Long-chain polyunsaturated fatty acids profile in plasma phospholipids of hyperphenylalaninemic children on unrestricted diet

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Article history:
Received 3 May 2010
Received in revised form 26 August 2010
Accepted 17 September 2010

Keywords:
Docosahexaenoic acid
Hyperphenylalaninemia
Fatty acids
Phenylketonuria

1. Introduction

Hyperphenylalaninemia (HPA) is a disorder mostly caused by the deficiency in the hepatic enzyme phenylalanine hydroxylase (PAH; McKusick 261600), that converts dietary phenylalanine (Phe) to tyrosine [1]. The range of disease severity observed among patients with this form of HPA is mainly due to allelic heterogeneity at the PAH locus. Combinations of mutations result in a spectrum of metabolic phenotypes, ranging from phenylketonuria (PKU, blood Phe levels > 360 μmol/L), which require dietary management, to mild hyperphenylalaninaemia (MHP, blood Phe levels ranging 120–360 μmol/L), which may exhibit a different LCPUFA profile from PKU or healthy children in plasma phospholipids.

Patients and methods: Forty-five MHP children (age 9–14 years) were age and sex matched with 45 PKU and 45 healthy children. Fatty acids were determined and expressed as % of total fatty acids.

Results: MHP children showed docosahexaenoic acid (DHA) levels higher than PKU children (mean difference, 0.2%; 95% confidence interval, 0.02%–0.38%), although difference was not significant after correction for multiple comparisons, and lower levels than healthy children (-0.8%; -1.01% to -0.59%). Concentration of n-3 PUFAs was higher in MHP than PKU children (0.6%; 0.4% to 0.8%).

Conclusions: The results suggest that low DHA levels in plasma phospholipids not only are evident in PKU but also may occur in MHP children, who are on unrestricted diet, as compared to healthy children.

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profile, especially DHA and AA, different from PKU children and/or healthy children.

2. Patients and methods

This observational study examined 45 MHP children age and sex matched with 45 PKU and 45 healthy children, consecutively admitted to the Department of Paediatrics, San Paolo Hospital, Milan, from July 2008 to December 2009. Inclusion criteria were: age at recruitment 9–14 years, weight at birth \( \geq 2500 \) g, gestational age 37–42 week inclusive, singleton birth, having Caucasian parents. Exclusion criteria were: having diabetes mellitus, endocrine diseases, chronic liver diseases, overweight/obesity according to the International Obesity Task Force [12]. Children with hyperlipidemia, according to Italian guidelines [13], were further excluded to prevent possible bias effects on plasma fatty acid status [14]. PKU children non compliant with the recommended diet were also excluded.

HPA children were detected by newborn screening test and diagnosed with PKU or MHP according to a predefined protocol [1]. They are periodically monitored at our Department since diagnosis. PKU children started the dietary treatment within six weeks of life based on Phe-free protein substitutes and vegetables with low Phe content. MHP children were allowed to follow an unrestricted dietary regimen and recommended routine dietary advice as used for healthy children. Starting from diagnosis, blood phenylalanine children was monitored monthly by means of dried blood spots on card mailed by families using the Guthrie test. Compliance was evaluated according to age adjusted reference threshold values of plasma Phe as suggested by Scrivier et al. [1].

Fasting blood samples were taken both in HPA and healthy children in the morning at 8 h \( \pm 30 \) min, within 3 days of recruitment. Plasma total cholesterol and triacylglycerol were measured using a dry multiplayer enzymatic method (Ectachem DT-60; Eastman Kodak Co, Rochester, NY).

A paediatrician described the investigation to the parents or legal guardian, and they signed an informed consent form. The Ethical Committee of the San Paolo Hospital, Milan, approved the study.

