Fatty acid and phospholipase A$_2$ plasma levels in children with autism

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Abstract
Objective: To evaluate fatty acid plasma levels, phospholipase A$_2$ activity, and the developmental profiles of children with autism vs. control subjects.
Methods: Twenty four children with autism underwent laboratory analysis for fatty acid quantification using gas chromatography and PLA$_2$ activity determination by fluorometric assay.
Results: No correlation was observed between the developmental quotient and fatty acid plasma levels. Phospholipase A$_2$ activity was significantly higher among autistic children compared with controls.
Conclusion: The study did not show a correlation between fatty acid and phospholipase A$_2$ plasma levels and the developmental profile of children with autism.
Keywords: Autistic disorder, fatty acids, phospholipase A$_2$.

Resumo
Objetivo: Avaliar os níveis plasmáticos de ácidos graxos, a atividade da fosfolipase A$_2$ e o perfil de desenvolvimento de crianças com autismo versus controles.
Métodos: Vinte e quatro crianças com autismo foram submetidas a exames laboratoriais para quantificação plasmática de ácidos graxos por cromatografia gasosa e para determinação da atividade de fosfolipase A$_2$ por ensaio fluorimétrico.
Resultados: Nenhuma correlação foi observada entre o coeficiente de desenvolvimento e os níveis plasmáticos dos ácidos graxos quantificados. A atividade da fosfolipase A$_2$ foi significativamente maior no grupo de crianças com autismo quando comparado ao grupo controle.
Conclusão: O estudo não demonstrou correlação entre os níveis plasmáticos de ácidos graxos e fosfolipase A$_2$ e o perfil de desenvolvimento de crianças com autismo.
Descritores: Transtorno autístico, ácidos graxos, fosfolipase A$_2$.

**Introduction**

Reduced levels of polyunsaturated fatty acids have been associated with some childhood mental disorders such as attention deficit hyperactivity disorder (ADHD) in boys, severe deficits in reading, spelling, and auditory memory, as well as dyslexia and developmental coordination disorder (DCD). Autism spectrum disorders (ASD) also seem to be associated with altered lipid metabolism in their pathogenesis. These alterations provoke changes in the structure and function of cell membrane phospholipids, similarly to what is observed in other developmental disorders. A significant reduction in the levels of polyunsaturated fatty acids in the red blood cell membrane of patients with autism reinforces this assumption.

Recently, El-Ansary et al. evaluated fatty acid profile in the plasma of 26 autistic children and 26 age-matched healthy children. The authors found increased levels of most saturated fatty acids, except for propionic acid, and reduced levels of most polyunsaturated fatty acids. Moreover, omega-3 supplementation in children with ASD appears to be safe and effective in improving behavior.

Also, children with ASD have been shown to present significantly higher phospholipase A$_2$ (PLA$_2$) activity. In fact, evidence suggests that the instability observed in fatty acid levels may be caused by an increase in PLA$_2$ activity, perhaps in association with the high oxidative stress found in these patients.

In this context, the objective of this study was to evaluate fatty acid plasma levels, PLA$_2$ activity, and the developmental profiles of children with autism compared with control subjects.

**Methods**

**Patients**

The study included children with autism and children who had no diagnostic features of any mental or behavioral disorder, or clinical symptoms that could indicate the occurrence of any other disease. Controls were matched for age and sex and were selected from the general population of the municipality of Juiz de Fora, MG, Brazil. The study protocol was approved by the Research Ethics Committee of Universidade Federal de Juiz de Fora (UFJF) (protocol no. 140/2007).

We initially selected 60 children aged 2 to 18 years with a diagnosis or possible diagnosis of autism from two local schools that agreed to participate in the study. Of these, 22 were excluded because they had other mental disorders, neurological or sensory impairment possibly related development failure, such as congenital rubella syndrome associated with severe mental retardation, cerebral palsy, deafness, or blindness. Of the 38 patients who comprised the sample, 33 (all < 13 years) underwent neuropsychological testing, and 24 children with autism were finally selected for inclusion in the final sample. These 24 children were subjected to laboratory analysis for fatty acid quantification and PLA$_2$ activity assessment.

