Metabolic Groups Related to Blood Vitamin Levels and Inflammatory Biomarkers in Brazilian Children and Adolescents

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Summary

Certain B-vitamins and vitamin A may be involved in inflammatory pathways associated with homocysteine and omega-3 fatty acids. The aims of this study were (i) to determine whether different metabolic profiles of B-vitamins and vitamin A in Brazilian children and adolescents were positively or negatively related to homocysteine and omega-3 fatty acids using k-means clustering analysis, (ii) compare nutrient intakes and metabolites between the different metabolic profiles, (iii) evaluate if the statistically significant metabolites found between the metabolic groups, can predict the variation of leukotriene A4 hydrolase (LTA4H) levels, a biomarker of low-grade inflammation, in the total studied population. This cross-sectional study included 124 children and adolescents, aged 9–13 y old. Dietary intake was assessed by the food frequency questionnaire and 24-hour recall. Biomarkers for vitamins B2, B6, B12, folate and vitamin A were measured in plasma. Omega-3 fatty acids and homocysteine were measured in red blood cells (RBC). Two different metabolic profiles were found. Thirty of these individuals had overall average higher riboflavin, pyridoxal, and vitamin B12 plasma levels (metabolic group 1) compared to 94 individuals (group 2). Group 2 had lower dietary intake of vitamin B2, vitamin A, and vitamin B12 and higher RBC levels of homocysteine. EPA and DHA erythrocyte levels were not different between metabolic groups. Multiple linear regression analyses showed that blood cobalamin, riboflavin, pyridoxal and homocysteine combined, explained 9.0% of LTA4H levels variation in the total studied population. The metabolic group that had low plasma levels of riboflavin, pyridoxal, and cobalamin also had a lower dietary intake of B-vitamin and higher RBC homocysteine. The combined levels of the riboflavin, pyridoxal, cobalamin and homocysteine biomarkers can predict the variation of LTA4H in the total population studied, but it is not clear how this regulation occurs.

Key Words

vitamin A, B-vitamin, metabolic profile, homocysteine, fatty acids, inflammation

Metabolomics analyses provide a dynamic depiction of metabolic status (1) and have been successfully used in several fields of research, including disease diagnosis (2), screening of biomarkers (3), and biochemical alterations associated with metabolites, such as levels of homocysteine, polyunsaturated fatty acids (4), and leukotrienes in human serum.

Leukotriene A4 (LTA4) is one of many derivatives from arachidonic acid in the eicosanoid pathway (5) and is metabolized by leukotriene A4 hydrolase (LTA4H) into leukotriene B4 (LTB4), that is known to facilitate chemotaxis of inflammatory cells, angiogenesis, and cell proliferation. LTA4H has been the focus of research related atherosclerotic genomic because of its role in low grade inflammation (6, 7), carotid intima media thickness (8), myocardial infarction, coronary artery disease and ischemic stroke (9–12).

Like leukotriene A4 hydrolase (LTA4H) (6, 7), increased total homocysteine (tHcy) has also been considered an independent predictor of low-grade inflammation, atherosclerosis process and all-cause mortality (13, 14). Homocysteine is a sulphur-containing amino acid whose metabolism is at the intersection of two pathways: remethylation to methionine, which requires folate and vitamin B12, and transsulfuration to cystathionine which requires vitamin B6.

Evidence suggests that low-grade inflammation has a fundamental role in all stages of the atherosclerotic

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process and several studies have confirmed that unbalanced nutrition can contribute to a state of low-grade inflammation during childhood (15–17), especially in those who are overweight (18).

Epidemiological and experimental evidence also indicates a role for certain B-vitamins in preventing or delaying onset of cardiovascular disease (CVD) (19). Although the mechanisms cannot be interrogated in human studies, active forms of folate, vitamin B12, vitamin B6, and vitamin B2 are involved in the reactions and metabolism of the S-containing amino acid, homocysteine. Circulating levels of tHcy are accepted as sensitive markers of folic acid and vitamin B12 status (20–22) but tHcy levels could also be related to levels of other metabolites. For example, the HELENA study (23) showed that plasma levels of folate, cobalamin, and tHcy were also associated with metabolites in the omega-3 fatty acid pathway. High red blood cell (RBC) levels of S-adenosylmethionine/S-adenosylhomocysteine (SAM/SAH) in children and adolescents have also been correlated with high vitamin A levels (24).