2.1. Fatty acids in plasma phospholipids

Peripheral venous blood, drawn in fasting conditions, was collected in a tube with anticoagulant (sodium citrate) and centrifuged at 4000 [rpm] for 10 min. Plasma obtained was kept at to \(-20\) °C till the moment of the analysis. Total lipids were extracted from plasma according to Folch et al. [15] by the addition of water and chloroform–methanol (2:1), in presence of BHT (butyl-hydrossil-toluene, 5 μg/mL) as antioxidant. After separation of the wateory phase, containing the water-soluble compounds, from organic phase, containing all the liposoluble compounds, the organic phase was removed. The phospholipid fraction was separated by thin-layer chromatography. Fatty acids were weighed and methylated by the addition of methanol/HCl 3 N. The tubes were placed in heater at 90 °C for 1 h. After addition of C17:0 as internal standard for the quantitative gas-analysis and methylation, fatty acid methyl esters were separated through the addition of H₂O and n-hexane and analysed by gas-capillary chromatography (serious HRGC Mega 2 Fisons fortified of FID, flame ionization detector). Separation of fatty acids of chain length in the range of 14 and 24 carbon atoms was performed by means of an Omegawax 320 capillary column (30 m, 0.32 mm i.D., 0.25 μm film thickness) with a temperature gradient from 60 to 150 °C to 10 °C/min, from 150 to 170 °C to 5 °C/min, from 170 to 230 °C to 2 °C/min. The mobile phase was made up by the inert gas helium. Methyl esters were identified by comparison with retention times of standard methyl esters (SIGMA, cod.189-19). The integration of chromatographic peaks was carried out through the employment of a dedicated software (Chromcard version 1.19). Fatty acids were then expressed as weight percentage.

2.2. Dietary assessment

Dietary intakes were calculated at recruitment through a weighed food diary of 3 days for MHP and healthy children, and by means of the individual dietary schedules (exactly indicating the daily amounts of allowed low-Phe foods and formulas) for PKU children. Although a 3-days food diary may be not the optimal procedure for representing long periods of child dietary intake, however it has been used extensively in the literature and recognized to be adequately reliable [16]. The same experienced dietician, unaware of the child, examined the dietary records. The intake of macronutrients and fatty acids were derived from the Italian Food composition tables [17].

2.3. Statistical analysis

The sample size was determined to detect a difference of at least 20% in the mean level of DHA between MHP and PKU children. Assuming in the PKU children a mean (SD) DHA level of 1.7 (0.4)% [6] and admitting a type I error level of 0.01 with a power of 90%, at least 42 patients per group are required. The Kolmogorov–Smirnov test was used to assess normality of distribution of continuous variables. Descriptive data are reported as mean (SD) or number of observations (percentage). Comparison among groups for continuous variables was performed by one-way ANOVA or the Kruskal–Wallis test, as appropriate. The Pearson’s \( r^2 \) or the Fisher’s exact test or the Kruskal–Wallis test, as appropriate, was used for comparing discrete variables. Significance of multiple comparisons was adjusted on the Bonferroni correction. All \( p \)-values less than 0.05 were considered to indicate statistical significance (two-tailed test). The SPSS software, version 15.0 (SPSS Inc, Chicago, IL) was used for all the statistical analyses.

3. Results

Out of the 135 participant children, 54 (18 per group) were girls and 81 (27 per group) boys, with a mean (SD) age of 12.3 (3.2) years. Mean (SD) blood phenylalanine ranged from 240 (130) to 470 (160) μmol/L in PKU children, and from 210 (103) to 330 (78) μmol/L in mild HPA children.

Plasma total cholesterol (mg/dl) differed among groups (\( p < 0.0001 \), with a mean (SD) value higher in MHP (157 [20]) and healthy (159 [21]) than PKU children (136 [15]) (\( p < 0.0001 \)). The mean (SD) triacylglycerol plasma level (mg/dl) was 87 (22), 94 (21) and 96 (20) in PKU, MHP and healthy children, respectively, and did not differ among groups (\( p = 0.107 \)).

