Low immune cell ARA and high plasma 12-HETE and 17-HDHA in iron-deficient South African school children with allergy

Running head: Low ARA status and high plasma 12-HETE and 17-HDHA in allergy.

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ABSTRACT

Allergy has been associated with altered fatty acid and inflammatory status. In this cross-sectional study of 321 rural iron deficient (ID) South African children (aged 6–11 years), a subsample (n=111) of children with parent-reported allergy data were divided into an allergic (n=30) and non-allergic (n=81) group and compared. PBMC arachidonic acid (ARA; *P*=0.010) and the PBMC ARA to dihomo-gamma-linolenic acid (DGLA) ratio (*P*=0.035) were lower in the allergic children. Plasma 12-hydroxyeicosatetraenoic acid and 17-hydroxydocosahexaenoic acid (17-HDHA) were higher (*P*=0.040 and 0.020, respectively) in the allergic group. Thus, a fatty acid composition and lipid mediator levels indicative of increased release of ARA from PBMC membranes, increased inflammation as well as the resolving thereof, were associated with parent-reported allergy symptoms. This study used baseline data of an intervention study which was registered at clinicaltrials.gov as NCT01092377.

***Keywords*:** Allergy, fatty acid composition, arachidonic acid, lipid mediator, inflammation, inflammation-resolving

Abbreviations

ARA	arachidonic acid
$cPLA_2$	cytosolic phospholipase 2
D5D	delta-5 desaturase
D6D	delta-6 desaturase
DGLA	dihomo-gamma-linolenic acid
DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
FADS	fatty acid desaturase
FAME	fatty acid methyl ester
HDHA	hydroxydocosahexaenoic acid
HEPE	hydroxyeicosapentaenoic acid
HETE	hydroxyeicosatetraenoic acid
ISAAC	International Study on Asthma and Allergy in Childhood
LA	linoleic acid
LCPUFA	long-chain polyunsaturated fatty acid
PBMC	peripheral blood mononuclear cells
PD1	protectin D1
PUFA	polyunsaturated fatty acid
RvD1	resolvin D1
tIgE	total immunoglobulin E

1. Introduction

Allergic disease, including rhinitis, asthma and eczema, is an increasing health problem worldwide, both in industrialized and developing countries [1, 2]. It is estimated that approximately 500 million people worldwide and over 30 million in Africa have allergic rhinitis and that about 200 million also have asthma as a co-morbidity [3]. In South Africa, the third phase of the International Study on Asthma and Allergy in Childhood (ISAAC III) found a prevalence for allergic rhinoconjuntivitis (rhinitis with itching and watery eyes), asthma and eczema of 18–20% in children aged 13–14 years [4].

It has been hypothesized that the rise in allergic disease could be due to changes in dietary habits [5]. In particular a higher intake of n-6 PUFA [6] and a lower relative intake of n-3 PUFA over recent decades may be important contributing factors [7, 8]. In contrast, some studies also report that lower status of the n-6 PUFA ARA is associated with allergic disease [9], and intervention with a combined docosahexaenoic acid (DHA; 22:6n-3) and arachidonic acid (ARA, 20:4n-6) supplemented formula delayed onset and reduced incidence of allergic disease in children up to three years of age [10]. Furthermore, variation in the fatty acid desaturase (FADS) genes coding for delta-5 (D5D) and delta-6 desaturase (D6D) has also been associated with allergy [11, 12].

There is, however, mechanistic evidence supporting opposing actions of n-6 and n-3 LCPUFA in allergic disease. When ARA, DHA and eicosapentaenoic acid (EPA, 20:5n-3) are released from phospholipid membranes mostly by cytosolic phospholipase A2 ($cPLA_2$), immunomodulatory metabolites can be produced [13-15]. While the n-6

LCPUFA metabolites were shown to have mainly pro-inflammatory properties, the n-3 LCPUFA metabolites mainly exert anti-inflammatory actions [16].

In addition to the role of PUFA in allergy, epidemiological studies have suggested that the intake of unnatural *trans*-fatty acids, such as elaidic acid (*trans*-18:1n-9), could be involved in allergic disease [17, 18]. *Trans*-fatty acids are produced by partial hydrogenation of unsaturated fatty acids and were used commonly in margarines and baked goods until recently [19]. Most countries, including South Africa, have now put legislation into place to reduce greatly and soon forbid the use of these fats due to their detrimental health effects [19-21].

