer (EG & G Berthold, Bald Wilberg, Germany), as described in detail previously.[15] Briefly,
171 200 microliters of 1:100 HBSS diluted blood were added to a 96 well microplate, either without
172 stimulus, or in the presence of a receptor-independent (phorbol 12-myristate 13-acetate, PMA) or
173 receptor-dependent (serum-treated zymosan particles, STZ) stimulus. Luminol was added to each
174 well to start the chemiluminescence reaction. Each measurement was carried out in at least four
175 replicates. Chemiluminescence was determined every 145 seconds at 37 °C for one hour.
176 Luminescence was expressed as relative light units per second (RLU/sec). Data were analyzed with

177 Winglow software (EG & Berthold). After subtraction of background signal, the signal intensity in
178 whole blood samples was corrected for the neutrophil population count.
179
180 Statistical analyses
181 Values are expressed as median with interquartile range unless stated otherwise. Differences
182 between numeric variables of patients and controls were analyzed using the nonparametric Mann-
183 Whitney U test. To evaluate whether immune status was correlated with fatty acid profile of the
184 PBMCs, all immune parameters were tested for the presence of a correlation with the value of the
185 Holman index in PBMCs by using Pearson's correlation test. In order to correct for multiple testing, a
186 p-value <0.01 was considered statistically significant. All statistical analyses were performed using
187 SPSS software (version 20.0; IBM SPSS, Inc., Chicago, IL, USA).
188

189 **Results**

190

191 Characteristics of HPN patients and healthy controls

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

202

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

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

211

212 Increased Holman index in HPN patients

213 The Holman index (i.e. the mead acid/arachidonic acid ratio) in plasma PC that is regarded as a
214 measure for EFA status and is suggestive for deficiency when increased above 0.2 is presented for all

215 subjects in Figure 1. A Holman index above 0.2 was not found in any of the HPN patients or controls.
216 The median (25th – 75th percentile) Holman index in plasma PC was significantly higher (p <0.01) in
217 patients (0.019 (0.015 – 0.028)) compared with controls (0.015 (0.011 – 0.017)).
218

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

220 The differences between groups in PBMC FA profiles were different from those of plasma PC (Table
221 1). PBMCs of patients and controls differed in n-6 and n-9 FA profiles. The relative amount of total n-
222 6 FA was significantly higher in patients, whereas that of oleic acid and total n-9 FA was lower in
223 patients. Minor, yet statistically significant differences were found between patients and controls in
224 palmitic, stearic, behenic, eicosatetraenoic, eicosapentaenoic, docosapentaenoic, eicosadienoic, cis-
225 vaccenic, total n-3 and total n-7 FA in PBMCs. The median (25th – 75th percentile) mead
226 acid/arachidonic acid ratio in PBMCs was not statistically different between patients (0.029 (0.011 –
227 0.076)) and controls (0.019 (0.013 – 0.040).
228

229 Increase in TNF-alpha production by PBMCs from HPN patients

230 Innate immune function was assessed by evaluating the expression of surface membrane activation
231 markers, and the stimulus-induced production of ROS and cytokines by leukocytes (Table 2). A 3.6-
232 fold increase in TNF-alpha production was found for PBMCs from patients compared to those from
233 controls, while IL-10 production was not different between groups. A 2.4-fold decrease in IL-10/TNF-
234 alpha ratio was found for PBMCs from patients compared to those from controls. No statistically
235 significant differences between patients and controls were found in the expression of the activation
236 marker L-selectin or of adhesion and degranulation markers of granulocytes and monocytes.
237 Receptor-independent (PMA) and receptor-dependent (STZ) induced ROS production were not
238 statistically different between groups.
239

240 No correlation between leukocyte functions and EFA status in PBMCs

241 To evaluate whether immune status was correlated with EFA status, cytokine production, ROS
242 production and expression of surface activation markers were tested for the presence of a
243 correlation with the mead acid/arachidonic acid ratio in PBMCs. None of the immune parameters
244 was significantly correlated with the mead acid/arachidonic acid ratio in PBMCs.
245

246 **Discussion**

247 In the present study we found no clinical or biochemical evidence for EFA deficiency in patients who
248 long-term and fully depend on OO-based parenteral nutrition. None of the patients or controls had a
249 Holman index above 0.2, meaning none of them met the criteria for the diagnosis of EFA deficiency.
250 Scaly skin lesions, the most prominent feature of EFA deficiency, were not seen in any of the
251 patients or controls. Accordingly, functional immunological parameters were not different between
252 groups, with the exception of evidence for increased inflammatory potential (TNF-alpha production)
253 in the patients.