Table 1 describes the daily nutrient intakes in HPA groups and healthy children. The three groups did not differ for the total energy intake. Compared to PKU, MHP children assumed higher protein (mean difference, \( \Delta 3.9\% \); 95% confidence interval, 95% CI, 2.9–5.0%) and lipid (2.0%; 0.3–3.7%) and lower carbohydrate (\(-7.1\%; -9.1\% \) to \(-5.2\%) MHP children showed higher intake of saturated fatty acids than PKU children (5.9%, 5.1–6.8%), and lower monounsaturated (\(-2.9\%; -3.3\% \) to \(-2.4\%) and polyunsaturated (\(-1.8; -2.1\% \) to \(-1.5\%) fatty acids. Cholesterol intake was 7.8 folds higher in MHP than PKU children (\( p < 0.0001 \)).
correction (0.02–0.38%) but it was no more significant after Bonferroni adjustment for multiple comparisons. The mean difference in DHA was no significant after correction for multiple comparisons.

In MHP children, a relationship was found between plasma phenylalanine concentration and DHA level in plasma phospholipids (Pearson’s correlation coefficient, r = 0.466, P = 0.039) but statistical significance of association was no more reached after adjustment for confounders (age, sex, BMI z-score, plasma total cholesterol and triacylglycerol) (P = 0.082).

### 4. Discussion

The main goal of the dietary treatment for PKU patients is to maintain plasma Phe levels within the safe range according to age [1]. Adequate control of blood phenylalanine may be effective in preventing central nervous system deficits associated with PKU [1]. Despite early and continuous treatment, PKU children may show reduced cognitive function compared with non-phenylketonuric siblings and/or matched healthy controls [18,19]. Indeed, while the dietary restriction in DHA may induce in PKU patients adverse effects on neural functions [3], it has been hypothesized a possible inhibitory effect of phenylalanine metabolites, especially phenylpyruvate and phenyllactate, on endogenous DHA synthesis [11] but at present there is lack of studies in humans assessing this issue. To clarify the possible role of hyperphenylalaninemia in determining the inhibition of LPCUFA synthesis it appears interesting to examine plasma DHA levels in HPA children on unrestricted dietary regimen. This may be remarkable especially in MHP children as they may exhibit a poor executive functioning and/or healthy children.

In this study, MHP children showed levels of n-3 PUFA and DHA, respectively, 22% and 12% higher than PKU children but difference in DHA was no significant after correction for multiple comparisons. Despite the daily nutrient intakes of MHP and healthy children were comparable for macronutrients and polyunsaturated fatty acids, MHP children exhibited significantly lower (around 30%) levels of DHA than healthy children. Compared to healthy controls, PKU children showed lower n-3 PUFA and DHA (23% and 37%, respectively) and higher 18:3n-3

### Table 2

| Daily nutrient intakes in HPA groups and in healthy children. Values are mean (SD)². |
|----------------------------------|----------------------------------|----------------------------------|----------------------------------|
|                                  | PKU children n = 45             | MHP children n = 45              | Healthy children n = 45          |
|                                  | Total (kcal) 2217 (391)         | 2252 (387)                       | 2261 (363)                       |
|                                  | Kcal /kg 70 (14)                | 73 (12)                          | 75 (12)                          |
|                                  | Protein (%) 9 (2)               | 13 (3)²                         | 14 (1)²                          |
|                                  | Carbohydrate (%) 64 (3)        | 57 (6)²                         | 56 (5)²                          |
|                                  | Lipid (%) 28 (3)               | 30 (5)²                         | 31 (4)²                          |
|                                  | Saturated (%) 7 (2)             | 13 (2)²                         | 13 (2)²                          |
|                                  | Monounsaturated (%) 15 (1)     | 12 (1)²                         | 12 (2)²                          |
|                                  | Polyunsaturated (%) 6 (1)      | 4 (1)²                           | 4 (1)²                           |
|                                  | 18:2n-6 (%) 5.1 (0.8)²         | 3 (1.2)²                         | 3.3 (0.4)²                       |
|                                  | 18:3n-3 (%) 0.5 (0.05)²        | 0.4 (0.1)²                       | 0.4 (0.1)²                       |
|                                  | Cholesterol (mg/d) 37 (6)      | 289 (59)²                        | 311 (62)²                        |

Different superscripts indicate significant difference between groups (P < 0.05) after the Bonferroni correction.