Besides poor PUFA status, iron deficiency, which is the most common nutrient deficiency worldwide, may also be associated with allergy. Poor fetal iron status has previously been hypothesized to be a risk factor to develop allergy [22]. In turn, food allergy may cause iron deficiency through intestinal chronic blood loss [23].

Allergic disease is poorly explored in African populations, even more so in relation to iron and fatty acid status and fatty acid derived immune mediator levels. Therefore, the aim of this study was to determine the prevalence of parent-reported allergy symptoms in a sample of 111 iron-deficient (ID) rural South African school children, and to compare iron status, total phospholipid fatty acid composition of peripheral blood mononuclear cells (PBMC), red blood cells (RBC) and plasma, as well as plasma lipid mediator concentrations of parent-reported allergic and non-allergic children.

2. Methods

2.1. Study design and subjects

This was a cross-sectional analysis utilizing baseline data from 321 ID 6–11-year-old children living in an area with low fish intake. These children were enrolled in a randomized, placebo-controlled intervention study aiming to determine the effects of iron and n-3 LCPUFA supplementation, alone and in combination, on cognition and infectious morbidity [24, 25]. The study was conducted at four primary schools in rural low-income villages in the Valley of a Thousand Hills in the Province of KwaZulu-Natal in South Africa, and a total of 321 children participated. The following inclusion criteria were used: 1) age 6–11 y; 2) hemoglobin > 8 g/dL; 3) iron deficiency, defined as either serum ferritin (SF) < 20 µg/L, serum transferrin receptor (TfR) > 8.3 mg/L, or zinc protoporphyrin (ZPP) > 70 µmol/mol haem in washed RBC; 4) apparently healthy, with no chronic illness; and 5) no consumption of iron or n–3 PUFA-containing supplements. Parents or guardians gave written informed consent and children gave verbal assent before the study started. The ethical committees of the North-West University (NWU-0061-08-A1), Potchefstroom, South Africa and the Swiss Federal Institute of Technology (ETH) Zürich (EK 2008-33), Switzerland, approved the study protocol. The study was registered at clinicaltrials.gov as NCT01092377.

2.2. Assessment of allergy symptoms

The ISAAC questionnaire was designed to determine the prevalence of allergic disease in children worldwide [26] and has a high positive predictive value in detecting allergic disease among children with symptoms [27-29]. In the present study, the ISAAC questionnaires were distributed via the children to the parents or caretakers of the total study cohort and 35% of questionnaires were completed and returned. These were used to classify the subsample of children (n = 111) for this cross-sectional analysis. The questionnaire was translated into the local language (Zulu) and verified by asking ten people who are fluent in both Zulu and English to explain what they understood from the questions and to answer the questions. The Zulu questionnaire was also further verified by having it translated back into English by an independent translator.

The questions of the ISAAC questionnaire are formulated to determine if a child has asthma (wheezing), rhinoconjunctivitis, and/or eczema and are designed to address the child's parents or caretaker for children aged 6–7 years. A set of questions is asked for each symptom, but the answers of only one or two specific questions are used to score a positive result [30]. The presence of asthma was estimated on the basis of a positive answer to the written question: "Has your child had wheezing or whistling in the chest in the past 12 months?" The presence of allergic rhinoconjunctivitis was estimated on the basis of positive answers to both these questions: "In the past 12 months, has your child had a problem with sneezing or a runny or blocked nose when he/she did not have a cold or the flu?" and if yes, "In the past 12 months, has this nose problem been accompanied by itchy watery eyes?" Eczema symptoms were estimated on the basis of positive answers to two questions: "Has your child had this itchy rash at any time in the past 12 months?" and, "Has this itchy rash at any time affected any of the following

places: the folds of the elbows; behind the knees; in front of the ankles; under the buttocks; or around the neck, ears, or eyes." These questions were preceded by the question "Has your child ever had a skin rash which was coming and going for at least 6 months?" A child was considered to have allergic disease if one of these conditions were present [30].

2.3. Laboratory measurements

Blood was collected in EDTA-coated and trace element free serum tubes (Becton Dickinson, New Jersey, United States of America) for the preparation of red blood cells (RBC), plasma and serum, and in cell preparation tubes for the preparation of peripheral blood mononuclear cells (PBMC). Hemoglobin concentrations were measured on site on an aliquot of whole blood by using the direct cyanmethemoglobin method [Ames Mini-Pak hemoglobin test pack and Ames Minilab, Bio Rad Laboratories (PTY) Ltd] by using Drabkin's solution and a standard miniphotometer. ZnPP was measured on washed RBCs by using a hematofluorometer (Aviv Biomedical) and 3-level control material provided by the manufacturer on the same day of blood sampling. After completion of the fieldwork, samples were transported on dry ice and stored at –80°C until analysis. SF and C-reactive protein (CRP) were measured by using an automated chemiluminescent immunoassay system (IMMULITE; DPCBuhlmann GmbH). Serum TfR was measured by using an enzyme immunoassay (Ramco Laboratories Inc). Body iron was calculated from the ratio of TfR:SF according to the equation of Cook *et al*. [31]. Serum zinc was measured by flame atomic absorption

