Essential fatty acid deficiency in malnourished children
Smit, Elsiena Neeltina

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2002

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Smit, E. N. (2002). Essential fatty acid deficiency in malnourished children: erythrocyte and breastmilk fatty acid compositions in different populations Groningen: s.n.

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Download date: 03-11-2018

Ella N. Smit1, M. Rebecca Fokkema2, Ingrid A. Martini3, Henk A. Wolti4, E. Rudy Boersma1 and Frits A.J. Muskiet2

1Department of Obstetrics/Pediatrics, Perinatal Nutrition and Development Unit, Groningen University and University Hospital; 2Department of Pathology and Laboratory Medicine, Groningen University Hospital; 3Laboratory Center, Groningen University Hospital; 4Department of Pediatrics, Groningen Martini Hospital, The Netherlands

Prostaglandins Leukot Essent Fatty Acids, accepted in modified form

Abstract

Background. Early suspicion of essential fatty acid deficiency (EFAD) or ω3-deficiency may rather focus on polyunsaturated fatty acid (PUFA) or long-chain PUFA (LCP) analyses than clinical symptoms. We determined cut-off values for biochemical EFAD and ω3-deficiency by measurement of erythrocyte 20:3ω9 (Mead acid), 22:5ω6/22:6ω3 and 22:5ω6/22:4ω6.

Methods. Cut-off values, based on 97.5 percentiles, derived from an apparently healthy omnivorous group (6 Dominica breast-fed newborns, 32 breast-fed and 27 formula+LCP-fed Dutch low-birth weight infants, 31 Jerusalem infants, 33 Dutch 3.5-years old infants, 69 omnivorous Dutch adults and 7 Dominica mothers) and an apparently healthy group with low dietary LCP intake (81 formula-fed Dutch low-birth weight infants, 12 Dutch vegans). They were validated by their application in an EFAD suspected group of 108, mostly malnourished, Pakistani children, three pediatric patients with chronic fat-malabsorption (abetalipoproteinemia, congenital jejunal and biliary atresia) and one patient with a peroxisomal disorder.

Results. Erythrocyte 20:3ω9, 22:5ω6/22:6ω3 and 22:5ω6/22:4ω6 proved age-dependent up to 0.2 years. Cut-off values for ages above 0.2 years were: 0.46 mol% 20:3ω9 for EFAD, 0.22 mol/mol 22:5ω6/22:6ω3 for ω3-marginality, 0.48 mol/mol 22:5ω6/22:6ω3 for ω3-deficiency and 0.33 mol/mol 22:5ω6/22:4ω6 for low 22:6ω3 precursor status. Increases beyond the 20:3ω9 and 22:5ω6/22:6ω3 cut-off values identified EFAD in 33.3% Pakistani children and 3 pediatric patients, ω3-deficiency in 35.2% Pakistani children and all 4 pediatric patients, and ω3-marginality in 60.2% Pakistani children. Increased 22:5ω6/22:4ω6 might be useful to detect a low status of 22:6ω3-precursors.

Conclusion. Present cut-off values may serve for PUFA supplement intervention until better concepts have emerged.

4.1.1. Introduction

Essential fatty acid (EFA) deficiency (EFAD) is a clinical condition that derives from inadequate status of fatty acids (FA) of both the ω6 and ω3 families. Isolated ω3-deficiency
is recognized as a separate condition next to EFAD, but isolated ω6-deficiency in humans is probably rare. Among the clinical features of EFAD and isolated ω3-deficiency are impaired growth, skin lesions, infertility, kidney abnormalities, fatty liver, polydipsia, increased susceptibility to infections, reduced learning and impaired vision [1-4]. These symptoms are nonspecific and may develop after long standing marginal EFA status [5]. Therefore, present clinical chemical cut-off values for EFAD and isolated ω3-deficiency are mostly based on the detection of disbalances between the ω3, ω6, ω7, ω9 and saturated FA families [6]. Such cut-off values do not necessarily relate to the presence of clinically detectable symptoms or disease development as gold standards, and a biochemical deficiency is therefore not to be confused with a clinically detectable deficiency.

The parent EFA linoleic (18:2ω6) and α-linolenic (18:3ω3) acids, and their long-chain polyunsaturated FA homologues of the ω3 and ω6 series (LCP; ≥C20 with at least 3 double bonds in methylene interrupted cis-configuration) are structural elements of membrane phospholipids and precursors of hydroxy FA and eicosanoids via the lipoxygenase and cyclooxygenase pathways [1,6]. Parent EFA derive exclusively from the diet, whereas LCP derive either from the diet or synthesis from parent EFA. Important food sources are vegetable oils like sunflower (18:2ω6) or soybean (18:2ω6 and 18:3ω3) oils, meat (arachidonic acid, 20:4ω6) and fish (eicosapentaenoic acid, 20:5ω3 and docosahexaenoic acid, 22:6ω3). LCP synthesis from 18:2ω6 and 18:3ω3 occurs by desaturation, chain-elongation and chain-shortening, with Δ6-desaturation as the first and rate-limiting step. Both 18:2ω6 (to 20:4ω6) and 18:3ω3 (to 22:6ω3 via 20:5ω3), but also non-essential oleic acid (18:1ω9) compete for conversion by Δ6-desaturase. This enzyme has preference for its substrates in the order 18:3ω3>18:2ω6>18:1ω9, implying that some combination of low 18:3ω3 and 18:2ω6 status is needed to allow 18:1ω9 to serve as a Δ6-desaturase substrate for the formation of 20:3ω9 (also known as ‘Mead acid’) and 22:3ω9. Mead acid incorporation into tissue lipids is associated with platelet hyperactivity [7], vasoconstriction [8] and altered cell-cell adhesion [9] but its feeding to rats has no adverse effects on health or growth [10].

