Clinical Nutrition xxx (xxxx) xxx

Contents lists available at ScienceDirect

Clinical Nutrition

journal homepage: http://www.elsevier.com/locate/clnu

Original article

Impact of multi-micronutrient supplementation on lipidemia of children and adolescents

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ARTICLE INFO

Article history:
Received 23 February 2019
Accepted 24 September 2019

Keywords:
Micronutrients
Vitamins
Lipoproteins
Lipidomics
Lipidemia
Cardiovascular health

SUMMARY

Background: Micronutrient supplementation has been extensively explored as a strategy to improve health and reduce risk of chronic diseases. Fat-soluble vitamins like A and E with their antioxidant properties and mechanistic interactions with lipoproteins, have potentially a key impact on lipid metabolism and lipidemia.
Objective: The impact of micronutrients on lipid metabolism requires further investigation including characterization of plasma lipidome following supplementation and any cause-effect on circulating lipids.
Design: In this study, we elucidate the effect and associations of a multi-micronutrient intervention in Brazilian children and teens with lipoprotein alterations and lipid metabolism.
Results: Our analysis suggests a combination of short and long-term impact of supplementation on lipid metabolism, potentially mediated primarily by α-tocopherol (vitamin E) and retinol (vitamin A). Among the lipid classes, levels of phospholipids, lysophospholipids, and cholesterol esters were impacted the most along with differential incorporation of stearic, palmitic, oleic and arachidonic acids. Integrated analysis with proteomic data suggested potential links to supplementation-mediated alterations in protein levels of phospholipases and pyruvate dehydrogenase kinase 1 (PDK1).
Conclusions: Associations between the observed differences in lipidemia, total triglyceride, and VLDL-cholesterol levels suggest that micronutrients may play a role in reducing these risk factors for cardiovascular disease in children. This would require further investigation.

1. Introduction

Micronutrients have critical functions in fetal programming [1] through aging [2]. Deficiencies in micronutrient status can severely impact growth and development [3], skeletal and extra skeletal processes [4], and cardiovascular health [5,6]. Supplementation of diet with one or combinations of vitamins and minerals have been explored with the aim of reducing chronic diseases [7–12], usually in adults. Specifically, the impact on cardiovascular health of micronutrients, particularly vitamins A, C and E [5,13–16], has recently been reviewed [17]. Vitamin E has also been used to treat nonalcoholic steatohepatitis in children [18] and nonalcoholic fatty liver disease in children and adolescents [19].

Fat-soluble vitamins, such as vitamin A precursors and vitamin E, act as free radical scavengers and are part of the antioxidant defense system [20] which also includes Cu, Zn and Mn. Deficiencies in any of these components decreases antioxidant protection. After absorption in the intestines, vitamin E and A are packaged into the chylomicrons on route to be incorporated into very low density lipoproteins (VLDL). Changes in lipid metabolism may alter any process dependent on these vitamins. Several other vitamins also play key mechanistic roles in lipid metabolism [21] specifically vitamin A [22,23], B12 [24], B6 [25],

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https://doi.org/10.1016/j.clnu.2019.09.010

Abbreviation list

PDK1	Pyruvate Dehydrogenase Kinase 1
VLDL	very low density lipoproteins
IDL	intermediate density lipoproteins
LDL	low density lipoproteins
HDL	high density lipoproteins
CE	cholesteryl ester
SE	sterol ester
DAG	diacylglycerol
TAG	triacylglycerol
Cer	ceramide
SM	sphingomyelin
LPC	lysophosphatidylcholine
LPE	lysophosphatidylethanolamine
LPI	lysophosphatidylinositol
LPS	lysophosphatidylserine
LPG	lysophosphatidylglycerol
LPA	lysophosphatidic acid
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PI	phosphatidylinositol
PS	phosphatidylserine
PG	phosphatidylglycerol
PA	phosphatidic acid
FDR	false discovery rate
LDL-c	LDL-cholesterol
V1	Visit 1
V2	Visit 2
V3	Visit 3
PLA2G2A	phospholipase A2 group IIA
PLA2G10	phospholipase A2 group 10
PLA2G5	phospholipase A2 group V
PLA2G1B	phospholipase A2 group 1B
PLA2G2E	phospholipase A2 group 2E