spectrometer (AA240FS; Varian Inc.) as previously described [32]. For reporting of prevalence and statistical analyses, ID was defined as either ZnPP >70 µmol per mol heme [33], TfR >8.3 mg/L (test kit reference value), or SF <15 µg/L [34]. Anemia was defined as Hb <11.5 g/L [35], and zinc deficiency as plasma zinc <65 µg/L. Inflammation was defined as CRP >5 mg/L, and SF values of all children with CRP >5 mg/L were excluded. Total IgE was measured in serum with the tIgE ELISA kit from Human Diagnostics (Wiesbaden, Germany). Fatty acid composition was determined in PBMC, RBC and plasma samples as previously described [24]. All samples used for fatty acid analysis were stored at −80ºC for less than 6 months before analysis. Briefly, phospholipids were extracted from PBMC, RBC and plasma with chloroform:methanol (containing 0.01% butylated hydroxytoluene) and methylated by using a modification of the method of Folch et al. [36]. External calibration was used for each fatty acid methyl ester (FAME) and corrected with a weighed heptadecanoic acid internal standard. The gas chromatography separation of FAME was carried out on a BPX 70 capillary column (60 m x 0.25 mm x 0.25 µm; SGE Analytic Sciences) by using helium as the carrier gas at a flow rate of 1.3 mL/min. FAME, including the FAME of the C18 trans fatty acids, *trans*-C18:1n-9 (elaidic acid), *trans*-C18:1n-7 (trans-vaccenic acid) and *trans*-C18:1n-6 (petroselaidic acid) were separated chromatographically The oven temperature was programmed from 130°C to 240°C, rose from 130°C to 200°C at 2°C/min, was held at 200°C for 4 min, and then rose at 5°C/min to 220°C. After the temperature was held isothermal at 220°C for 5 min, it was increased by 10°C/min to 240°C, where it was retained for 5 min. The total analysis time was 53 min. Quantitation was performed with Masshunter mass spectrometry data software (B.06.00, Agilent Technologies) after the

192 data were transferred from ChemStation mass spectrometry data software (Agilent
193 Technologies, G1701EA version E.02.00.493). In Masshunter, target and qualifier
194 transitions were selected for each FAME from the full scan data obtained, and a target
195 to qualifier ratio between 80% and 120% was accepted. Further quality control
196 measures entailed spiking and extraction of biological samples with fatty acids to obtain
197 exact chromatographic retention times and target to qualifier ratios within the biological
198 matrices. The relative composition (weight %) of fatty acids was reported as previously
199 described [24].

200 Ratios between fatty acids in the desaturation and elongation pathway were used to
201 reflect enzyme activity differences in this pathway between groups (Figure 1). Although
202 gamma-linolenic acid (GLA, 18:3-n6) is the direct product of D6D, it is rapidly elongated
203 to dihomo-gamma-linolenic acid (DGLA, 20:3n6) [37] resulting in very low GLA
204 concentrations (RBC mean = 0.05%). Therefore, as previously described [38],
205 ARA:DGLA and DGLA:LA ratios were used to estimate D5D and D6D activities,
206 respectively.

207 Lipid mediators were analysed with liquid chromatography mass spectrometry.
208 Plasma 17-HDHA; 5- and 12-hydroxyeicosapentaenoic acid (HEPE); 5-, 8-, 11-, and
209 12-HETE and PGE_2 and PGD_2 were measured. As outlined in Fig. 1, 17-HDHA is
210 produced from DHA and is the precursor of the D-series resolvins and protectins, which
211 are inflammation-resolving immune modulators [39, 40]. PGE_2, PGD_2 and HETE are
212 mainly pro-inflammatory and are derived from AA, whereas HEPE and its products
213 have anti-inflammatory functions and are derived from EPA [39, 40]. Lipid mediators
214 were extracted from plasma with solid phase extraction (SPE) using Strata-X