Hence, B-vitamins, fatty acids and vitamin A may also modulate key pathways involved in inflammatory processes (25). Therefore, using the plasma vitamins retinol, β-carotene, riboflavin, pyridoxal, 5-MTHF, and cobalamin in an unsupervised clustering approach, that considers the nutrient-nutrient interaction, may help us identify metabolic groups that may be related to RBC homocysteine and omega-3 fatty acids, both known to be involved in a low-grade inflammation and atherosclerosis process. Describing individuals with better or worse metabolic profile, would help health professionals to better treat them and avoid CVD in the future. This strategy identifies individuals who deserve to be monitored and to receive nutritional counseling.

Several algorithms and tools have been developed which utilize available metabolic data to find novel heterogeneous metabolic pathways. The k-means algorithm is an approach that begins by randomly initializing k centroids and then assigning each metabolite to the closest centroid. It was used in this study because it is most appropriate for the task, is very well accepted, and requires only the number of clusters and feature matrix as input (26).

The aims of this study were (i) to determine whether different metabolic profiles of B-vitamins and vitamin A are positively or negatively related to RBC homocysteine and omega-3 fatty acids concentrations using k-means clustering analysis, (ii) compare nutrient intakes and metabolites between the different metabolic profiles in children and adolescents, and (iii) evaluate if the statistically significant metabolites found between the metabolic groups, can predict the variation of leukotriene A4 hydrolase (LTA4H) levels, a biomarker of low-grade inflammation and atherosclerosis, in the total studied population. Our hypothesis was that individuals with the highest levels of some plasma B-vitamins and vitamin A (metabolic group) will have the lowest RBC homocysteine and highest RBC levels of omega-3 fatty acids and that these metabolic groups will be related to intake of specific nutrients.

**MATERIALS AND METHODS**

Population and study design. Participants in this study were children and adolescents (9 to 13 y old) who were included based on their assent and written informed consent of their guardians. Data collection was performed in 2013 and 2014, at the Ribeirão Preto Medical School Hospital (HCRP), University of São Paulo, Brazil. The study was approved by the internal Ethics Committee (Process HCRP No. 14255/2010) and by the National Research Ethics Commission (No. 00969412.6 CAAE. 0000.5440). The trial was registered on ClinicalTrials.gov (NCT01823744).

Exclusion criteria were individuals: (i) with one or more episodes of axillary temperature higher than 37°C in the 15 d preceding the blood collection, (ii) with three or more episodes of liquid stools in the 24 h before assessment, (iii) with intake of any kind of vitamin or mineral supplement, (iv) on a supervised diet for reducing weight or any other type of dietary restriction, (v) with a diagnosis of chronic disease that may interfere with data collection, (vi) that participated in another clinical trial in the 4 wk preceding the study, and (vii) outliers values for RBC homocysteine and omega-3 fatty acids concentrations (EPA and DHA).

Dietary intake and metabolomics data described in this cross-sectional, convenient sample size study were from a crossover N-of-1 intervention study (27) where metabolites in plasma, RBC, and nutritional status were assessed at baseline (visit 1), after 6 wk (5 d a week only) of daily supplementation of 12 vitamins and 5 minerals (visit 2), and after 6 wk of a washout period (visit 3). To avoid the influence of supplements on plasma metabolite concentrations, metabolomics data from visit 1 only were used in the analysis reported here. However, three 24 h recall were used to reduce inconsistencies in reported diet intakes. Therefore, all the methodology and data in this study are similar to Mathias et al. (27), except that metabolomic and food frequency questionnaire (FFQ) data were only from baseline (visit 1). Twenty graduate students were formally trained as interviewers for nutritional assessment for this project. Principal component analysis (PCA) of intake data indicated no bias between interviewers.

This is a cross-sectional, convenient sample size study. A total of 280 children and adolescents (151 in 2013 and 149 in 2014) from 3 schools met the inclusion and exclusion criteria and were included in the study. Characteristics of the schools are detailed in Mathias et al. (27). One hundred and forty-seven individuals met criteria for under and over reporters of energy intake. After K-means clustering analysis with K=5 for this project. Principal component analysis (PCA) of intake data indicated no bias between interviewers.