and D [26]. Potential associations between multi-micronutrient uptake and lipid profiles have also been conducted in children [27] and adults [28]. However, these studies analyzed changes in HDL, LDL, VLDL levels but not specific lipid species which limits mechanistic insights into changes in lipid metabolism. In this study, we investigated the effects and associations of a multi-micronutrient intervention with lipoproteins alterations. Plasma lipidome and proteome were analyzed in samples from a 6-week multivitamin/mineral intervention [29] in 9–13 year olds in Brazil.

2. Materials and methods

2.1. Clinical trial

The clinical trial including the study design has previously been described in detail by *Mathias* et al. [29]. Briefly, healthy children and adolescents (aged between 9 and 13 years, 11 months and 29 days) from three schools in the west side of Ribeirão Preto (Administrative district 2), Brazil, were provided micronutrients (Nestrovit™) that included vitamin A, vitamin E, folate, vitamin B1, vitamin B2, niacin, vitamin B6, vitamin B12, vitamin D3, vitamin C, biotin, vitamin B5 and calcium, phosphorus, iron, magnesium and zinc for 5 days a week for 6 weeks (March to June, 2013 and 2014) in each of 2 successive years. Summary of the dose of each supplemented micronutrient is provided in Supplementary Table 1. The dose of 2 bars for ages 9–11 and 3 for ages 11 + corresponded to approximately 100% daily recommended allowances but less than upper tolerable level for most nutrients (supplementary table and *Mathias* et al. [29]). Six of the authors from the *Mathias* et al. [29] study individually monitored supplement intake at the beginning of each school period to ensure the supplement was taken by the individual. The adherence rate was 98% over both years. We report data (Supplementary Data 1) from the first year of the study, which included a six week intervention period followed by a six week washout period. Visit 1 (V1) is defined as the visit before the start of the intervention period. Visit 2 (V2) is defined as the visit upon completion of the six week intervention period and visit 3 (V3) is defined as the visit upon completion of the six week washout period. Assessments at each visit included anthropometrics, blood biochemistry, vitamins, lipidomics, proteomics and dietary habits. Dietary habits were also assessed (details in *Mathias* et al. [29]). 141 children and adolescents were enrolled in baseline with 135 participants completing the six week intervention and subsequent six week washout part of the study. Table summarizing the demographic information and general health information of the participants is provided in Supplementary Table 2. Energy, carbohydrate, lipid, fiber and protein intake assessments of the participants is provided in Supplementary Data 2 following the methods described in *Mathias* et al. [29]. The trial was registered on ClinTrials.gov (NCT01823744).

2.2. Lipidomics analysis

We conducted comprehensive lipidomics analysis on fasted (12 h) plasma samples. Lipid extraction and analysis were performed as reported previously [30]. In summary, plasma was diluted 50 fold with 150 mM ammonium bicarbonate aqueous solution. 180 μL diluted plasma was pipetted in a polypropylene deep well plate and 810 μL methyl tert-butyl ether/methanol (7:2, v/v) containing internal standards was added to extract lipids. The internal standard mixture contained cholesterol-d6, cholesteryl ester (CE) 20:0, diacylglycerol (DAG) 17:0/17:0, triacylglycerol (TAG) 17:0/17:0/17:0, ceramide (Cer) 18:1;2/17:0, sphingomyelin (SM) 18:1;2/12:0, lysophosphatidylcholine (LPC) 12:0, lysophosphatidylethanolamine (LPE) 17:1, lysophosphatidylinositol (LPI) 13:0, lysophosphatidylserine (LPS) 13:0, lysophosphatidylglycerol (LPG) 17:1, lysophosphatidic acid (LPA) 17:0, phosphatidylcholine (PC) 17:0/17:0, phosphatidylethanolamine (PE) 17:0/17:0, phosphatidylinositol (PI) 16:0/16:0, phosphatidylserine (PS) 17:0/17:0, phosphatidylglycerol (PG) 17:0/17:0, and phosphatidic acid (PA) 17:0/17:0. The plate was shaken for 15 min at 4 °C and centrifuged for 5 min at 300 g to separate phases. 100 μL of the organic phase was transferred to a 96-well plate and dried under vacuum. Lipid extract was reconstituted in 40 μL of 7.5 mM ammonium acetate in chloroform/methanol/propanol (1:2:4, V/V/V) and immediately analyzed.