(Phenomenex, Torrance, United States of America). The method was modified for Strata-X SPE columns from a previously described method [41]. Briefly, plasma samples (1 ml) were diluted with water and adjusted to 15% methanol (v/v), to a final volume of 4.6 ml. Internal standards, PGB_2-d4 and 12-HETE-d8 (500 pg of each), were added to each sample. The samples were incubated on ice for 30 min and then centrifuged at 3000 rpm for 8 min to remove any precipitated proteins. The resulting clear supernatants were acidified with 1N hydrochloric acid to pH 3.0 and immediately applied to SPE cartridges that had been preconditioned with 2 ml methanol followed by 4 ml water. The cartridges were then washed with 10 ml 15% (v/v) methanol, 10 ml water, and 4 ml hexane in succession. Lastly, the lipid-derived immune modulators were eluted with 6 ml methyl formate. A vacuum manifold (Phenomenex) was used to perform the SPE and the vacuum was regulated that single drops could be seen from each cartridge. The methyl formate was evaporated under a fine stream of nitrogen and the residue was dissolved in 20 µl acetonitrile, flushed with nitrogen and stored, at the most, for four days at –20°C before analysis with LCMSMS.

Samples were analysed with an Agilent Technologies 6410 MSMS, coupled to an Infinity 1260 HPLC pump (Santa Clara, United States of America). The instrument was operated in negative electrospray ionisation mode. At least two transitions were monitored for each compound, using a dynamic multiple reaction monitoring (MRM) method. The collision and fragmentor voltages were optimized with flow injection analysis. The gas temperature and flow was set at 350°C and 12 l/min respectively, and the capillary voltage at 4000 V. Chromatographic analysis was performed on a C18 column (Poroshell, 2.7µ, 100 x 2.1 mm; Agilent Technologies) with a flow rate of 0.4

ml/min and column temperature of 50°C. Sample injections were performed with an Agilent G1367B autosampler. The sample chamber temperature was set at 5°C and the injection volume was 15 µl, subsequent to mixing 5 µl of sample (in acetonitrile) with 10 µl water in the autosampler, just before injection. The analysis was performed using an acetonitrile-based system with a flow program, by mixing two solvents (A and B) with programmed ratio changes for optimal separation of compounds. Solvent A was water/glacial acetic acid, 99:1 (v/v) and solvent B was 100% acetonitrile.

Data were quantified with Masshunter B05 02, using external calibration for each compound and two internal standards to correct for losses and matrix effects during sample preparation and analysis. An example of a typical plasma lipid mediator chromatogram, including transitions of each compound, is depicted in supplemental Fig. 1.

2.4. Statistical analyses

Statistical analyses were performed using IBM SPSS Statistics (version 21; IBM Corporation). All data were checked for the presence of outliers (±3 SD from the mean) and normal distribution. Non-parametric data were log-transformed for statistical analysis. Normally distributed data were expressed as means ± SD and non-normally distributed data as medians and range (min to max). Differences in characteristics among the total study sample, subgroups with and without allergy data, as well as between the allergic and non-allergic children, were examined by using ANCOVA, adjusted for age, gender, school, body iron and serum zinc for continuous variables and

the chi-square test for categorical variables. Differences in tIgE, iron status indices, serum zinc, CRP, the total phospholipid fatty acid composition of PBMC, RBC and plasma, and in plasma lipid mediators between the allergic and non-allergic group were examined with ANCOVA, adjusted for age, gender, school, body iron (not included for iron status indices) [31] and serum zinc (not included for serum zinc). Associations between different fatty acids were determined with Pearson's or Spearman's rank correlation for normal and non-normally distributed data, respectively. The level of significance was set at a p-value less than 0.05.

3. Results

In total, ISAAC questionnaires were sent to the parents or caretakers of 296 children, and 111 were completed and returned. Based on the ISAAC questionnaire, 27% of the children with available ISAAC data had parent-reported allergy symptoms, consisting of a mixture of rhinoconjuntivitis (14.6% [n = 12/82]), asthma (25.4% [n = 18/71) and eczema symptoms (11.1% [n = 7/63]). Allergic and non-allergic children did not differ significantly with respect to sex distribution, age and anthropometric indices (Table 1). Allergic children showed a trend to have a higher prevalence of ID based on SF levels < 15 µg/L (P = 0.089) and a trend to have a higher prevalence of CRP concentrations greater than 5 mg/L (P = 0.087) (Table 1). The median tIgE in the sample was 131.7 IU/ml (0.0 – 921.0 IU/ml), and did not differ (P = 0.811) between allergic and non-allergic children (Table 1). There was no difference between the serum zinc concentrations of allergic and non-allergic children (Table 1).