Data collection. All participants were assessed for anthropometric, pubertal (28), and economic status (29). Height and weight were measured by a dietitian immediately after blood collection, according to the procedures described by Jellife (30) and World Health Orga-
nization (WHO). Body mass index (BMI) was calculated and used for nutritional status classification according to WHO (31). 12 hour-fasting blood samples were taken immediately after check-in procedures were completed. Blood was collected in EDTA tubes for clinical biochemistry, metabolomics, and RBC fatty acid profiling. All samples were coded at the time of collection, centrifuged, aliquoted, and frozen at $-80^\circ$C for further analyses. Trained phlebotomists performed blood draws.

**Dietary assessment.** The nutritive value of the foods was analyzed for energy (kcal), carbohydrate (g), protein (g), lipids (g), total cholesterol (mg), omega-3 fatty acids (g), and vitamins A (mg), B2 (mg), B12 (mg), B6 (mg), folic acid (mg). Diet data were double-entered into the program to reduce inconsistencies.

Diet Win Professional® software version 2011 (www.dietwin.com.br) was used to analyze dietary intake data. It has more than 5,000 food items from 6 food composition databases (TACO/Brazil, IBGE, USDA, CENEXA, Germany, and General Directory of Food) and more than 1,300 recipes. Dietary intakes were assessed by three 24 h dietary recalls at baseline (visit 1), and then 6 (visit 2) and 12 (visit 3) wk later. One parent or guardian was also present for the dietary intake assessment. Two-week days and one weekend were considered per each child. The 24 h recall was used in a stepwise method adapted (32) to assess a participant’s food intake. To estimate the usual intake using multiple 24 h recalls, intra-individual variability was removed (33) using the multiple source method (MSM) (34).

The usual intake was also evaluated using the Brazilian FFQ (35). The children and adolescents and their legal representatives were asked about frequency of food intake and portion sizes during the previous month using a book (36) which has photos of small, medium, and large portion sizes of foods typically consumed in Brazil. Intakes of foods not described in this tool were also incorporated in the FFQ. The estimated average requirement (EAR) and the adequate intake (AI) from Dietary Reference Intake were used to determine if the population nutrient intake was adequate (37, 38). The data reported in the FFQ were transformed into daily frequencies according to Araújo et al. 2010 (39). After determining these scores, they were multiplied by the size of food portion consumed by the participant.

**Laboratory analyses.** Fasted (12 h) blood samples (total of 21 mL) were taken immediately after check-in procedures were completed. Blood was collected in EDTA tubes for metabolomics, RBC fatty acid profiling, and in ACD tubes for clinical biochemistry. All samples were coded at the time of collection, centrifuged, aliquoted, and frozen at $-80^\circ$C for further analyses. Clinical biochemistry analyses were done immediately after the blood draw in the HCRP Laboratory using standard procedures on a Weiner Lab CT 600i (Diamond Diagnostics). Metabolomic analysis of EDTA samples were done at the Nestlé Institute of Health Sciences, Nestlé Research Center laboratories (Lausanne, Switzerland), and at Vitas Analytical Services (Oslo, Norway). Analysis of plasma metabolite levels were fully described in Mathias et al. (27) and in protocols.io: dx.doi.org/10.17504/protocols.io.vd4e28w.

In brief, vitamin B2 (riboflavin) and vitamin B6 (pyridoxine) were measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) operating in positive electrospray ionization (ESI+) mode using published methods (40). 5-Methyltetrahydrofolate
(5-MTHF) was analyzed by reverse phase ultra-high-performance liquid chromatography (RP-UHPLC) with mass spectrometry detection (MS) operating in positive electrospray ionization (ESI+) at unit resolution. Retinol and β-carotene were analyzed with ultra-performance liquid chromatography (UPLC).

Homocysteine was measured by LC-MS/MS and the methodology is fully described in Da Silva et al. (41). Vitamin B12 was analyzed by Vitas Analytical Services (Oslo, Norway) through AM-396 and MonoBind ELISA (Folate/Vitamin B12 Anemia Panel V AST test system, Monobind, Lake Forest, CA 92630, USA).

Leukotriene A4 hydrolase (LTA4H), was measured through SomaLogic proteomics assay® (SomaLogic Inc., 2945 Wilderness Place, Boulder, CO 80301, USA), using DNA Aptamers, which are the equivalent of monoclonal antibodies. In brief, the specific protein to be measured binds tightly to its cognate SOMAmer (slow off-rate modified aptamer) binding molecule, which includes a photo-cleavable biotin (PCB) and fluorescent label at the 5’end. Bound protein-SOMAmer complexes are captured onto streptavidin coated beads (SA) by PCB on the SOMAmer. Unbound proteins are washed away. Bound proteins are tagged with biotin. The PCB is cleaved by UV light and the protein-SOMAmer complexes are released into solution. SOMAmers are quantified by hybridization. Each probe spot is proportional to the amount of SOMAmer recovered, which is proportional to the amount of protein present in the original sample (42, 43).