Both 20:3ω9 and the so-called triene-tetraene (20:3ω9/20:4ω6) ratio are widely used as markers for biochemical EFAD [6,11,12]. The total plasma 20:3ω9/20:4ω6 ratio has for many years been the EFAD ‘gold standard’. With time, the upper-limit has been reduced from 0.4 to 0.2 [13]. Mead acid increases prior to a 20:4ω6 decrease, since tissues and notably brain [6] tend to conserve 20:4ω6 at developing EFA deficiency [10,14]. Total plasma may also not be the preferred compartment, since its FA profile derives from at least four different lipid classes, which are located in a variety of lipoproteins with different functions, origins, targets, turnover rates and interindividual compositions. Erythrocyte (RBC) FA contents might provide a more reliable parameter of cellular EFA-status, which reflects bone marrow FA availability and plasma-RBC phospholipid exchange processes of the preceding 2-3 months. RBC FA derive solely from RBC plasma membrane phospholipids, contain the full range of LCP, are well defined with respect to their dietary dependence [6] and relate to the FA composition of brain [15,16]. RBC FA might on the other hand be somewhat dependent on RBC age-distribution [17].

Isolated ω3-deficiency has attracted attention since its recognition in a 6 years old girl who received long-term ω3 FA poor total parental nutrition [18]. Isolated ω3- (or ω6-) deficiency does not necessarily cause augmented 20:3ω9, because of the 18:1ω9
desaturation suppressing effect of the remaining sufficient 18:2ω6 (or 18:3ω3).

We established cut-off values, based on 97.5 percentiles, of RBC 20:3ω9 and 22:5ω6/22:6ω3 for assessment of biochemical EFAD and ω3-deficiency and investigated the added value of RBC 20:4ω6/22:6ω3 and 22:5ω6/22:4ω6 as parameters of ω3 status. The study population was composed of apparently healthy subgroups of different ages consuming either an LCP-containing omnivorous diet or a low-LCP diet (i.e. formula-fed infants and vegans). The cut-off values were validated by investigating their ability to detect EFAD and ω3-deficiency in a group of mostly malnourished Pakistani infants with known low EFA and/or ω3 status, in 3 patients with chronic fat-malabsorption of various causes, and in one patient with a peroxisomal disorder. The Pakistani data were also used for the comparison of the diagnostic sensitivities of RBC 22:5ω6/22:6ω3, 20:4ω6/22:6ω3 and 22:5ω6/22:4ω6 as indices of ω3-status.

4.1.2. Subjects and Methods

4.1.2.1 Study groups

The RBC FA data of this study are a collection of results from studies conducted by our research group during the past 10 years. The data derived from employment of the same pre-analytical and analytical methods for RBC FA analyses [29,30] in a single laboratory. The selected study population was composed of two apparently healthy groups and two EFAD-suspected groups (Table 1). Details regarding their dietary backgrounds can be obtained from the respective papers.