Samples were analyzed by direct infusion in a QExactive mass spectrometer (Thermo Fisher Scientific) equipped with a TriVersa NanoMate ion source (Advion Biosciences). In brief, 5 μL of extract was infused during 5 min and analyzed in both polarities in a single acquisition. Cholesterol, CE, DAG, and TAG were monitored in FTMS positive mode while phospholipids were monitored in negative mode. For each polarities, MS full scans (Rm/z = 200 = 140 000) were followed by a MS/MS data-independent analysis over a similar mass range with a resolving power of 17 500 (at $m/z = 200$) and an isolation width of 1.0 Da.

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All data was analyzed with in-house developed lipid identification software based on LipidXplorer. Data post-processing and normalization were performed on an in-house developed R based package.

2.3. Assessment of plasma 25-Hydroxy Vitamin D3 levels

25-Hydroxy Vitamin D3 levels were measured using published methods [31] at Vitas® (Oslo, Norway).

2.4. Proteomics analysis

For protein measurement, samples were analyzed using DNA-aptamer based recognition on the SOMAscan platform (SomaLogic, Boulder, CO, USA), as described by Gold et al. [32]. Median normalized relative fluorescence units (RFUs) were log2-transformed before applying principal component analysis and linear models [33]. Statistical analyses were performed in R 3.1.3 (R Foundation for Statistical Computing).

2.5. Baseline data processing

Of the 141 (V1) children and adolescents enrolled in the study, 136 participants completed all the assessments (i.e. participation and analysis in V2 and V3). Outliers in these participants were identified using principal component analysis for clinical and vitamin parameters separately and for each time point (as described earlier in *Mathias* et al. [29]). Outliers were defined as samples falling outside of the 99% confidence ellipse at any time point. Six individuals were identified as statistical outliers for clinical variables (namely Fat free mass (% of body weight), Fat mass (% of body weight), Glucose (mg/dL), Total Cholesterol (mg/dL), LDL Cholesterol (mg/dL), HDL Cholesterol (mg/dL), Triglycerides (mg/dL), Mean corpuscular volume (fl), Mean corpuscular hemoglobin (pg), Basophils (% of total white blood cells), Platelets (number of cells x 103/ul), Albumin (g/dL), Calcium (mmol/L), Iron (mg/dL) and Phosphate (mg/dL)). Six individuals were statistical outliers for circulating vitamin levels. These individuals were excluded from further analyses, thus leaving 123 participants for the lipidomics analysis. Additionally, we used the lipid measurements as a final filtering criterion. If any lipid species contained greater than 25 percent of missing values for any visit, they were not considered for further analysis. If greater than 25 percent of lipid measurements were not available for any individual, they were not considered for further analysis. Considering this work to be an extension to the *Mathias* et al. [29] study, where the exclusion criterion for vitamin and clinical variables were set to be 25% of missing values the same cut-off was used to avoid type II errors (false negatives) due to an insufficient sample size (n) and to be consistent with the previous analysis. Accordingly, four individuals were removed for further analysis. Ultimately, we retained 119 individuals for the analysis presented in this study.

2.6. Statistical and correlation analysis

The statistical comparisons were made between two visits (i.e. between V1−V2, V1−V3, V2−V3) individually. We used the Wilcoxon signed rank test, which is a nonparametric test for paired observations, for between visit comparisons. P-values were corrected for multiple testing calculating the false discovery rate (FDR) adjusted p-values using the Benjamini−Hochberg procedure [34] and the significance threshold was set to 0.05.

To assess the correlations between the different measurements, we used Spearmans Rank correlation. The P-values were similarly corrected for multiple testing calculating the false discovery rate (FDR) adjusted p-values using the Benjamini−Hochberg procedure [34] and the significance threshold was set to 0.05.