RBC omega-3 fatty acids (eicosapentaenoic fatty acid – EPA, and docosahexaenoic acid – DHA) were analyzed by gas-liquid chromatography. Reagents and methods are fully described in Masood et al. (44) and Destaillats et al. (45). C-reactive protein was measured by immunoturbidimetric method (commercial kit PCR ultrasensible®, Turbitest line AA, Wiener lab 2000 Rosario, Argentina).

The prevalence of deficiencies and insufficiencies in this study were determined using CDC cutoff references values and international references (46) and are reported in Mathias et al. (27), as follow: plasma retinol (0.2 μg/mL), β-carotene (6 to 11 y, 0.13 μg/mL; 12 to 19 y, 0.09 μg/mL), riboflavin (20.1 nmol/L), pyridoxal (21.1 nmol/L). 5-MTHF (91 nmol/L), and cobalamin (728 pg/mL).

Dyslipidemias were defined for Brazilian children and adolescents (47) as total cholesterol >200 mg/dL and/or LDL-c >130 mg/dL, and/or triglycerides >100 for 9 y old or >130 mg/dL for 10 to 13 y old. tHcy levels under 15 μmol/L were considered normal values (24).

Statistical analyses. All statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS), version 20.0®. All variables were log-transformed for correlation analyses. Due to outliers related to under and over reporters, the participants who ate lower than 0.79×basal metabolic rate or higher than 2.4×basal metabolic rate were excluded from all analysis (48, 49).

The plasma vitamins retinol, β-carotene, riboflavin, pyridoxal, 5-MTHF and cobalamin were analyzed using K-means clustering and ANOVA to identify individuals with similar metabolic profiles. Linear regression covariance analyses (ANCOVA) adjusted for age, sex, BMI, and for energy were applied to compare groups. Because β-carotene and retinol plasma concentrations may be influenced by lipoprotein variability and result in variation in the plasma level of these nutrients, analyses of these metabolites were adjusted by including total cholesterol in the ANCOVA analyses. Because essential linolenic fatty acid and linoleic fatty acid compete for the same desaturase enzymes and result in variation of omega-3 fatty acids, analyses of EPA and DHA were adjusted by including linoleic and linolenic acids in ANCOVA model (50, 51).

T-test was applied to compare age and BMI, and Q-square was used to compare gender and overweight proportions between metabolic groups. The percentage of individuals within each group who were below EAR/AI for nutrient intake and below cutoff values for plasma vitamins were also analyzed according to Q-square test.

Multiple Linear Regression was applied to evaluate if the statistically different metabolites in the metabolic groups, could predict the variation of leukotriene A4 hydrolase (LTA4H) in the total studied population. All variables were log transformed for this analysis. The p-value<0.05 was considered significant for all statistical analyses.

RESULTS

The mean age was 11.7 (SD 1.1) and mean body mass index (BMI) was 19.6 (SD 4.2). The study sample was 46.9% male, 1.36% severely thin, 10.9% thin, 52.4% eutrophic, 18.4% overweight and 17% obese. No differences in energy and macronutrients intakes were found when we compared intake for energy, carbohydrate, protein and lipid by FFQ (1,953 kcal/d, 560, 265 g±86, 70 g±24, and 65 g±21) and 24 h (p=0.32).

K-means clustering identified groups of individuals with similar metabolite profiles. No distinct groups were found at K>2 but two groups at K=2 consisted of 124 out of 147 individuals. Metabolic group 1 had 30 participants and metabolic group 2 had 94 participants.

Table 1 shows demographics, nutritional status and biomarkers of these two metabolic groups. Pubertal status was similar between groups (mean 2.52, SD 0.90; p=0.43). There was no difference in proportions of overweight individuals between groups (p=0.76). Individuals in group 2 were characterized by a dietary intake containing less vitamin B2 (FFQ: p=0.03), vitamin A (24 h recall; 0.04), and vitamin B12 (24 h recall; p=0.03).