The apparently healthy subjects were either assigned to an omnivorous group or a group with low dietary LCP intake. The omnivorous group comprised 6 Dominica newborns (studied at two occasions), 32 human milk fed low-birth weight (LBW) Dutch infants (studied at three occasions) and 27 LBW Dutch babies who received formula with LCP (of which 1 was studied at one, 1 at two and 25 at three occasions), 31 Jerusalem infants, 33 Dutch 3.5-years old infants, 69 omnivorous Dutch adults and 7 Dominica mothers, totaling 326 data points. The low-dietary-LCP group comprised 81 LBW Dutch infants who received formula without LCP (studied at three occasions) and 12 Dutch adults consuming a vegan diet, totaling 255 data points. All LBW (≤ 2.50 kg) Dutch babies were born in the Groningen Martini Hospital, The Netherlands. They comprised a group of healthy, predominantly preterm, babies (gestational ages at birth 31-40 weeks; 66% ±37 weeks) who participated in various studies on the effect of diet (i.e. breastmilk and formula with and without LCP) on LCP status. Their RBC FA were determined around postnatal days 11, 21 and 42 [21-23]. The 7 healthy Dominica mothers and 6 of their healthy babies (cord blood) were studied at delivery. These exclusively breast-fed infants were also investigated in the 20-22 days postnatal period [24]. The group of healthy breast-fed Jerusalem infants (ages 1
Table 1. Erythrocyte fatty acids and ratios.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Omnivorous group</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age &lt;0.2 years</td>
<td>119</td>
<td>3.38</td>
<td>14.61</td>
<td>2.52</td>
<td>1.01</td>
<td>5.58</td>
<td>0.83</td>
<td>0.19</td>
<td>2.68</td>
<td>0.41</td>
<td></td>
</tr>
<tr>
<td>Dominica newborns</td>
<td>6</td>
<td>6.87</td>
<td>13.10</td>
<td>2.90</td>
<td>1.12</td>
<td>4.06</td>
<td>0.60</td>
<td>0.28</td>
<td>3.38</td>
<td>0.38</td>
<td></td>
</tr>
<tr>
<td>Dutch LBW infants</td>
<td>68</td>
<td>3.38 (0.003-0.003)</td>
<td>4.06 (0.01-0.04)</td>
<td>4.48 (0.05-0.06)</td>
<td>4.74 (0.17-0.26)</td>
<td>5.90 (0.17-0.26)</td>
<td>5.90 (0.17-0.26)</td>
<td>5.90 (0.17-0.26)</td>
<td>5.90 (0.17-0.26)</td>
<td>5.90 (0.17-0.26)</td>
<td>5.90 (0.17-0.26)</td>
</tr>
<tr>
<td>Dominica infants</td>
<td>6</td>
<td>4.06 (0.05-0.07)</td>
<td>4.48 (0.05-0.07)</td>
<td>4.74 (0.05-0.07)</td>
<td>5.90 (0.17-0.26)</td>
<td>5.90 (0.17-0.26)</td>
<td>5.90 (0.17-0.26)</td>
<td>5.90 (0.17-0.26)</td>
<td>5.90 (0.17-0.26)</td>
<td>5.90 (0.17-0.26)</td>
<td>5.90 (0.17-0.26)</td>
</tr>
<tr>
<td>Dutch LBW infants + Dominica infants</td>
<td>61</td>
<td>4.06 (0.05-0.05)</td>
<td>4.48 (0.05-0.05)</td>
<td>4.74 (0.05-0.05)</td>
<td>5.90 (0.17-0.26)</td>
<td>5.90 (0.17-0.26)</td>
<td>5.90 (0.17-0.26)</td>
<td>5.90 (0.17-0.26)</td>
<td>5.90 (0.17-0.26)</td>
<td>5.90 (0.17-0.26)</td>
<td>5.90 (0.17-0.26)</td>
</tr>
<tr>
<td>Age &gt;=0.2 years</td>
<td>128</td>
<td>8.37</td>
<td>14.64</td>
<td>2.76</td>
<td>0.78</td>
<td>4.61</td>
<td>0.27</td>
<td>0.17</td>
<td>3.02</td>
<td>0.29</td>
<td></td>
</tr>
<tr>
<td>Jerusalem</td>
<td>19</td>
<td>8.37 (0.21-0.50)</td>
<td>14.64 (0.21-0.50)</td>
<td>2.76 (0.21-0.50)</td>
<td>0.78 (0.21-0.50)</td>
<td>4.61 (0.21-0.50)</td>
<td>0.27 (0.21-0.50)</td>
<td>0.17 (0.21-0.50)</td>
<td>3.02 (0.21-0.50)</td>
<td>0.29 (0.21-0.50)</td>
<td></td>
</tr>
<tr>
<td>Dutch 3.5 years old infants</td>
<td>33</td>
<td>10.10</td>
<td>14.37</td>
<td>2.95</td>
<td>0.66</td>
<td>2.87</td>
<td>0.27</td>
<td>0.23</td>
<td>4.91</td>
<td>0.23</td>
<td></td>
</tr>
<tr>
<td>Dutch omnivorous adults + Dominica mothers</td>
<td>69</td>
<td>10.10 (3.48-3.53)</td>
<td>14.37 (3.48-3.53)</td>
<td>2.95 (3.48-3.53)</td>
<td>0.66 (3.48-3.53)</td>
<td>2.87 (3.48-3.53)</td>
<td>0.27 (3.48-3.53)</td>
<td>0.23 (3.48-3.53)</td>
<td>4.91 (3.48-3.53)</td>
<td>0.23 (3.48-3.53)</td>
<td></td>
</tr>
<tr>
<td>Onion group</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age &lt;0.2 years</td>
<td>243</td>
<td>5.76</td>
<td>13.74</td>
<td>3.02</td>
<td>1.19</td>
<td>3.91</td>
<td>0.69</td>
<td>0.31</td>
<td>3.59</td>
<td>0.39</td>
<td></td>
</tr>
<tr>
<td>Dutch LBW infants</td>
<td>81</td>
<td>5.76 (0.02-0.04)</td>
<td>13.74 (0.02-0.04)</td>
<td>3.02 (0.02-0.04)</td>
<td>1.19 (0.02-0.04)</td>
<td>3.91 (0.02-0.04)</td>
<td>0.69 (0.02-0.04)</td>
<td>0.31 (0.02-0.04)</td>
<td>3.59 (0.02-0.04)</td>
<td>0.39 (0.02-0.04)</td>
<td></td>
</tr>
<tr>
<td>Dutch LBW infants</td>
<td>81</td>
<td>9.10</td>
<td>12.58</td>
<td>2.91</td>
<td>1.13</td>
<td>3.65</td>
<td>0.61</td>
<td>0.32</td>
<td>3.55</td>
<td>0.39</td>
<td></td>
</tr>
<tr>
<td>Dutch LBW infants</td>
<td>81</td>
<td>9.10 (0.02-0.04)</td>
<td>12.58 (0.02-0.04)</td>
<td>2.91 (0.02-0.04)</td>
<td>1.13 (0.02-0.04)</td>
<td>3.65 (0.02-0.04)</td>
<td>0.61 (0.02-0.04)</td>
<td>0.32 (0.02-0.04)</td>
<td>3.55 (0.02-0.04)</td>
<td>0.39 (0.02-0.04)</td>
<td></td>
</tr>
<tr>
<td>Age &gt;=0.2 years</td>
<td>12</td>
<td>10.23</td>
<td>13.75</td>
<td>2.75</td>
<td>0.51</td>
<td>3.89</td>
<td>0.25</td>
<td>0.13</td>
<td>3.67</td>
<td>0.18</td>
<td></td>
</tr>
<tr>
<td>Dutch vegan adults</td>
<td>12</td>
<td>10.23 (19-61)</td>
<td>13.75 (19-61)</td>
<td>2.75 (19-61)</td>
<td>0.51 (19-61)</td>
<td>3.89 (19-61)</td>
<td>0.25 (19-61)</td>
<td>0.13 (19-61)</td>
<td>3.67 (19-61)</td>
<td>0.18 (19-61)</td>
<td></td>
</tr>
<tr>
<td>EFAD suspected group</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age &gt;=0.2 years</td>
<td>108</td>
<td>8.66</td>
<td>14.38</td>
<td>2.89</td>
<td>1.03</td>
<td>2.47</td>
<td>0.39</td>
<td>0.43</td>
<td>5.66</td>
<td>0.36</td>
<td></td>
</tr>
<tr>
<td>Pakistani infants</td>
<td>108</td>
<td>8.66 (25-57)</td>
<td>14.38 (25-57)</td>
<td>2.89 (25-57)</td>
<td>1.03 (25-57)</td>
<td>2.47 (25-57)</td>
<td>0.39 (25-57)</td>
<td>0.43 (25-57)</td>
<td>5.66 (25-57)</td>
<td>0.36 (25-57)</td>
<td></td>
</tr>
</tbody>
</table>
Table 1. Erythrocyte fatty acids and ratios.