2.7. Ethics

The trial was registered on ClinTrials.gov (NCT01823744). Enrolled participants signed the statement of informed assent and a parent of each participant signed informed consent. Participants received Institutional Review Board (Brazilian National Ethics Committee, CONEP 00969412.6.0000.5440) approved compensation as well as breakfast and lunch following blood draws on each asses visit.

3. Results

3.1. Clinical study

The primary findings of the clinical trial including the study design has previously been described in detail by *Mathias* et al. [29] and *Toffano* et al. [35]. Briefly, the multi-micronutrients used in the study significantly and reproducibly impacted the levels of total cholesterol, LDL-cholesterol (LDL-c), glucose and nine circulating organic vitamin forms (including FMN, nudifloramide, pantothenic acid, pyridoxal, α-tocopherol, 5-methyltetrahydrofolic acid, folate, vitamin B12 and γ-tocopherol). The intervention did not significantly impact the plasma levels of circulating 25-Hydroxy Vitamin D3 levels (details in materials and methods), with the median levels of 57 nmol/L for V1, 65 nmol/L for V2 and 52 nmol/L for V3 (individual participant measurements are provided in Supplementary Table 3 and Supplementary Data 3. The decrease of average levels of blood lipids and glucose suggested that one or a combination of vitamins and minerals in the supplement influenced lipid metabolism, similar to findings in earlier studies [26,36−46]. We extended these findings to how the intervention affected lipid metabolism.

3.2. Lipidomics assessment

We conducted untargeted lipidomics of intact lipid species and identified DAGs (diacylglycerols), SEs (sterol esters), TAGs (triacylglycerols), free cholesterol, LPCs (lysophosphatidylcholines), LPAs (lysophosphatidic acids), LPEs (lysophosphatidylethanolamines), PIs (phosphatidylinositols), PCs (phosphatidylcholines) among others. After processing and cleaning detailed in materials and methods, all species reliably detected across different visits were used in this analysis. Subsequently, we also filtered lipids within each visit, based on 25 percent of missing measurements. Accordingly, some lipid species such as SE 20:0, TAG 48:3, TAG 54:2, PI 34:1, PE 38:4, PC 37:3, PC 40:4 were excluded from further analysis. In terms of the participants, we retained 119 participants for final lipidomic analysis (details in materials and methods).

Ten SEs, 7 TAGs, free cholesterol, 7 LPCs, 3 PI and 15 PCs were significantly different between at least one inter-visit comparison (Fig. 1). Overall, we observed statistically significant reduction in lipid levels upon intervention and also during washout. Fifteen lipids were reduced significantly between V1 and V2, suggesting a direct and rapid impact of intervention. These included 6 SEs, 3 PIs, 2 PCs, 2 LPCs, free cholesterol, and 1 LPE (Fig. 1). SEs levels were reduced the most (7.49−12.67% based on mean fold changes, Fig. 1).

Seventeen different lipids were statistically changed between V2 and V3 including 3 which also reduced between V1−V2 (SE 18:1, SE 20:3 and PC 36:2). These included 4 SEs, 7 TAGs and 6 PCs. The magnitude of reduction was 10.18−15.59% in SE levels, 12.51−21.25% in TAG levels and 8.18−14.69% in PC levels. Similarly, a slower but long-term impact of intervention and/or a new steady