Control children were selected from the same schools. The group initially comprised 32 children aged 2 to 12 years with no symptoms of mental disorders, no demands for health care services, and not using any drug. After neuropsychological testing, 24 children were selected to comprise the final control group and were subjected to laboratory analysis.

Neuropsychological assessment was performed by specialists according to diagnostic criteria set forth in the Diagnostic and Statistical Manual of Mental Disorders, 4th edition, Text Revision (DSM-IV-TR). Evaluation started with a clinical interview, following application of inclusion and exclusion criteria. In a second clinical interview, patients underwent psychiatric mental status examination according to Brazilian guidelines. Additional interviews were conducted whenever necessary to complete these steps.

Neuropsychological assessment was performed using the Psychoeducational Profile – Revised (PEP-R), an instrument created to assess developmental age in children with autism. The PEP-R assesses development and a range of behaviors characterized as an inventory of skills and behaviors designed to identify patterns of uneven and idiosyncratic learning. The development scale includes the following dimensions: gross motor coordination, fine motor coordination, visual motor coordination, perception, imitation, cognitive performance, and verbal cognition. The scale was applied by a neuropsychologist in a single meeting that lasted about 1 hour. The range of behavior scale, used to identify atypical behaviors, was applied but not used for correlation analysis purposes in the present study (results varied widely, probably because of the use of medications, and therefore were not related to the developmental age of children). The total score obtained for each patient or control yielded a certain age of development (AD). The quotient of development (QD) was calculated for patients using the formula QD = AD/chronological age in months x 100.

Computed tomography (CT) scans of the skull were obtained to exclude the presence of any neurological disease with symptoms similar to those found in children with autism.

**Blood collection**

Blood samples (10 mL) were collected by venipuncture of the peripheral forearm vein of autistic and control children using a 21G scalp vein set (Becton Dickinson, Franklin Lakes, USA). Blood was collected into two tubes.
one containing anticoagulant (lithium heparin) and another without anticoagulant, centrifuged at 1,811 x g for 15 minutes at room temperature, and then stored in a freezer at -70 ºC until laboratory analysis. All materials were coded to preserve patient anonymity, and all procedures followed the principles of good laboratory practice.

### Fatty acid quantification

Fatty acid levels were determined according to the method originally described by Folch et al.¹⁵ and modified by El-Ansary et al.⁸ Briefly, 200 μL of sample was extracted in the presence of internal standards and esterified fatty acid using 3N hydrochloric acid methanolic solution in sealed bottles under nitrogen. Samples were incubated at 100 ºC for 45 minutes. Fatty acid methyl esters were extracted with n-hexane, and fatty acid composition of the extract was analyzed using a gas chromatograph (Hewlett-Packard HP 5890 Series II Plus, Analytical Direct, Wilmington, USA) equipped with a flame ionization detector and using a capillary column of 30 m x 0.25 mm x 0.25 μm (Supelco-Omegawax, Sigma-Aldrich, St. Louis, USA). The flow rate of helium was 1.2 mL/minute, with a separation/flow ratio of 50:1. Furnace temperature was 205 ºC. Injector and detector temperatures were 260 and 262 ºC, respectively. Two internal standards, C15:0 and C23:0, were added during analysis. Fatty acids (arachidonic acid [ARA], eicosapentaenoic acid [EPA], and docosahexaenoic acid [DHA]) were identified by comparison of retention times with authentic standards. Quantification of each fatty acid was expressed as weight percentage of total fatty acids present in the sample. Analyses were performed in duplicate.

### PLA₂ activity

PLA₂ activity was determined by fluorometric assay using pyrene-labelled phospholipid analog 1-octosanyl-2-(pyren-1-yl)-hexanoyl-sn-glycerophosphomethanol (C28-O-PHPM) as substrate. A total of 40 μL of serum was incubated at 37 ºC for 90 minutes in a buffer (final volume: 200 μL) containing 30 μm of C28-O-PHPM and 14 μm of Tris/hydrogen chloride, pH 7.4. Following incubation, the reaction was stopped and the released pyrenylhexanoic acid extracted and quantified as described by Ross et al.¹⁶ Serum blank reactions were stopped at zero time.