<table>
<thead>
<tr>
<th>Pediatric patients</th>
<th>n</th>
<th>Age (years)</th>
<th>18:2ω6 (mol%)</th>
<th>20:4ω6 (mol%)</th>
<th>22:4ω6 (mol%)</th>
<th>22:5ω6 (mol%)</th>
<th>22:6ω3 (mol%)</th>
<th>20:3ω9 (mol/mol)</th>
<th>22:5ω6/22:6ω3 (mol/mol)</th>
<th>20:4ω6/22:6ω3 (mol/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jejunal atresia with apple peel syndrome</td>
<td>1</td>
<td>0.67</td>
<td>4.58</td>
<td>13.12</td>
<td>2.02</td>
<td>1.78</td>
<td>1.35</td>
<td>3.14</td>
<td>1.32</td>
<td>9.72</td>
</tr>
<tr>
<td>Abetalipoproteinemia</td>
<td>1</td>
<td>5</td>
<td>2.22</td>
<td>12.34</td>
<td>4.38</td>
<td>1.87</td>
<td>1.43</td>
<td>2.23</td>
<td>1.31</td>
<td>8.63</td>
</tr>
<tr>
<td>Biliary atresia</td>
<td>1</td>
<td>0.67</td>
<td>8.70</td>
<td>11.85</td>
<td>3.24</td>
<td>1.23</td>
<td>0.96</td>
<td>0.56</td>
<td>1.28</td>
<td>12.34</td>
</tr>
<tr>
<td>Peroxisomal disorder</td>
<td>1</td>
<td>1.5</td>
<td>11.53</td>
<td>12.79</td>
<td>2.65</td>
<td>0.65</td>
<td>0.69</td>
<td>0.35</td>
<td>0.94</td>
<td>18.54</td>
</tr>
</tbody>
</table>

Ages are medians (range), Erythrocyte fatty acids are medians (2.5-97.5 percentiles). LBW, low-birth-weight; LCP, long chain polyunsaturated fatty acids; EFAD, essential fatty acid deficiency. 1, Human milk or formula+LCP fed infants; 2, formula (without LCP) fed infants; 3, data for n=11 (one outlier excluded). One Pakistani infant aged <0.2 years is not included in this Table. The cut-off value for EFAD was established by taking the mean of the 97.5 percentiles of the Jerusalem infants >=0.2 years and Dutch 3.5 years old infants. Two cut-off values were established for RBC 22:5ω6/22:6ω3, 20:4ω6/22:6ω3 and 22:5ω6/22:4ω6. The first was defined as the border between ω3-sufficiency and ω3-marginality and derived from the mean 97.5 percentiles of the Jerusalem infants and the Dutch omnivorous adults plus Dominica mothers. The second was defined as the border between ω3-marginality and ω3-deficiency and derived from the 97.5 percentile(s) of the entire low-dietary-LCP group (RBC 22:5ω6/22:6ω3) or of the several low-dietary-LCP groups (RBC 20:4ω6/22:6ω3 and 22:5ω6/22:4ω6).
6 months) were recruited from the ‘Spafford mother and child health clinic’ in East-Jerusalem (unpublished data). The 3.5 years old healthy Dutch children participated in a study on the effect of perinatal exposure to PCB’s and dioxins on neurological development [25]. The adult Dutch vegans and 15 of the adult omnivores participated in a study on the LCP status of the vegan diet [26]. The remaining 54 adult Dutch omnivores donated blood for the establishment of RBC FA reference values (unpublished results).

The EFAD and/or ω3-deficient groups were composed of 109 Pakistani infants and 4 pediatric patients. The, mostly malnourished, Pakistani infants (ages 2-60 months) were recruited from the Federal Government Service Hospital in Islamabad [27,28]. They were classified as malnourished according to the Gomez classification, i.e. a weight below 75% of average for age, using the reference data from the United States National Center for Health Statistics. Of these children, 58.7% were breast-fed (70.3% malnourished, 29.7% not malnourished) and 38.5% were formula-fed (85.7% malnourished, 14.3% not malnourished). Nutritional status of 2.8% was unknown. Three pediatric patients suspected of EFAD and/or ω3-deficiency were diagnosed with abetalipoproteinemia (genetically confirmed), jejunal atresia (with apple peel syndrome) and biliary atresia. The other patient was diagnosed with a peroxisomal disorder, characterized by increased plasma 26:0/22:0 ratio, pipecolic acid concentration and phytanic acid.

Approval for the intervention studies with LBW babies was obtained from the medical ethical committee of the Groningen Martini Hospital. The study protocols of the 3.5 years old Dutch children and omnivorous and vegan Dutch adults were approved by the medical ethical committee of the Groningen University Hospital. All study protocols were in agreement with local ethical standards and the Helsinki declaration of 1975, as revised in 2000.