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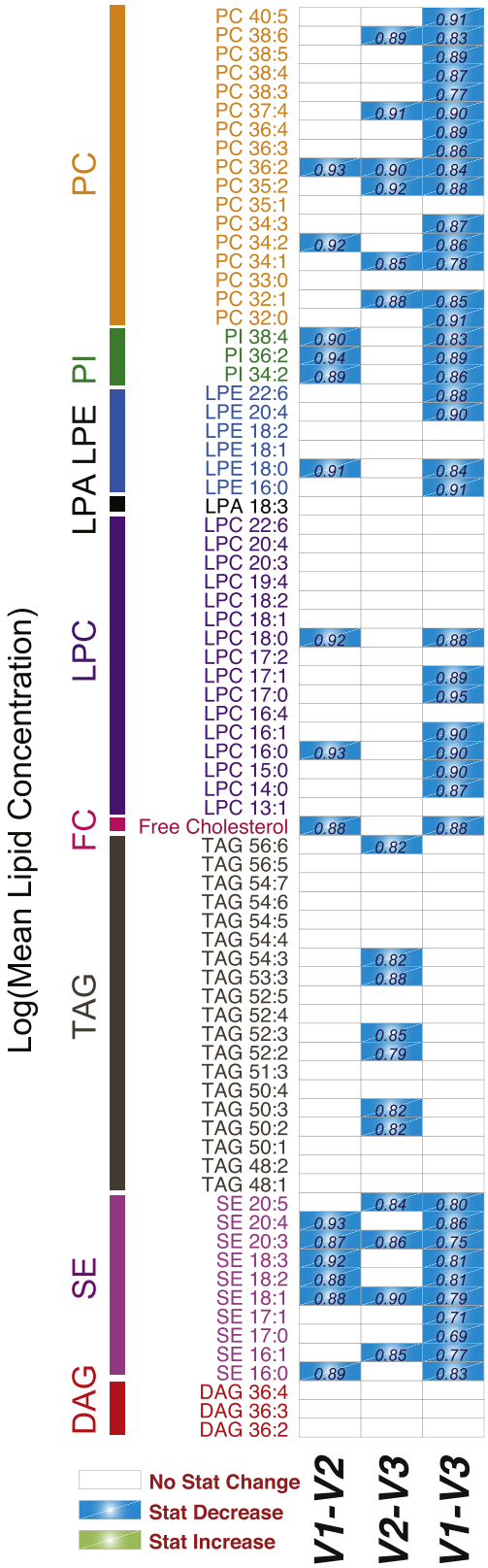

Fig. 1. Changes in lipid levels measured across visits. Increase or decrease in lipid levels in cross-visit comparisons are indicated by blue (decrease) or green (increase) filled boxes. Mean fold changes of lipids across visits are indicated by the numbers in the boxes. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

state between V1 and V3, was found with 40 significantly different lipids, including 10 which were different between V2–V3 and 15 lipids which differed between V1–V2. These included 10 SEs, 15 PCs, 7 LPCs, free cholesterol, 4 LPE and 3 PIs. The magnitude of reduction was 13.59–31.38% in SE levels, 9–23.21% in PC levels, 4.62–12% in LPC levels. All analyzed PCs, LPCs, and SEs had reduced levels between V1 and V2 and their levels were further reduced between V2–V3, indicating a potential long-term impact of the intervention. For example, the level of SE 20:3 was 12.68% lower at V2 compared to V1 and 25.29% reduced between V1 and V3. In comparison, free cholesterol levels were 12.25% lower between V1–V2 but 11.82% between V1–V3.

3.3. Relationship of lipid species to other parameters

The intervention led to reduction of the LDL-cholesterol levels measured through standard clinical chemistry methods [29]. While the intervention did not impact HDL-cholesterol, VLDL-cholesterol and triglycerides levels (defined by no statistically differences between V1 and V2), we observed that total cholesterol levels and total free cholesterol were lower after the intervention along when measured by untargeted lipidomics. To understand the relationships between lipoproteins and the specific lipid species, we analyzed the correlations between the changes in different lipid species in VLDL, LDL and HDL at each of the visits. Changes of levels of triglyceride and VLDL cholesterol levels at different visits were similarly correlated to changes in DAGs, TAGs, which reduced significantly during the washout phase (Fig. 2). Changes in composition of DAGs and TAGs are consistent with the changes in lipoprotein particles reported previously [47]: VLDL was enriched in TAGs, HDL had higher levels of phospholipids. HDL-cholesterol measurements were negatively correlated to the DAGs only during washout phase. LDL-cholesterol and total cholesterol were similarly correlated to SE 18:2 (Fig. 2) at visit 3.