K-means clustering of participants was based on vitamin plasma levels for retinol, β-carotene, pyridoxal, riboflavin, cobalamin, and 5-MTHF. This technique randomly assigns each metabolite to the closest centroid and not all biomarkers will necessarily be statistically different between the two groups. Only riboflavin
Table 1. Nutritional status, nutrient intakes, plasma vitamins, red blood cell omega 3 fatty acids, red blood cell homocysteine levels, percentage of dyslipidemia and C-reactive protein between two different metabolic groups.*

| Variables | Metabolic Group 1 | Metabolic Group 2 | p-valueb |
|-----------|-------------------|-------------------|----------|
|           | n=10              | n=94              |          |
| Age (y)   | 11.6±0.9          | 11.6±1.13         | 0.82     |
| Sex (M/F)%| 26.6/73.3         | 52.1/47.8         | 0.01     |
| BMI (kg/m²) | 18.4±2.9         | 19.6±4.3          | 0.08     |
| Overweight (%) | 16.7            | 36                | 0.76     |

|          | 24 h              |                   |          |
|          |                   |                   |          |
| Energy (kcal/d) | 1.793±515      | 1.993±560          | 0.18     |
| Lipid (g/d) | 64.2±18.2        | 68.0±21.7         | 0.38     |
| Omega 3 (g/d) | 1.0±0.4         | 1.1±0.5          | 0.97     |
| Vitamin A (µg/d) | 515±293       | 437±324          | 0.05     |
| Folate (µg/d) | 109.6±74.0     | 107.4±73.0       | 0.31     |
| Vitamin B2 (mg/d) | 1.74±0.7      | 1.67±0.8         | 0.03     |
| Vitamin B6 (mg/d) | 1.3±0.5        | 1.4±0.6         | 0.91     |
| Vitamin B12 (µg/d) | 3.1±1.4        | 3.1±1.9         | 0.40     |

|          |                   |                   |          |
|          | Biomarkers        |                   |          |
| Retinol (µg/mL) | 0.4±0.1         | 0.4±0.1         | 0.50     |
| β-carotene (µg/mL) | 0.26±0.16      | 0.21±0.11       | 0.18     |
| Riboflavin (nmol/L) | 18.1±10.4     | 13.1±8.3        | 0.01     |
| Pyridoxal (nmol/L) | 8.9±2.9       | 8.0±3.0         | 0.04     |
| 5-MTHF (nmol/L) | 20.6±11.4      | 22.9±14.7       | 0.57     |
| Cobalamin (pg/mL) | 783±210        | 384±102         | 0.01     |
| EPA (mg/dL) | 0.4±0.2         | 0.5±0.2         | 0.12     |
| DHA, mg/dL | 3.8±1.9         | 4.4±2.1         | 0.09     |
| Linoleic fatty acids (mg/dL) | 15.3±4.7     | 16.6±4.2        | 0.04     |
| Homocysteine µmol/L | 2.5±0.6        | 2.9±0.8        | 0.04     |
| C-reactive protein (mg/dL) | 0.1±0.2       | 0.1±0.2        | 0.72     |
| % of dyslipidemia | 20              | 14              | 0.41     |

* Metabolic groups were derived from K-means clustering technique and ANOVA, using retinol, β-carotene, riboflavin, pyridoxal, 5-MTHF, and cobalamin plasma levels as variables.
** p<0.05.
*All results are presented in mean and SD although some were non-continuous variables.
†p value based on t-test, Q-square, and linear regression covariance analyses adjusted for sex, for energy intake, for plasma cholesterol, for linoleic fatty acid, accordingly.
BMI: Body Mass Index.
FFQ: Food Frequency Questionnaire.
5-MTHF: 5 methyltetrahydrofolate; DHA: red blood cells docosahexaenoic fatty acid; EPA: red blood cell eicosapentaenoic fatty acid.

(p=0.01), pyridoxal (p=0.04) and cobalamin (p=0.01) were different between the two groups. EPA and DHA erythrocyte levels were not different between metabolic groups. Levels of tHcy were in the normal range (under 15 µmol/L) for all participants but were higher in group 2 than in group 1 (p=0.04). The two confounding variables, C-reactive protein and percentage of dyslipidemia, may affect biomarkers plasma levels, but there
were no differences between groups ($p=0.72$ and $p=0.41$, respectively).