4.1.2.2 Blood sampling, processing and analyses

EDTA-anticoagulated blood samples were collected by venepuncture and immediately cooled in melting ice. The samples were centrifuged at 800 g for 10 min in a cooled centrifuge. Plasma and buffy coat were removed and the RBC washed three times with isotonic saline. The RBC were finally suspended to a hematocrit of about 50%. For the analysis of RBC FA, 200 µl of this suspension was transferred to a 15-ml Teflon-stoppered tube, containing 1 mg butylated hydroxytoluene (antioxidant) and 50.0 µg margaric acid (17:0; internal quantification standard). Preserved RBC samples were stored at –20 °C. The samples from Dominica, Jerusalem and Pakistan were transported to The Netherlands in dry ice. FA methyl esters were prepared by acid-catalyzed transmethylation. They were separated by gas chromatography on an apolar capillary column and detected with a flame ionization detector [29]. The RBC FA composition was calculated by assuming that equal peak areas give rise to equal weight amounts [30]. Data were expressed as mol% (FA composition) or mol/mol (FA ratios). The within-run and day-to-day precisions for 15 RBC FA have been described in detail [29]. They vary characteristically between 1.9-12.0% and 1.1-17.6%, dependent on FA abundance. The between-series precisions (in %) of some of the presently evaluated RBC FA amount to: 3.7 (18:2ω6), 5.3 (20:4ω6), 8.0 (22:5ω3) and 14.9 (22:6ω3).
RBC FA data of the apparently healthy omnivorous and low-dietary-LCP groups (Table 1) were used for establishment of cut-off values. Age-dependency was investigated by Spearman rank test at p<0.05. Cut-off values were defined as the 97.5 percentiles (P97.5), according to recommendations of the International Federation of Clinical Chemistry [31]. The cut-off value for RBC 20:3ω9 served for classification into EFA sufficient (≤P97.5) and EFAD (>P97.5). The RBC 22:5ω6/22:6ω3 20:4ω6/22:6ω3 and 22:5ω6/22:4ω6 were used for classification into ω3-sufficient (≤P97.5 of omnivorous group), ω3-marginal (>P97.5 of omnivorous group but ≤P97.5 of low-dietary LCP group) and ω3-deficient (>P97.5 of low-dietary LCP group). These cut-off values were validated in the Pakistani children and 4 pediatric patients by their classification as EFAD+ω3-deficient, EFAD+ω3-marginal, EFAD+ω3-sufficient, EFA-sufficient+ω3-deficient, EFA-sufficient+ω3-marginal or EFA-sufficient+ω3-sufficient.

### 4.1.3. Results

#### 4.1.3.1 RBC 20:3ω9 and 22:5ω6/22:6ω3 cut-off values for EFA and ω3-status

RBC 20:3ω9 of all subjects, Dominica newborns excluded, proved age-dependent (r=-0.709, p<0.0001, Figure 1 top), but became age-independent from 0.2 years. Since the P97.5 of the four subgroups aged ≥0.2 years seemed age-dependent (Table 1), we decided to take the average of the Jerusalem infants and Dutch 3.5 years old infants to find a value of 0.46 mol% as the upper limit of EFA sufficiency for all subjects aged ≥0.2 years. The RBC 22:5ω6/22:6ω3 of the omnivorous group, Dominica newborns excluded, was age-dependent (r=-0.668, p<0.0001, Figure 1 bottom). The RBC 22:5ω6/22:6ω3 of the omnivorous group was still age-dependent beyond the age of 0.2 years (r= -0.546, p<0.0001), but the P97.5 values of the Jerusalem infants and the Dutch omnivorous adults plus Dominica mothers proved remarkably similar (Table 1). Despite the higher P97.5 of the Dutch 3.5 years old infants (see Discussion), we decided to take the average of the Jerusalem infants ≥0.2 years and the Dutch omnivorous adults plus Dominica mothers to find a value of 0.22 mol/mol as the upper limit of ω3-sufficiency for subjects aged ≥0.2 years. The RBC 22:5ω6/22:6ω3 of the low-dietary-LCP group was age-independent (Figure 1 bottom) and its 4 subgroups had similar P97.5 values (Table 1). We therefore decided to average their P97.5 to find a value of 0.48 mol/mol as the upper limit of ω3-marginality for all ages.

#### 4.1.3.2 Validation of RBC 20:3ω9 and 22:5ω6/22:6ω3 cut-off values

The 0.46 mol% RBC 20:3ω9 cut-off value for EFAD and the 0.22 and 0.48 mol/mol 22:5ω6/22:6ω3 cut-off values for ω3-marginality and ω3-deficiency were evaluated by investigating their influence on the classification of all subjects aged ≥0.2 years, i.e. 140 apparently healthy controls, 108 of the 109 malnourished Pakistani children and the four pediatric patients (Figure 2). Because of the definition of the cut-off values at a P97.5, it is obvious that 2.5 percent of several apparently healthy subgroups were classified as EFAD,
Figure 1. ■, Omnivores; □, Low-dietary-LCP group; ●, Pakistan EFAD+ω3-deficient; ▲, idem +ω3-marginal; ●, idem +ω3-sufficient; ○, Pakistan EFA-sufficient +ω3-deficient; Δ, idem +ω3-marginal; ◊, idem +ω3-sufficient; *, Patients.