We also investigated the correlations between the changes in lipids and circulating organic vitamin forms. Following the intervention, α-tocopherol was highly correlated to several PCs, PIs, TAGs, SEs and DAGs (Fig. 3). α-tocopherol and retinol were similarly correlated to several SEs when comparing V1 to V3, while the positive correlations between α-tocopherol and PIs and PCs remained. During the washout phase (V2–V3), α-tocopherol and retinol were strongly positively correlated to several lipids across different classes, including free cholesterol levels. γ-Tocopherol was positively correlated to PC 36:2, PC 34:2 and PI 36:2 during the intervention phase and several TAGs, DAG 36:4 and PC 34:3 during washout phase. α-β carotene was positively correlated to LPC 14:0 and SE 17:0 during the washout phase. 5-methyltetrahydrofolic acid was positively correlated to PC 38:4 during the intervention phase and with SE 17:0 during the washout phase (Fig. 3).

Overall, correlation analysis highlights the potential key role of fat soluble vitamins (e.g. A, E) more than the water soluble vitamins (e.g. B, C) and its potential interactions with the different lipids classes, specifically SEs, PCs, TAGs, DAGs and PIs. Fatty acid composition analysis of the differentially observed lipid species suggested that intervention led to reduction in incorporation of linoleic acid (C 18:2), palmitic acid (C 16:0), oleic acid (C 18:1), arachidonic acid (C 20:4), stearic acid (C 18:0), palmitoleic acid (C 16:1) (Fig. 4), in the decreasing order of number of species impacted.

3.4. Plasma proteomics analysis

Plasma proteins were analyzed in each participant at each visit using aptamer-based detection [32]. In general, the differentially observed proteins highlighted alterations in the host immune