The percentage of individuals within each group who were (i) below EAR/AI for nutrient intake and (ii) below cutoff values for plasma vitamins were analyzed. A higher percentage of individuals in group 2 ate lower amounts of vitamin A assessed by FFQ ($63.8\%$ vs $43.3\%$; $p=0.04$), and had lower plasma levels of $\beta$-carotene ($10.6\%$ vs $0\%$; $p=0.02$) and riboflavin ($90.4\%$ vs $63.3\%$; $p=0.001$). All participants had plasma levels for pyridoxal and 5-MTHF below reference levels although 5-MTHF presented no statistical difference between groups. Dietary intake for vitamin B6 was above recommendation for all individuals regardless of group membership based on the 24 h dietary recall.

Multiple linear regression analyses applied to all subjects showed that, (i) although plasma cobalamin was the only biomarker statistically correlated with LTA4H, the B-vitamins were statistically correlated with each other in the regression model; and (ii) that cobalamin, riboflavin, pyridoxal and homocysteine combined, explained $9.0\%$ of LTA4H variation (ANOVA = 0.03) (Tables 2 and 3).

**DISCUSSION**

Characterizing healthy metabolic groups in children and adolescents may help prevent future non-communicable disease in this age-specification population. Brazilian children and adolescents with lower plasma status of riboflavin, pyridoxal, and cobalamin (metabolic group 2) (i) also had higher RBC levels of homocysteine, (ii) were eating foods containing less vitamin B2, vitamin A, and vitamin B12, (iii) had higher percentage of individuals eating lower amounts of vitamin A assessed by FFQ, and (iv) had higher percentage of individuals with plasma levels below cutoff for $\beta$-carotene and riboflavin. The big differences found comparing FFQ and 24 h dietary recall for vitamin B12, vitamin A and folate intakes are expected and explained by lower correlations for vitamin B12 intake between these two instruments (52), and by low to moderate agreement between these two instruments for foods consumed occasionally, such as fruits and dark green vegetables, which are good sources of vitamin A and folate (53–55).

Higher riboflavin and cobalamin plasma levels were consistently associated with decreased levels of $tHcy$. Table 2. Pearson correlation for leukotriene A4 hydrolase, homocysteine and plasma B-vitamins in all studied population.

| Variables$^a$ | Leukotriene A4 hydrolase |  | B-vitamins and homocysteine |
|---------------|---------------------------|-------------------------------------------------|
| Pyridoxal (nmol/L) vs. LTA4H | 0.18 | 0.06 | 0.37 $^*$ |
| Riboflavin (nmol/L) vs. LTA4H | 0.16 | 0.09 | 0.027 |
| Cobalamin (pg/mL) vs. LTA4H | 0.36 | 0.01* | 0.01* |
| Homocysteine ($\mu$mol/L) vs. LTA4H | $-0.005$ | 0.48 | $-0.01$ |

$^a$ All variables were log transformed.

$^*$ $p<0.05$.

Table 3. Biomarkers statistically different between metabolic groups, homocysteine and the association with leukotriene A4 hydrolase in all studied population. Results of multiple linear regression analysis.

| Variables$^a$ | Unstandardized B coefficient | $p$-value | 95% Confidence Interval |
|---------------|-------------------------------|-----------|-------------------------|
| Pyridoxal (nmol/L) | 0.012 | 0.49 | $-0.024$–$0.048$ |
| Riboflavin (nmol/L) | $-0.027$ | 0.81 | $-0.25$–$0.20$ |
| Cobalamin (pg/mL) | 0.38 | 0.01* | 0.10–0.66 |
| Homocysteine ($\mu$mol/L) | 0.236 | 0.37 | $-0.29$–$0.76$ |

$^a$ All variables were log transformed.

$^b$ Leukotriene A4 hydrolase as dependent variable; Pyridoxal, riboflavin, cobalamin and homocysteine as independent variables; ANOVA = 0.03; Durbin-Watson = 2.05.

$^*$ $p<0.05$. 
and reduced CVD risk (56–60). Significant decreases in tHcy concentration occur after vitamin supplementation (vitamin B12, vitamin E, vitamin A and folic acid) in pediatric heart transplant patients with tHcy levels higher than normal (61, 62). Others have shown that intake of vitamin B6 below EAR/AI was associated with low pyridoxal plasma levels which may increase homocysteine levels (63).