Erythrocyte 20:3ω9 (top) and 22:5ω6/22:6ω3 (bottom) as a function of age. RBC, erythrocyte; EFA, essential fatty acid; EFAD, EFA deficiency. RBC 20:3ω9 is a marker for EFA status and RBC 22:5ω6/22:6ω3 is a marker of ω3 status. Notice the log scale of the x-axis. For subject numbers in subgroups see Table 1. RBC 20:3ω9 and 22:5ω6/22:6ω3 of the omnivorous group became age-independent from 0.2 years. RBC 22:5ω6/22:6ω3 of the low-dietary-LCP group was age-independent. For subjects aged ≥0.2 years, the RBC 20:3ω9 cut-off value for EFAD was 0.46 mol%; their RBC 22:5ω6/22:6ω3 cut-off values for ω3-marginality and deficiency were 0.22 and 0.48 mol/mol, respectively. For cut-off values of subjects <0.2 years, see Table 1. The arrow indicates a Pakistani child aged <0.2 years, whose data were not used for the evaluation of the cut-off values. For diagnosis of patients 1-4, see Table 1.

124
Figure 2. Classification of controls and malnourished Pakistani infants according to EFA and ω3 status.

EFA, essential fatty acid; EFAD, EFA deficiency. All subjects were above 0.2 years of age. For subject numbers in subgroups see Table 1. The applied cut-off values were 0.46 mol% RBC 20:3ω9 for EFAD, 0.22 mol/mol RBC 22:5ω6/22:6ω3 for ω3-marginality and 0.48 mol/mol RBC 22:5ω6/22:6ω3 for ω3-deficiency (see Figure 1). The distribution of the Pakistani infants was: 15.7% EFAD+ω3-deficient, 16.7% EFAD+ω3-marginal, 0.9% EFAD+ω3-sufficient, 19.5% EFA-sufficient+ω3-deficient, 43.5% EFA-sufficient+ω3-marginal, and 3.7% EFA-sufficient+ω3-sufficient.

ω3-marginal or ω3-deficient. Of the 108 Pakistani infants 33.3% were found to be EFAD and 66.7% EFA-sufficient. Thirtyfive (35.2) percent were ω3-deficient, 60.2% ω3-marginal and 4.6% ω3-sufficient. When combined they were classified as 15.7% EFAD+ω3-deficient, 16.7% EFAD+ω3-marginal, 0.9% EFAD+ω3-sufficient, 19.5% EFA-sufficient+ω3-deficient, 43.5% EFA-sufficient+ω3-marginal and 3.7% EFA-sufficient+ω3-sufficient. The RBC 20:3ω9 and 22:5ω6/22:6ω3 data of the four pediatric patients suspected of EFAD/ω3-deficiency are shown in Table 1 and Figure 1. The three patients with chronic fat-malabsorption had both increased 20:3ω9 and 22:5ω6/22:6ω3, indicating EFAD+ω3-deficiency. The patient with the peroxisomal disorder was classified as EFA sufficient+ω3-deficient, with a 20:3ω9 below the cut-off value in combination with increased 22:5ω6/22:6ω3, due to very low 22:6ω3.
4.1.3.3  \textit{RBC 20:4\omega6/22:6\omega3 and 22:5\omega6/22:4\omega6 for \omega3-status}

Figure 3 illustrates the values of 20:4\omega6/22:6\omega3 (top) and 22:5\omega6/22:4\omega6 (bottom) for establishment of \omega3-status. The P97.5 of RBC 20:4\omega6/22:6\omega3 and 22:5\omega6/22:4\omega6 for the omnivorous and low-dietary-LCP groups proved age-dependent beyond the age of 0.2 years, except for the 22:5\omega6/22:4\omega6 of omnivores. The cut-off values of RBC 20:4\omega6/22:6\omega3 and 22:5\omega6/22:4\omega6 were, analogous to 22:5\omega6/22:6\omega3, based on the P97.5 of the Jerusalem infants ≥0.2 years and the Dutch omnivorous adults plus Dominica mothers (upper limit of \omega3-sufficiency) and the P97.5 of the four low-dietary-LCP subgroups (upper limit of \omega3-marginality). Interconnection of these cut-off values allowed classification of the Pakistani children and the four patients by visual inspection. It showed that the use of RBC 20:4\omega6/22:6\omega3 and 22:5\omega6/22:4\omega6 caused a shift towards lower prevalence of \omega3-deficiency and higher prevalence of \omega3-marginality and \omega3-sufficiency in the Pakistani group. With 20:4\omega6/22:6\omega3 cut-off values the distribution became 14.7% \omega3-deficient, 75.2% \omega3-marginal and 10.1% \omega3-sufficient and with 22:5\omega6/22:4\omega6 cut-off values it became 0.9% \omega3-deficient, 68.8% \omega3-marginal and 30.3% \omega3-sufficient. Three of the 4 pediatric patients were classified as \omega3-deficient and one as \omega3-marginal (abetalipoproteinemia) with use of RBC 20:4\omega6/22:6\omega3, whereas use of RBC 22:5\omega6/22:4\omega6 cut-off values classified one patient (jejunal atresia) as \omega3-deficient, two patients as \omega3-marginal and one patient (peroxisomal disorder) as \omega3-sufficient.