* Statistically significant.
² Values adjusted for age and sex.
² Significance of difference among groups.

### Table 2

| LPCUFA (%) in plasma phospholipids. Values are mean (SD)². |
|----------------------------------|----------------------------------|----------------------------------|
|                                  | PKU on diet n = 45              | MHP on free diet n = 45          | Healthy children n = 45          |
|                                  | Saturated 45.4 (2.9)³           | 48.4 (2.6)³                      | 48.7 (2.5)³                      |
|                                  | Monounsaturated 17.7 (1.5)      | 14.3 (1.6)³                      | 13.4 (1.2)³                      |
|                                  | Polyunsaturated 38.0 (2.4)      | 37.3 (2.3)                       | 37.9 (2.4)                       |
|                                  | 18:2n-6 (%) 20.7 (2.6)         | 20.9 (2.7)                       | 21.4 (2.5)                       |
|                                  | 20:4n-6 (%) 8.4 (1.5)          | 8.7 (1.4)                        | 8.9 (1.3)                        |
|                                  | n-6 PUFA 34.4 (2.8)           | 34.2 (2.6)                       | 34.2 (2.5)                       |
|                                  | 18:3n-3 (%) 0.15 (0.09)        | 0.13 (0.07)                      | 0.11 (0.04)                      |
|                                  | 22:6n-3 (%) 1.7 (0.4)²         | 1.9 (0.5)²                       | 2.7 (0.5)²                       |
|                                  | n-3 PUFA 2.7 (0.6)²            | 3.3 (0.5)²                       | 3.5 (0.5)²                       |

Different superscripts indicate significant difference between groups (P < 0.05) after the Bonferroni correction.

* Statistically significant.
² Values adjusted for age, sex, BMI z-score, plasma total cholesterol and triacylglycerol.
² Significance of difference among groups.
(36%), that is in accordance with previous studies [4–6]. These results suggest that independently of the dietary regimen, MHP children may exhibit DHA levels in plasma phospholipids not appreciably different from PKU children, but worse than in healthy subjects. Additionally, the weak relationship found between plasma phenylalanine concentrations and DHA levels in MHP children might suggest a putative toxic effect of phenylalanine metabolites in disturbing fatty acid metabolism, as found in non-humans studies [11]. Trials aimed to assess the possible role of phenylalanine metabolites in disturbing fatty acid metabolism would be desirable.

Caution has to be paid in inferring this conclusion to the general HPA population, due to some limitations. Firstly, this study was not able to estimate the dietary intake of single fatty acids and then to assess the underlying potential relationship between dietary intakes and plasma fatty acids profile. Indeed, this would be an important issue to investigate as it may be not excluded that plasma fatty acids levels may relate to dietary intake and biologic processing of fats. Another limitation is that recording a 3-days food diary may be not an optimally accurate procedure for representing long-term dietary intake of fatty acids in MHP or healthy children. However, adoption of this approach did not introduce possibly in this study a major bias, as crucial results were based on plasma fatty acids.

On the whole, within the limitations of the present study, one can conclude that low DHA levels in plasma phospholipids not only are evident in PKU but also may occur in MHP children, who are on unrestricted diet, as compared to healthy children. Additional investigations need to assess and elucidate the underlying biochemical mechanism and pathways involved in regulating the fatty acids profile in the HPA population. Lastly, since adequate levels of DHA have been recognized to affect positively the neural function in healthy subjects, randomised adequately powered trials should be conducted to evaluate the potential role of a DHA supplementation on neural outcomes of hyperphenylalaninemic subjects, independently of dietary regimen.

References