The associations between B-vitamins and omega-3 fatty acids are still unclear. The physiological mechanisms proposed by others (23, 64–67) are usually based on positive correlations between folate and specific fatty acids but additional research is needed to explain the mechanisms for the above vitamins. We did not find any association between the metabolic groups and omega-3 fatty acids. The results can be explained by the fact that energy, lipid and folate intakes, folate plasma levels, and omega-3 fatty acids intakes were not different between individuals assigned to the 2 metabolic groups.

The average levels of RBC tHcy in our study were very low, although higher in metabolic group 2, and in agreement with the Canadian Health Measures Survey and Canadian Laboratory Initiative on Pediatric Reference Intervals (CALIPER) (68). The CALIPER and others studies are establishing reference intervals for disease biomarkers, but major challenges still exist, including the need for a large and representative sample size, participant recruitment, cost, and genetic inter-individuality (69–72). The reference intervals (68) for tHcy have been between 1.7 to 10.6 μmol/L for 9 to 25 y old individuals, consistent with our data for the Brazilian population. Since tHcy levels were normal, even in metabolic group 2, and the participants were clinically stable, we were not expecting high values for plasma C-reactive protein (15).

This cluster analysis showed that lower plasma levels of riboflavin, pyridoxal, and cobalamin were related to higher RBC levels of tHcy and with lower dietary intake of vitamin B2, vitamin A, and vitamin B12. Therefore, differences in RBC tHcy were found between groups of participants categorized by distinct nutritional status for vitamin A, vitamin B2, vitamin B6, and vitamin B12, emphasizing the need to considerate person’s inter-variability in responses in future researchers. In addition, multiple regression analysis showed that 9.0% of LTA4H variation, a potent predictor of low-grade inflammation and atherosclerosis (8, 11), can be explained by combined blood riboflavin, pyridoxal, cobalamin and homocysteine levels in the total studied population. It shows the importance of characterizing healthy metabolic profiles in children and adolescents to help prevent chronic diseases and atherosclerosis in the future.

This is the first study associating B-vitamins and homocysteine with LTA4H. Nevertheless, the multiple linear model described a positive correlation, which does not mean cause-effect relationship. It is not possible to conclude which compound is influencing each other’s expression, since high LTA4H levels may mobilize B-vitamins to sites of inflammation, therefore, further studies are needed to explain the role of these metabolites regulating this potent biomarker of low-grade inflammation. The anti-inflammatory attributes of B-vitamins are promising. Vitamin B2 has been shown to improve omega-3 fatty acids, known to be anti-inflammatory compounds (67). Vitamin B6 can also be mobilized to sites of inflammation where it may serve as a co-factor in pathways producing metabolites with immunomodulating effects (73). It is important to note that all participants had plasma levels for pyridoxal below reference levels. Vitamin B12 has also been associated with inflammatory markers, such as interleukin 6 (74). However, there is no study evaluating the clinical significance of LTA4H variation. Hence, the explanation for the positive correlation among B-vitamins, homocysteine and LTA4H requires additional research to deconvolute how genetic variation of LTA4H (5, 11) (as well as other components of the immune system) interact with these plasma biomarkers.

The limitations of the present study included a non-population-based sampling, the small sample size, and the cross-sectional design of the study. The strengths were the categorization of individuals with different values for plasma B-vitamins variables (riboflavin, pyridoxal, and cobalamin) considering the nutrient-nutrient interaction and the exclusion of under and over-reporters.

In conclusion, the metabolic group with low plasma levels of B-vitamin biomarkers (riboflavin, pyridoxal, and vitamin B12) had also a lower dietary intake of B-vitamin and higher RBC homocysteine levels, and should be directed to nutritional counseling. The combined biomarkers riboflavin, pyridoxal, cobalamin and homocysteine levels can predict 9.0% of LTA4H variation in total studied population and, therefore, should be monitored in children and adolescents to avoid future non-communicable diseases, but further studies are needed to elucidate the role of these B-vitamins in regulating LTA4H.

Authorship

JPM and JK formulated the research question and the study design: MORVA, MGM, CACL, RGS, RBDT, JMC, EH, TTB, JSCJ, JK and JPM performed data collection; ACF A, FVU, SM, JK and JPM analyzed and interpreted the data; ACF A, FVU, SM, JK and JPM wrote the original draft, edited and revised the manuscript. All authors read and approved the final manuscript.

Disclosure of state of COI

SM and JK were employees of Nestlé Institute of Health Sciences (NIHS) when this study was conducted. JK is currently employed by Vydiant. Other authors do not have any conflict of interest.

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