4.1.4. \textit{Discussion}

Suspicion of low EFA or \omega3 FA status may rather focus on clinical chemical tests than the presence of clinical symptoms. Early diagnosis is important, since it is increasingly recognized that subclinical micronutrient deficiencies may cause disease in the long run. We established cut-off values for RBC 20:3\omega9 and 22:5\omega6/22:6\omega3 for establishment of biochemical EFAD, \omega3-marginality and \omega3-deficiency and investigated the added value of 20:4\omega6/22:6\omega3 and 22:5\omega6/22:4\omega6 as parameters of \omega3 status. The cut-off values are based on the 97.5 percentiles of two major groups of apparently healthy subjects, who either consumed diets with LCP (breast-fed babies, babies receiving LCP-enriched formula, omnivorous infants and adults) or diets with very little LCP (babies receiving formula without LCP and vegans). Recruitment of healthy LBW, predominantly preterm, babies for establishment of cut-off values may be questioned. However, preterm and term babies have similar RBC LCP contents at birth and do not exhibit major differences in postnatal RBC LCP courses upon the same feeding regimen [6]. Newborns are known to have high 20:3\omega9 and 22:5\omega6/22:6\omega3 [32], and it was found that both the postnatal decline of 20:3\omega9 and 22:5\omega6/22:6\omega3 in omnivorous subjects reached stable levels from the age of about 0.2 years (2.4 months). No 22:5\omega6/22:6\omega3 decrease seems to take place when the diet is virtually devoid of LCP, since infants who received formula without LCP and adult vegans had remarkably similar 22:5\omega6/22:6\omega3 ratios (Table 1). We therefore defined two levels for \omega3-status, i.e. one based on the RBC 22:5\omega6/22:6\omega3 P97.5 of omnivores (upper limit of \omega3-sufficiency) and a second based on the P97.5 of formula-fed infants and vegans (upper limit \omega3-marginality). The term ‘marginality’ was introduced to indicate that there is no evidence that the well-known low \omega3-status of vegans [26] should be regarded as a state of
Figure 3. ■, Omnivores; □, Low-dietary-LCP group; ●, Pakistan EFAD+ω3-deficient; ▲, idem +ω3-marginal; ○, idem +ω3-sufficient; ◊, Pakistan EFA-sufficient +ω3-deficient; △, idem +ω3-marginal; ◊, idem +ω3-sufficient; *, Patients.

Erythrocyte 20:4ω6/22:6ω3 (top) and 22:5ω6/22:4ω6 (bottom) as a function of age. RBC, erythrocyte; EFA, essential fatty acid; EFAD, EFA deficiency. RBC 20:4ω6/22:6ω3 and 22:5ω6/22:4ω6 are markers of ω3 status. Notice the log scale of the x-axis. for subject numbers in subgroups see Table 1. Cut-off values for both 20:4ω6/22:6ω3 and 22:5ω6/22:4ω6 were age-dependent beyond the age of 0.2 years, except for the 22:5ω6/22:4ω6 cut-off value for omnivores. Classification of the ω3-status of the Pakistani infants by symbol is based on the 22:5ω6/22:6ω3 cut-off value (see Figure 1). Application of the 20:4ω6/22:6ω3 and 22:5ω6/22:4ω6 cut-off values in the Pakistani group gave rise to a shift towards lower prevalence of ω3-deficiency and higher prevalence of ω3-marginality and ω3-sufficiency compared with the use of the 22:5ω6/22:6ω3 cut-off value. The arrow indicates a Pakistani child aged <0.2 years, whose data were not used for the evaluation of the cut-off values. For diagnosis of patients 1-4, see Table 1.
ω3-deficiency. Data of the 3.5 years old Dutch infants were not used for the calculation of the upper limit of ω3-sufficiency. These children exhibited expected higher RBC 22:5ω6/22:6ω3 P97.5, compared with the Jerusalem infants ≥0.2 years and the Dutch omnivorous adults plus Dominica mothers (Table 1). Fish intake of 1-4 years old Dutch children is known to be 70% lower than that of Dutch adults on a g/kcal/day basis [33] and many of them should probably be regarded to consume a vegan diet with regard to LCP ω3 intake.

The finally selected cut-off values for 20:3ω9 (i.e. 0.46 mol%) and 22:5ω6/22:6ω3 (i.e. 0.22 and 0.48 mol/mol, Figure 1) cannot be easily validated, since there is no 'gold standard' for the diagnosis of EFAD or ω3-deficiency. We therefore decided to validate these outcomes in, mostly malnourished, North-Pakistani infants who are known to have very low intakes of vegetable oils and fish, causing high incidences of EFAD and ω3-deficiency with occasional symptoms consistent with these conditions [27,34]. It was found that the encountered high percentages EFAD, ω3-marginality and ω3-deficiency are indeed consistent with their diets (Figure 2). Malnutrition is, however, by far more complex than EFAD alone, and we felt that current cut-off values required further confirmation in non-malnutrition cases of EFAD and ω3-deficiency. It was found that the cut-off values enabled detection of EFAD and ω3-deficiency in patients with inherited disfunctional chylomicron assembly (abetalipoproteinemia) and congenital atresia of the jejunum and biliary ducts. The patient with the peroxisomal disorder had high 22:5ω6/22:6ω3, due to very low 22:6ω3. The low 22:6ω3 levels of patients with peroxisomal disorders are considered to derive from their low 24:6ω3 to 22:6ω3 retroconversion capacity, due to insufficient peroxisomal β-oxidation [35]. The value of the present cut-off values should however be investigated more closely in larger groups of patients with miscellaneous causes of chronic fat-malabsorption (e.g. cystic fibrosis, liver disease), increased EFA demand (cancer, trauma) and inborn errors that affect EFA-metabolism (such as the Zellweger’s syndrome).