For the purpose of investigating the differences in fatty acid composition between the allergic and non-allergic children, the phospholipid fatty acid composition was measured in PBMC of 45, in RBC of 103 and in plasma of 87 children (Table 2). PBMC ARA (P = 0.010) and the PBMC ARA to DGLA ratio were lower in the allergic children (P = 0.035). PBMC elaidic acid (*trans*-C18:1n-9) tended to be higher (P = 0.075) in the allergic group, and correlated negatively with ARA (n = 44, r_P = –0.453, P = 0.002) (Figure 2).

Plasma lipid mediators were measured in 76 children (Table 3). 12-HETE and 17-HDHA were higher (P = 0.040 and 0.020, respectively) in the allergic group.

4. Discussion

The main finding of this cross-sectional analysis in a small sample of ID rural South African children with a high prevalence of parent-reported allergy is that allergic children had a lower PBMC total phospholipid ARA composition, together with higher ARA-derived 12-HETE levels. Another novel finding from our study is that the DHA-derived anti-inflammatory lipid-mediator and precursor of the D-series resolvins, plasma 17-HDHA, was higher in the allergic children.

There are three possible mechanistic explanations for the lower PBMC ARA observed in the allergic children in this study. Firstly and most strongly supported by these data, it is possible that lower ARA composition was caused by the increased release of ARA from PBMC membranes. This hypothesis is supported by the observation that the pro-inflammatory ARA-derived 12-HETE was higher in plasma of the allergic children. Clinical evidence demonstrates that the platelet-derived 12-lipoxygenase (LOX) metabolite of ARA, 12-HETE, is involved in allergic inflammation [42, 43].

Although unexpected, because of the anticipated pro-inflammatory profile usually associated with allergy, we propose that higher concentrations of 17-HDHA in the plasma of the allergic children may indicate an activated resolving mechanism of ongoing inflammation caused by allergy. In line with this, a previous study found that 17-HDHA and its product, resolvin D1, strongly reduced the production of total immunoglobulin E (tIgE) in human B cells, which is elevated in allergic disease [13]. Our study was, however, limited because resolvins and protectins were outside the quantitation limit of our methodology.

Our finding of lower PBMC ARA (17%) in the children with reported allergy symptoms is in agreement with others who found 47% lower ARA and 70% lower DHA in T cell phospholipid membranes of 6-month to 12-year old children with atopic dermatitis compared to non-atopic controls [8, 44]. Even though the current study had a limited number of PBMC samples (n = 45), we still observed significant differences in ARA and the ARA:DGLA ratio between the allergic (n = 14) and non-allergic (n = 31) children. Of the three lipid pools studied, PBMC phospholipids are most closely linked to the cellular responses involved in allergy. The overall relative composition of DHA in PBMC of the children studied (1.5%) is low compared to the ~3% that others have reported [45-47]. The relative composition of DHA in RBC (3.7%), in contrast, is comparable to those reported (3 – 4%) in other studies [46, 48].

Second, D5D activity may be impaired in the allergic children, which is supported by our finding of a significantly lower PBMC ARA:DGLA ratio in this group, which is in line with findings of previous studies [11, 12]. Lindskou *et al.* also found a reduced ratio of ARA:DGLA in mononuclear cells of patients with atopic dermatitis [12]. Furthermore, as reviewed by Lattka *et al*., polymorphisms in the fatty acid desaturase (FADS) gene cluster, specifically FADS1 encoding D5D, have been associated with allergic disease [11]. The findings of the current study could be extended by investigating possible genetic variation of the FADS1 gene and D5D enzyme activity in relation to allergy in this population group.

Another mechanistic explanation for lower PBMC ARA in the allergic group is that ARA could have been displaced from PBMC membranes by elaidic acid (*trans-C*18:1-n9), as this *trans*-fatty acid correlated negatively and with medium strength with PBMC

ARA. Elaidic acid did not correlate with DHA, and it is possible that DHA may be less affected due to the relatively lower levels of DHA naturally occurring in PBMC membranes [49]. The mean percentage PBMC elaidic acid (2.7%) in the study sample was very high in comparison with the 0.5% of total *trans*-C18:1 reported in 6-month to 12-year old Caucasian allergic children in Italy [44]. Furthermore, the PBMC phospholipid *trans*-fatty acid, elaidic acid, tended to be higher in the allergic children, which supports the hypothesis that *trans*-fat might be associated with allergic disease [17, 18]. The median intake of snacks (unknown brands of chips and cookies) that could possibly contain *trans*-fat in a different subsample of this study population was 10.0 g ranging from 0.0 to 47.5 g per day and intake of these snacks was known to be high from a very young age in this particular area in South Africa (Valley of a Thousand Hills) [50, 51].