254

255 We found significantly lower relative plasma PC concentrations of LA and alpha-linolenic acid in
256 HPN patients than controls. About 90 percent of the total amount of EFA in plasma PC consisted of
257 LA in both groups. We found average relative concentrations of LA in the plasma PC of 14.7 percent
258 in the patients and 24.7 percent in the controls, which is in line with previous reports, ranging from
259 11 to 24 percent in patients and from 22 to 30 percent in controls.[7,10-14,16,17] The lower LA in plasma PC
260 of HPN patients was compensated for by a higher average relative concentration of oleic acid (17.5
261 vs 10%). The different relative proportions of oleic acid and LA in plasma PC of patients and controls
262 probably reflect the differences in relative supply of those two fatty acids in the TPN regimen
263 (patients) compared with the diet (controls). Alpha-linolenic acid represents only a small proportion
264 of the total amount of EFA present in plasma, as has previously been described for both HPN
265 patients and healthy controls.[7,10-14,16,17]

266

267 EFA deficiency is traditionally defined as a mead acid/arachidonic acid ratio (Holman index) above
268 0.2 in plasma, since a low EFA intake will eventually lead to increased mead acid synthesis. Although
269 a significant difference between patients and controls was found with a maximum Holman index in
270 patients of 0.051 and in controls of 0.030, none of the patients or controls met the criterion for EFA

271 deficiency. A Holman index above 0.2 is sporadically described [7,11], but most HPN patients like our
272 patients have a Holman index lower than 0.2.[7,11-14]
273
274 To establish the clinical relevance of the fatty acid profile, we evaluated patients for the presence of
275 the most prominent clinical sign of EFA deficiency, scaly skin lesions, on the day that blood was
276 withdrawn for analysis. We did not find such evidence in any of the patients or controls.
277 Interestingly, clinical signs of EFA deficiency have been described in HPN patients with a Holman
278 index lower than 0.2. For instance, Jeppesen reported an median (25th -75th percentile) Holman index
279 for HPN patients without skin problems of 0.10 (0.04 – 0.028), while for HPN patients with skin
280 problems a Holman index of 0.05 (0.02 – 0.20) was found.[12] However, these skin problems were self-
281 reported, and might not be related to the EFA status of the patients.
282
283 Besides the effects of EFA status on clinical symptoms, we were also interested in the effect of the
284 EFA status at the cellular level, since it is known that EFA deficiency impairs cellular aspects of the
285 immune response. [8] A previous study showed no major differences in FA profile between
286 neutrophils and monocytes so the PBMC lipids were considered to be representative for immune
287 cells.[10] We therefore analyzed fatty acid profiles of PBMCs and performed functional tests to
288 evaluate immune function. The FA profile of PBMCs was different from that in plasma PC: while the
289 concentration of n-6 FA was lower and that of n-9 FA higher in plasma PC of patients compared to
290 controls, the opposite was found in PBMCs. Differences between plasma and PBMC FA profiles have
291 been described before, and may be explained by the possibility that cells exert a significant level of
292 control over their plasma membrane composition, and a second explanation may be that cells can
293 metabolize PUFAs and thereby modify the FA composition of their plasma membrane. [10,15]
294
295 PBMC function was evaluated by measuring the stimulus (PHA)-induced production of pro- and anti-
296 inflammatory cytokines, yielding a 3.6-fold increase in production of the pro-inflammatory cytokine

297 TNF-alpha in patients compared to controls. Since the IL-10/TNF-alpha ratio was only 2.4 times
298 lower in patients, the difference in TNF-alpha seems to be partly neutralized by the anti-
299 inflammatory IL-10. The increased TNF-alpha production was not related to EFA status, since no
300 correlation was found between TNF-alpha and the mead acid/arachidonic acid ratio in PBMCs. Other
301 immune functions were not correlated to this ratio of fatty acids.
302
303 Differences in stimulus-induced cytokine production may have consequences in clinical situations
304 where the immune system needs to be triggered, like during infection. However, we found no
305 evidence of differences between patients and controls with regard to other markers of immune
306 function (stimulus-induced ROS production, expression of surface activation markers), a finding that
307 is in line with previous work in this field. [18]
308
309 It was not unexpected that the healthy controls had no evidence of EFA deficiency, since EFA are
310 abundantly present in the western diet. Although some HPN patients have been described with an
311 increased Holman index, most patients with lipid containing parenteral nutrition did not meet the
312 criterion of a Holman index above 0.2 before. [11,13] The LA caloric intake of about 6.5 percent present
313 in OO-based HPN seems to be adequate for our patients to have a Holman index below 0.2.
314
315 Limitations of the present study should be taken into account. First, besides scaly skin lesions, no
316 other, but also less prominent, clinical features of EFA deficiency, like infection susceptibility or
317 wound healing were evaluated in our study population. Secondly, only innate immune functions
318 were evaluated, since these seem to be particularly affected in HPN patients, as is exemplified by
319 the increased risk for pneumonia and wound infections in mildly malnourished surgical patients on
320 PN.[19] Furthermore, the limited power because of small study groups precludes the detection of
321 subtle changes in FA profile and immune function.
322

323 In conclusion, we found no clinical or biochemical evidence that HPN patients who fully and long-

324 term depend on olive oil based lipids have an increased risk for EFA deficiency.

325

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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.

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401

402

403 **Figure legends**

404

405 **Figure 1**: Mead acid/arachidonic acid ratio (Holman index) in plasma phosphatidylcholine of HPN
406 patients and healthy controls. Essential fatty acid deficiency is characterized by a Holman index >
407 0.2. [4] All values are presented as medians. * A p-value of <0.01 was considered to be statistically
408 significant.

409

410 **Tables**

411

412 **Table 1**: Fatty acid composition of plasma phosphatidylcholine (PC) and PBMCs as percentage by
413 weight of total fatty acids. All data are presented as median with interquartile range. A p-value of
414 <0.01 was considered statistically significant.

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

416 [b] HPN patients are statistically different from healthy controls with a p-value <0.01.

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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).

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