We used the same method for the calculation of cut-off values for RBC 20:4ω6/22:6ω3 and 22:5ω6/22:4ω6 as employed for 22:5ω6/22:6ω3. We subsequently compared the added value of the former two ratios to detect low ω3 status in the Pakistani children and the 4 pediatric patients. It was found that RBC 20:4ω6/22:6ω3 is age-dependent beyond the age of 0.2 years and that this ratio is also less sensitive for the detection of ω3-deficiency in the Pakistani children and pediatric patients, compared with 22:5ω6/22:6ω3. The RBC 22:5ω6/22:4ω6 ratio on its turn was age-independent from 0.2 years in omnivores, but proved less sensitive for the detection of low ω3-status compared with both the 22:5ω6/22:6ω3 and 20:4ω6/22:6ω3 ratios in the Pakistani children and the pediatric patients. Low sensitivity of the 22:5ω6/22:4ω6 ratio is disappointing, since analogous to 20:3ω9 for EFAD, this ratio may be regarded as a ‘functional’ marker [36] that detects competition between FA of the ω3 and ω6 series in favor of ω6. Moreover, the intakes of both 22:5ω6 and 22:4ω6 from the diet are probably very low, which leaves this ratio to be a closer reflection of enzymatic activity than ratios that contain potentially diet-derived LCP such as 22:6ω3 and 20:4ω6. The most plausible explanation for the discrepancy is that each of the presently investigated ratios reflects different aspects of ω3 status. Increased 22:5ω6/22:6ω3 and increased 20:4ω6/22:6ω3 may both predominantly indicate low dietary 22:6ω3 intake and/or absorption. Increased 22:5ω6/22:4ω6 may predominantly point at low status of ω3 FA up to 22:5ω3, collectively referred to as 22:6ω3 precursors. We propose

The two groups of Dutch infants with ages below 0.2 years exhibited deviating 22:5ω6/22:6ω3 cut-off-values with advancing age (Table 1; Figure 1 bottom). Their deviation with time is due to the intake of 22:6ω3 from breastmilk or LCP-enriched formula, and the lack of dietary 22:6ω3 intake by feeding formula without LCP [6,37]. There is evidence that enrichment of formula with 22:6ω3 improves early visual development, notably in premature infants [37,38]. This effect is transient, but has nevertheless been the basis to add 22:6ω3 to formula for premature infants in many countries. Enrichment of formulas for term infants is also gaining increasing support. Whether 0.01-0.17 years (0.5-2.0 months) old infants with RBC 22:5ω6/22:6ω3 ratios in the about 0.43-0.48 mol/mol range should be classified as ω3-marginal, or even ω3-deficient, seems therefore rather a matter of opinion than a scientifically proven fact. It is in this context also interesting to point at the very low cord blood RBC 22:5ω6/22:6ω3 ratio of the breast-fed Dominica newborns (median 0.19; P97.5 0.26 mol/mol) (Figure 1 bottom; Table 1), with similarly low 22:5ω6/22:6ω3 around postnatal day 21 (0.19; 0.27 mol/mol; data not shown in Table 1) and the even lower 22:5ω6/22:6ω3 of their mothers (0.09; 0.16 mol/mol; data not shown in Table 1). The Dominica mothers were known to have high fish intake and consequently high ω3-status, as also witnessed by their high breastmilk 22:6ω3 contents [24]. In contrast, the much higher RBC 22:5ω6/22:6ω3 of Dutch formula- and breast-fed infants and Dutch adults, is probably related to the typical North-European diet with low intake of fish and high dietary 18:2ω6/18:3ω3 ratio due to the predominant use of 18:2ω6-rich oils [39]. Intake of both fish and 18:3ω3 have been associated with lower risk of coronary artery disease in omnivores [40,41], and it seems therefore attractive to lower the 22:5ω6/22:6ω3 cut-off level for ω3-marginality (for omnivorous subjects) to that of the 97.5 percentile of the Dominica infants and their mothers. Such a cut-off value would classify the majority of the Western subjects as ω3-marginal and thereby illustrates the difficulty to define a border between ω3-sufficiency and marginality. It seems that any cut-off value for ω3 or 22:6ω3 status assessments may eventually have to be based on hard clinical evidence and not on a 97.5 percentile of an apparently healthy omnivorous population with inherently high risk of cardiovascular disease.

In conclusion, we calculated cut-off values for assessment of biochemical EFA status by measurement of RBC 20:3ω9, RBC 22:5ω6/22:6ω3 and RBC 22:5ω6/22:4ω6. The cut-off values are based on 97.5 percentiles of apparently healthy populations and apply for subjects aged ≥0.2 years (2.4 months). They amount to 0.46 mol% RBC 20:3ω9 for EFAD, 0.22 mol/mol RBC 22:5ω6/22:6ω3 for ω3-marginality, 0.48 mol/mol RBC 22:5ω6/22:6ω3 for ω3-deficiency and 0.33 mol/mol RBC 22:5ω6/22:4ω6 for low status of ω3 fatty acids up to 22:5ω3. It is important to realize that values beyond these cut-off values have not been validated on the basis of clinically detectable symptoms, but that they rather point at...
states of altered substrate competition (20:3ω9, 22:5ω6/22:4ω6) and altered LCPω6/LCPω3 balance (22:5ω6/22:6ω3), which might be consistent with subclinical deficiencies or imminent clinical deficiencies. Employment of present cut-off values indicated high prevalence of biochemical EFAD and ω3-deficiency in mostly malnourished North-Pakistani infants with very low intakes of vegetable oils and fish, in three patients with chronic fat-malabsorption and in one patient with a peroxisomal disorder. In view of lack of toxicity, increasing concern of the (long-term) consequences of low micronutrient status and relatively low costs, we suggest to use these cut-off values, for the decision of dietary supplement intervention until better concepts have emerged.

Acknowledgments

We thank Prof. B. Steinmann and colleagues (Zurich University Children’s Hospital, Switzerland) for allowing us to study the patient with abetalipoproteinemia. Dr. M. Volmer is gratefully acknowledged for his aid in statistical analyses.

References

11. Holman RT. The ratio of trienoic:tetraenoic acids in tissue lipids as a measure of essential fatty acid requirement. J Nutr 1960;70:405-10