In this sample, the prevalence of parent-reported allergy symptoms was 27%, which generally agrees with the allergy prevalence of 18–20% in South African children aged 13–14 years reported by the ISAAC study [4]. Due to the limitation that only 35% of questionnaires were received back from the parents of children participating in the main study, overestimation of allergy prevalence cannot be ruled out.

In the current study, tIgE were generally very high and did not differ significantly between the allergic and non-allergic children. Possible reasons are that 1) the presence of helminth infection and its contribution to tIgE was not determined in our study, and 2) it was recently found in black South African (Xhosa) children aged 6 months to 10 years that sensitization and raised IgE levels, according to internationally-derived cut-off values for the most commonly used clinical allergy tests (skin prick tests,

specific IgE and multiplexed ImmunoCAP® from Thermo Fisher, Sweden), were significantly less predictive for actual food allergy (specifically peanut allergy) [52]. Thus, ethnic-specific cut-offs might be necessary.

The main limitation of this cross-sectional study was the small sample size, and the limited availability of PBMC. It was, however, unavoidable since only one PBMC sample per child was available, because the second subsample was allocated for other analyses.

5. Conclusion

Our data show that rural ID South African children with parent-reported allergy symptoms have low PBMC ARA and high ARA-derived plasma 12-HETE compared with their non-allergic counterparts. These data indicate that increased release of ARA from PBMC phospholipid membranes to be metabolised to pro-inflammatory lipid mediators such as 12-HETE might, at least partially, be a mechanism underlying allergy. Furthermore, the novel finding of higher plasma 17-HDHA in children with allergy indicates that resolution of allergic inflammation might be in progression.

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388

389 **Conflict of interest**

390

391 PCC, JB and CMS have received speaking honoraria from Unilever. LM has no conflict
392 of interest.

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Table 1

Characteristics of all children participating in main study, children with and without ISAAC data as well as allergic and non-allergic children[1]

	All children (*n* = 236–321)	Children with ISAAC data (n = 72–111)	Children without ISAAC data (n = 164–210)	Allergic children (*n* = 18–30)	Non-allergic children (*n* = 54–81)
Age (y)	8.9 ± 1.3[a]	9.2 ± 1.3	8.8 ± 1.3	9.3 ± 1.4[a]	9.1 ± 1.4
Ratio male:female (%)	51:49	45:55	54:45	57:43	41:59
Height (m)	1.28 ± 0.09	1.30 ± 0.09	1.27 ± 0.08	1.31 ± 0.08	1.29 ± 0.09
Weight (kg)	27.8 (17.9–48.1)[b]	29.0 (19.3–48.1)	27.1 (17.9–48.1)	28.9 (20.2–47.2)[b]	29.1 (19.3–48.1)
Anthropometric indices [*n* (%)]					
Stunting (HAZ < –2SD)	18 (5.8)	6 (5.4)	12 (6.0)	1 (3.3)	5 (6.3)
Mildly stunted (HAZ < –1SD ≥ –2SD)	99 (32.1)	37 (33.6)	62 (31.3)	11 (36.7)	26 (32.5)
Underweight (WAZ < –2SD)	7 (2.9)	2 (2.7)	5 (3.0)	0 (0.0)	2 (3.7)
Overweight (BAZ > 1SD < 2SD)	64 (21.1)	22 (20.4)	42 (21.4)	4 (13.3)	18 (23.1)
Obese (BAZ ≥ 2SD)	21 (6.9)	5 (4.6)	16 (8.2)	0 (0.0)	4 (5.1)
Plasma tIgE (IU/ml)	131.7 (0.0–921.0)	135.9 (0.0–921.0)	114.7 (0.0–887.0)	104.6 (0.0 – 740.1)	135.9 (0.0–740.0)
Blood hemoglobin (g/dL)	12.1 ± 0.8	12.1 ± 0.7	12.1 ± 0.8	12.2 ± 0.7	12.1 ± 0.7
Serum ferritin (µg/L)[c]	19.1 (3.1–73.1)	21.1 (5.4–64.6)	18.9 (3.1–73.1)	18.8 (5.4 – 43.8)	20.5 (6.2–64.6)
Serum transferrin receptor (mg/L)	5.7 (2.4–11.8)	5.7 (2.5–9.7)	5.7 (2.4–11.75)	5.3 (2.5 – 9.2)	5.6 (2.5–9.7)
Body iron stores (mg/kg)	3.0 ± 2.5	3.2 ± 2.3	2.9 ± 2.6	2.8 ± 2.3	3.3 ± 2.3
Zinc protoporphyrin (µmol/mol heme)	75.0 (33.0–215.0)	72.0 (40.0–141.0)	75.0 (33.0–171.0)	72.0 (48.0 – 112.0)	74.0 (40.0–161.0)
C-reactive protein (mg/L)	0.4 (0.0–17.8)	0.3 (0.0–3.9)	0.3 (0.0–4.9)	0.4 (0.0–9.9)	0.3 (0.0–17.8)
Serum zinc (µg/L)	73.3 (45.8–106.2)	74.7 (45.8–104.1)	72.6 (46.2–106.2)	70.6 (55.9–102.2)	75.5 (45.8–104.1)
Deficiencies [*n* (%)]					
Anemia (Hb < 11.5 g/dL)	65 (20.6)	18 (16.2)	47 (23.0)	3 (10.0)	15 (18.5)
Iron deficiency based on SF (<15.0 µg/L)	78 (28.1)	24 (25.0)	54 (29.7)	9 (37.5)	15 (20.8)
Iron deficiency based on TfR (>8.3 mg/L)	36 (11.3)	12 (10.9)	24 (11.5)	3 (10.0)	9 (11.3)
Iron deficiency based on ZnPP (>70 µmol/mol heme)	197 (62.7)	64 (58.7)	133 (64.9)	18 (60.0)	46 (58.2)
Shortage of body iron (negative values)[d]	33 (12.0)	11 (11.6)	22 (12.2)	4 (16.7)	7 (9.9)
Iron deficiency anemia (Hb < 11.5 g/dL and SF < 15 µg/L)	25 (9.2)	7 (7.3)	18 (10.2)	2 (8.3)	5 (6.9)
Zinc deficiency (SZn <65 µg/L)	70 (24.8)	22 (22.4)	48 (26.1)	8 (32.0)	14 (19.2)