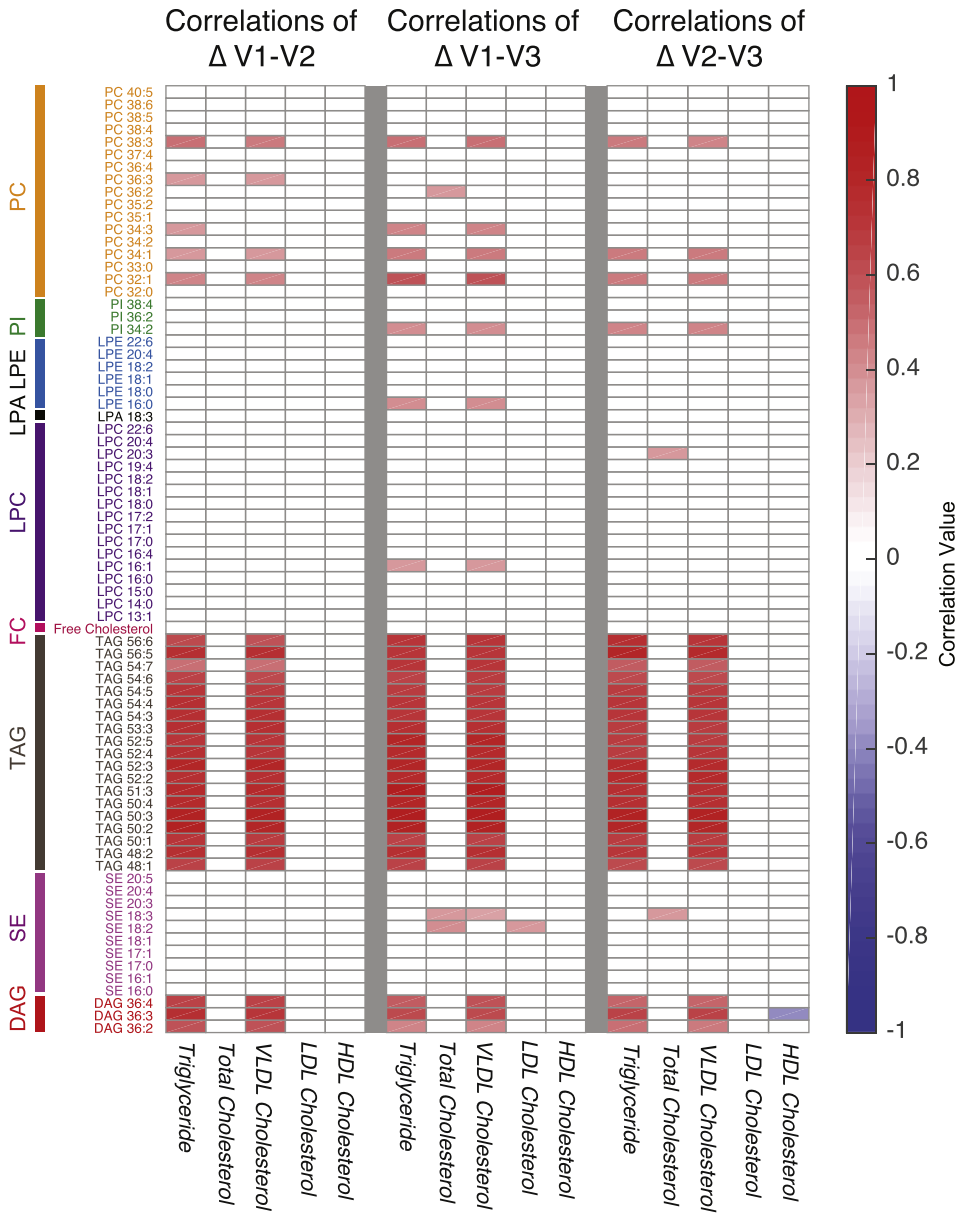

Fig. 2. Correlations of changes in measured lipid species (deltas between the visits) against changes in cholesterol measurements.

system, cytokine signaling, altered protein and lipid metabolism. We specifically focused on the potential connections to lipid metabolism. In that regard, we identified approximately 30 proteins, which were specifically impacted by the intervention (marked by statistically different levels between V1 and V2, P values after FDR correction < 0.05). These proteins were implicated in impacting phospholipid metabolism, glycerophospholipid metabolism, sphingolipid de novo biosynthesis and metabolism, synthesis of PA/PC and acyl chain remodeling of PI/PG/PS/PC/PE using the Reactome database [48]. Phospholipases (PLA2G2A, PLA2G10, PLA2G5), which mediate hydrolysis of phospholipids to fatty acids, were changed by the intervention. For example, PLA2G2A (phospholipase A2 group IIA) levels increased following the supplementation ($p = 0.0108$). In contrast, PLA2G10 (phospholipase A2 group 10 - $p = 0.00004$) and PLA2G5 (phospholipase A2 group V - $p = 0.0058$) levels decreased after the supplementation ($p = 0.00004$). PLA2G2A levels were not statistically different after the washout as compared to the baseline, indicating that vitamin supplements are needed for the effect. However, PLA2G10 ($p = 2.06e-08$) and PLA2G5 ($p = 0.0002$) levels were significantly lower after washout (V1−V3), indicating a long-term impact of supplementation. Long term (i.e. changes between V1 and V3 and not between V1 and V2) also altered the levels of phospholipases like PLA2G1B (phospholipase A2 group 1B, V3>V1, $p = 0.0279$) and PLA2G2E (phospholipase A2 group 2E, V1>V3, $p = 0.0166$). These changes may be directly due to the supplementation as described in earlier studies [48]. Additionally, we observed a decrease in PDK1 (pyruvate dehydrogenase kinase 1) levels upon supplementation (V1>V2, $p = 0.0024$) which recovered during washout (V3>V2, $p = 0.0023$). PDK1 plays a key role in regulating the formation of acetyl-coenzyme A from pyruvate. Potentially, these alterations in the protein levels could be a reason to alter the in-flow of fatty acids and potential alterations to the pool of different lipids (as demarcated in Fig. 4).