### Statistical analysis

Results were analyzed using descriptive statistics. Correlations between parameters were assessed using Pearson's correlation coefficient. Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) version 13.0 for Windows.

### Results

#### Patients

Mean age in the group of autistic patients (n = 24) was 7.4±2.9 years, and in the control group (n = 24), 7.2±1.8 years (age range in both groups: 2-12 years). A total of 18 children were male in each of the groups (boy to girl ratio = 3:1).

In the week preceding blood collection, none of children had started the use of any new medication. Of the 24 autistic children, 21 (87.5%) were making use of psychotropic drugs to treat autism symptoms, under either monotherapy (n = 7) or polytherapy (n = 14). The drugs most frequently prescribed were risperidone and carbamazepine, used in combination with each other or with other drugs. Control children were not on any medication. None of the children in any group were using supplements containing omega-3 and/or omega-6.

In the patient group, nine children (37.5%) had a history of seizures during development. In the control group, none of the children reported the occurrence of seizures.

Application of the PEP-R scale of development yielded a total score based on the sum of points in the “Passed” category (1 point for each item in that category). Children diagnosed with autism had a mean score of 31.12±23.75, compared to 120.79±19.21 in the control group. Mean score in the control group was 3.8 times higher than the mean score in the patient group (p < 0.001). Mean AD was 66.54±15.97 in the control group and 16.64±8.91 in the patient group.

Patients were divided into two groups according to QD values for the assessment of correlations between the developmental profile of children and the parameters assessed. In the subset of QD < 15 (n = 11), mean QD was 10.42±4.18, and in the subgroup with QD > 15 (n = 12), mean QD was 29.26±13.18.

#### Fatty acid quantification

ARA plasma levels (weight percentage of total fatty acids) were significantly elevated in autistic children (16.82±1.04 mmol L⁻¹) compared with children in the control group (12.26±1 mmol L⁻¹) (p < 0.001). EPA plasma levels were significantly reduced in autistic children (0.76±0.20 mmol L⁻¹) when compared to controls (1.24±0.27 mmol L⁻¹) (p < 0.001). The same was observed for plasma levels of DHA in the group of autistic children (3.67±0.65 mmol L⁻¹) compared with the control group (4.95±0.65 mmol L⁻¹) (p < 0.001).

Analysis of correlations between plasma levels of fatty acids ARA, EPA, and DHA in both groups showed increased rates of ARA:EPA and ARA:DHA in autistic...
children ($p < 0.05$) and reduced rates of EPA:ARA and EPA:DHA in the autistic group ($p < 0.005$) when compared to control children.

Among autistic children, no correlation was observed between QD and plasma levels of ARA ($p = 0.81$, $r = -0.05$), EPA ($p = 0.39$, $r = -0.18$), and DHA ($p = 0.70$, $r = -0.08$). Also, analysis of possible correlations between the two subgroups of autistic children, namely those with QD $< 15$ ($n = 11$) and with QD $> 15$ ($n = 13$) and plasma levels of ARA ($p = 0.90$), EPA ($p = 0.92$) and DHA ($p = 0.15$) failed to find significant results.

**PLA$_2$ activity**

PLA$_2$ activity was significantly higher in autistic children ($170±10$ pMol min$^{-1}$ mL$^{-1}$, $n = 24$) when compared to the control group ($132±8$ pMol min$^{-1}$ mL$^{-1}$, $n = 24$) ($p < 0.05$).

**Discussion**

The demographic characteristics of our sample, with a larger share of male children, at a 3:1 ratio, corroborate the already known higher incidence of autism in boys, with ratios ranging from 3.5 to 4 boys for every girl. Even though this ratio may vary depending on the degree of intellectual function, among the six girls with autism in our study, one had a developmental age of 34, and all others, < 18. Among the girls in the control group, one showed a developmental age of 73, compared to 77 for all the others (data not shown). When autistic children were divided according to QD, four girls and seven boys were included in the subgroup with more severe developmental deficits (QD $< 15$). The subgroup with higher QD ($> 15$), in turn, included two girls and 11 boys. These data suggest a change in sex ratio, with a greater representation of women, especially when considering children with larger deficits in learning patterns, as indicated by Klin and by Mercadante & Rosário.