Acute-phase protein [*n* (%)]					
C-reactive protein (>5 mg/L)	22 (7.2)	7 (6.6)	15 (7.5)	4 (13.8)	3 (3.9)

Abbreviations: HAZ: height-for-age ȥ score; WAZ: weight-for-age ȥ score; BAZ: BMI-for-age ȥ score; Hb: haemoglobin; SF: serum ferritin; SZn: serum Zinc; tIgE: total immunoglobulin E; TfR: transferrin receptor; ZnPP: Zinc protoporphyrin.

[1]Differences between the children with and without ISAAC data, as well as the allergic and non-allergic children were examined with ANCOVA, adjusted for gender, age, school, total body iron (not included for iron status indices) and serum zinc (not included for serum zinc) for continuous variables and by using the chi-square test for categorical variables ($P<0.05$). Allergic children showed a trend to have a higher prevalence of ID based on SF levels < 20 µg/L ($P = 0.089$) and to have a CRP concentration greater than 5 mg/L ($P = 0.087$).

[a] Mean ± SD (all such values).

[b] Median; minimum to maximum in parentheses (all such values). Data were log-transformed to perform statistical analysis.

[c] Serum ferritin values of all children with CRP>5 mg/L were excluded.

[d] Total body iron was calculated on the basis of Cook et al. [31].

Table 2

Peripheral blood mononuclear cell (PBMC), red blood cell (RBC) and plasma total phospholipid fatty acid composition (weight percentage) of allergic and non-allergic children[1].

	PBMC			**RBC**			**Plasma**		
	Allergic children	Non-allergic children		Allergic children	Non-allergic children		Allergic children	Non-allergic children	
	n = 8 – 14	n = 28 – 31		n = 20 – 28	n = 49 – 75		n = 19 – 25	n = 47 – 62	
			P-value			*P*-value			*P*-value
Elaidic (*trans*-C18:1n-9)	3.02 ± 1.21[a]	2.51 ± 0.98	0.075	0.17 (0.06 – 0.27)	0.17 (0.04 – 0.74)	0.126	0.22 (0.11 – 0.87)	0.22 (0.06 – 0.77)	0.789
LA (C18:2n-6)	7.65 ± 2.88	7.23 ± 1.25	0.258	12.91 ± 1.17	13.33 ± 1.03	0.603	23.03 ± 2.35	23.52 ± 2.63	0.563
DGLA (C20:3n-6)	1.46 (0.94 – 1.90)[b]	1.56 (1.01 – 2.33)	0.739	1.44 (1.03 – 1.84)	1.30 (0.93 – 1.71)	0.109	2.72 (1.54 – 4.54)	2.58 (1.33 – 4.09	0.880
DGLA:LA ratio	0.21 ± 0.06	0.22 ± 0.06	0.477	0.10 (0.07 – 0.16)	0.10 (0.07 – 0.14)	0.079	0.12 (0.07 – 0.24)	0.11 (0.06 – 0.20)	0.739
ARA (C20:4n-6)	18.11 (13.33 – 23.12)	21.79 (15.11 – 28.46)	0.010	15.04 ± 1.04	14.88 ± 1.25	0.428	13.48 ± 2.06	14.06 ± 1.90	0.198
ARA:DGLA ratio	12.50 (9.08 – 23.69)	13.79 (8.56 – 19.64)	0.035	10.78 ± 2.08	11.60 ± 1.69	0.427	4.86 (3.14 – 10.00)	5.46 (3.07 – 11.38)	0.603
Total n-6 LCPUFA	23.72 ± 2.90	26.90 ± 4.01	0.089	22.12 ± 1.45	21.56 ± 1.69	0.202	17.80 ± 2.30	18.38 ± 2.28	0.076
EPA (C20:5n-3)	0.13 (0.05 – 0.41)	0.11 (0.02 – 0.54)	0.813	0.16 (0.08 – 0.38)	0.17 (0.03 – 0.38)	0.670	0.18 (0.09 – 0.40)	0.18 (0.02 – 1.33)	0.802
DHA (C22:6:n-3)	1.36 (0.88 – 2.16)	1.51 (0.96 – 2.91)	0.377	3.76 ± 0.55	3.75 ± 0.74	0.573	3.12 ± 0.58	3.44 ± 0.68	0.118
Total n-3 LCPUFA	2.70 (1.83 – 4.35)	2.97 (1.88 – 4.43)	0.783	5.60 ± 0.69	5.50 ± 0.98	0.629	3.93 (3.08–5.34)	4.30 (2.86–7.13)	0.215

Abbreviations: ARA: arachidonic acid; DGLA: dihomo-gamma-linolenic acid; LA: linoleic acid; LCPUFA: long-chain polyunsaturated fatty acid.

[1] Differences between the allergic and non-allergic groups were examined with ANCOVA, adjusted for gender, age, school, total body iron and serum zinc. $P < 0.05$ was considered significant. Non-parametric data were log-transformed before analyses. Data were expressed as percentage of total fatty acids.

[a] Mean ± SD (all such values).

[b] Median; minimum to maximum in parentheses (all such values).

Table 3

Plasma lipid-mediator concentrations of allergic and non-allergic children[1].

	Lipid-mediators		
	Allergic children	Non-allergic children	
	n = 16 – 20	n = 46 – 59	
			P-value
17-HDHA	2.18 ± 2.11[a]	1.74 ± 1.67	0.020
5-HEPE	1.25 ± 1.30	0.95 ± 0.57	0.310
12-HEPE	0.81 ± 1.19	0.48 ± 0.53	0.742
5-HETE	6.11 ± 3.59	5.47 ± 2.76	0.355
8-HETE	1.31 ± 0.87	1.19 ± 0.54	0.136
11-HETE	1.61 ± 0.72	1.60 ± 0.49	0.421
12-HETE	32.83 ± 30.68	23.83 ± 17.61	0.040
PGE_2	4.61 ± 4.27	5.01 ± 5.10	0.730
PGD_2	2.11 ± 1.75	2.41 ± 2.11	0.800

Abbreviations: HDHA: hydroxydocosahexaenoic acid; HETE: hydroxyeicosatetraenoic acid, HEPE: hydroxyeicosapentaenoic acid.

[1] Differences between the allergic and non-allergic groups were examined with ANCOVA, adjusted for gender, age, school, total body iron and serum zinc. Data were log-transformed before analyses. $P < 0.05$ was considered significant. Data were expressed in pg/µl.

[a] Mean ± SD (all such values).

Figure captions

Fig. 1. N-6 and n-3 polyunsaturated fatty acid desaturation and elongation pathways and selected immune modulating metabolites.

Fig. 2. Scatter plot representing the negative association between PBMC arachidonic acid and elaidic acid (*trans*-C18:1n-9), r_P = –0.453 and *P* = 0.002 (n =44).

Supplemental Fig. 1. Typical plasma lipid mediator chromatogram with multiple reaction monitoring (MRM) transitions. Target and qualifier transitions are shown for each compound.