According to Aman et al., it is estimated that 46% of children with global development disorders are treated with psychotropic drugs to control symptoms. In our study, of the 24 children in the case group, 21 (87.5%) were using psychotropic drugs, and 14 children were using two or more psychotropic drugs to reduce symptoms associated with the autistic spectrum. Even though we have included solely children with autism and no other ASD, the frequency of prescriptions seems high and could be related to a poor implementation of health care routines, combined with a scarcity of specialized care in Brazil.

With regard to fatty acid quantification results, high plasma levels of ARA and low levels of EPA and DHA were observed in the group of autistic children, which is consistent with the evidence of altered lipid metabolism in neuropsychiatric disorders. It has been proposed that such imbalance is related to changes in the structure and function of cell membrane phospholipids. In particular, the levels of EPA have been associated with the production of eicosanoids, which have anti-inflammatory, antithrombotic, and vasodilator properties and were reduced in the autistic children of our study.

The production of eicosanoids, a group of biologically active molecules derived mainly from ARA and released from the membrane by the PLA$_2$ enzyme, seems to be particularly related to physiological dysfunctions characteristic of ASD. This group includes prostaglandins, leukotrienes, and thromboxanes, which act as second messengers, regulating a variety of physiological processes, and as mediators in inflammation. In addition, eicosanoids play important roles in the neurobiology of ion channels, receptors, release of neurotransmitters, synaptic plasticity, and neuronal gene expression. Prostaglandins E$_2$ and D$_2$ have sedative properties, and are involved in the control of the sleep-wake cycle. Elevated concentrations of ARA, such as those observed in our study, could be associated with pathological conditions.

DHA has a role in inhibiting neuronal apoptosis and regulating neuronal excitability, and it may also modulate abnormal electrical impulses in the neurons. A deficit of these fatty acids could be related to the increased susceptibility to seizures observed in patients with ASD and was observed in our group of autistic children.

Our results are consistent with those reported by Bell et al. Those authors also found an increase in ARA:EPA ratio in erythrocytes and plasma of autistic children vs. age-matched children with normal development. However, our findings are discordant with those of El-Ansary et al., who observed reduced ARA:DHA and higher EPA:ARA ratios in autistic patients. These differences can probably be explained by fluctuations in EPA and DHA levels as a result of different dietary patterns, as the levels of ARA remain relatively stable.

Omega-3 supplementation has been associated with better results in behavioral assessment scales applied to autistic children. In addition, patients who receive EPA supplementation have been shown to present a reduced PLA$_2$ activity when compared with patients not supplemented. These findings confirm that the children participating in our study were not using any dietary supplement.

The high levels of PLA$_2$ activity observed in our autistic children are consistent with the report of Bell et al. and provide further evidence of an association between accelerated phospholipid metabolism and biochemical alterations of the lipid membrane. In addition, El-Ansary et al. indicated a relationship between high levels of saturated fatty acids and high oxidative stress. Bell et al. also suggested that the increase in PLA$_2$ activity
was associated with high oxidative stress, resulting in instability of the fatty acid profile. According to Tostes et al., the increased levels of plasma nitric oxide observed in autistic children seem to corroborate this hypothesis.

Among the limitations of the study is the fact that we did not exclude children under treatment. The use of psychotropic drugs may have interfered with the results of laboratory tests and with the children's behavior during assessment. However, Molloy et al. evaluated the levels of different cytokines (IFN-γ, IL-2, IL-4, IL-5, IL-10, and IL-13) in children with global development disorders and found statistically similar levels of these cytokines regardless of the use of psychotropic drugs (65% of the children were under treatment). Additionally, no significant inhibition of PLA activity was observed in the group of autistic patients, even though this secondary inhibition in response to neuroleptic drugs has been observed in both in vitro and in vivo animal experiments.

In sum, autistic children show abnormalities in phospholipid metabolism and developmental profile. However, the present study did not show a correlation between fatty acid and phospholipase A2 plasma levels and the developmental profile of children with autism.

References


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