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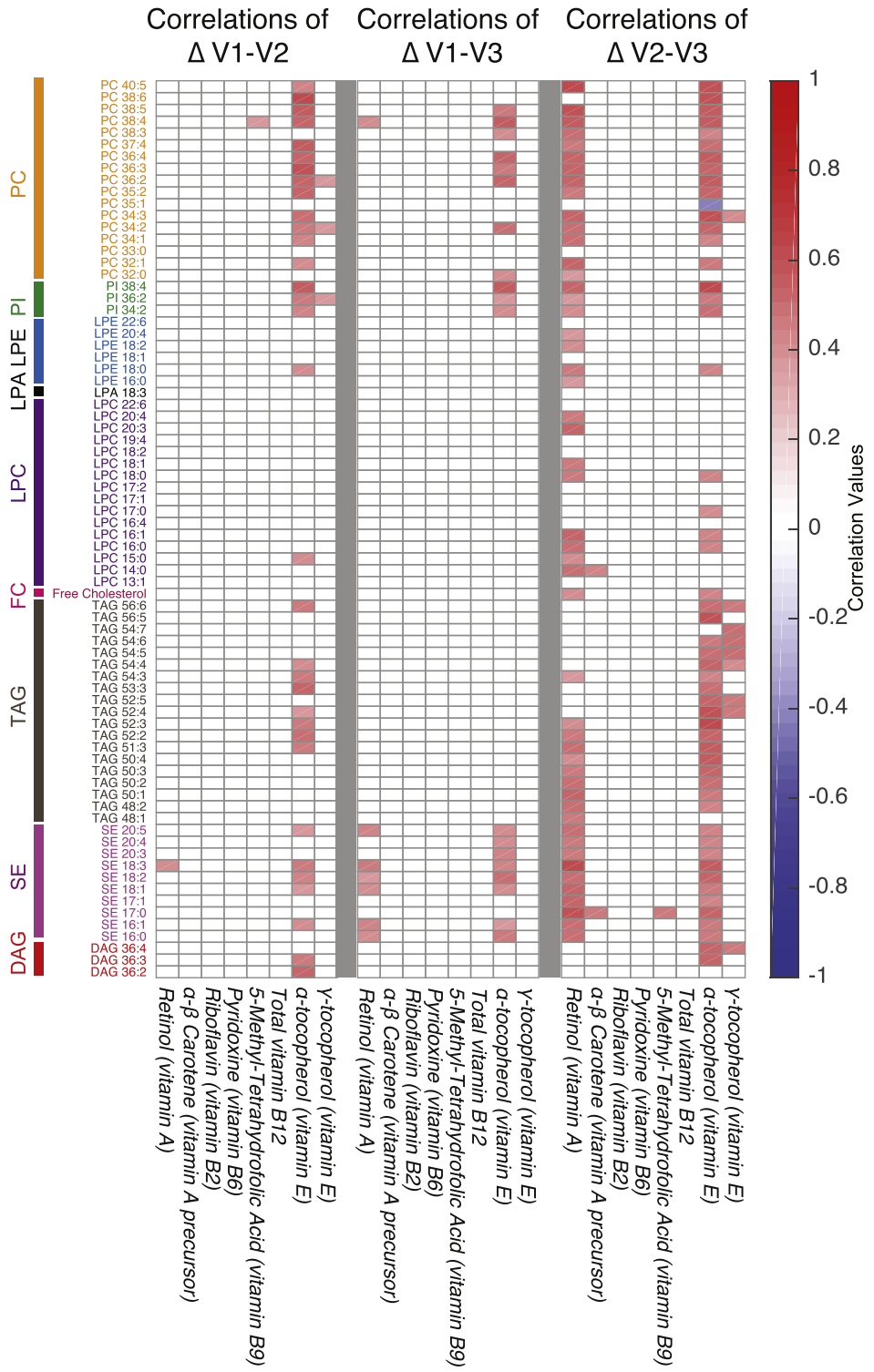

Fig. 3. Correlations of changes in measured lipid species (deltas between the visits) against changes in circulating vitamin forms.

4. Discussion

Micronutrients play a significant role in health and disease [1–16] with effects on clinical measures of plasma lipid levels [20–28]. However, changes in lipid species due to vitamin supplementation has not been well described. We focused on evaluating the impact of multi-micronutrient supplementation (vitamin A, vitamin E, folate, vitamin B1, vitamin B2, niacin, vitamin B6, vitamin B12, vitamin D3, vitamin C, biotin, vitamin B5 and calcium, phosphorus, iron, magnesium and zinc) to the circulating lipoprotein levels and lipidemia, thus highlighting the in-depth impact of micronutrient intervention on lipid metabolism and

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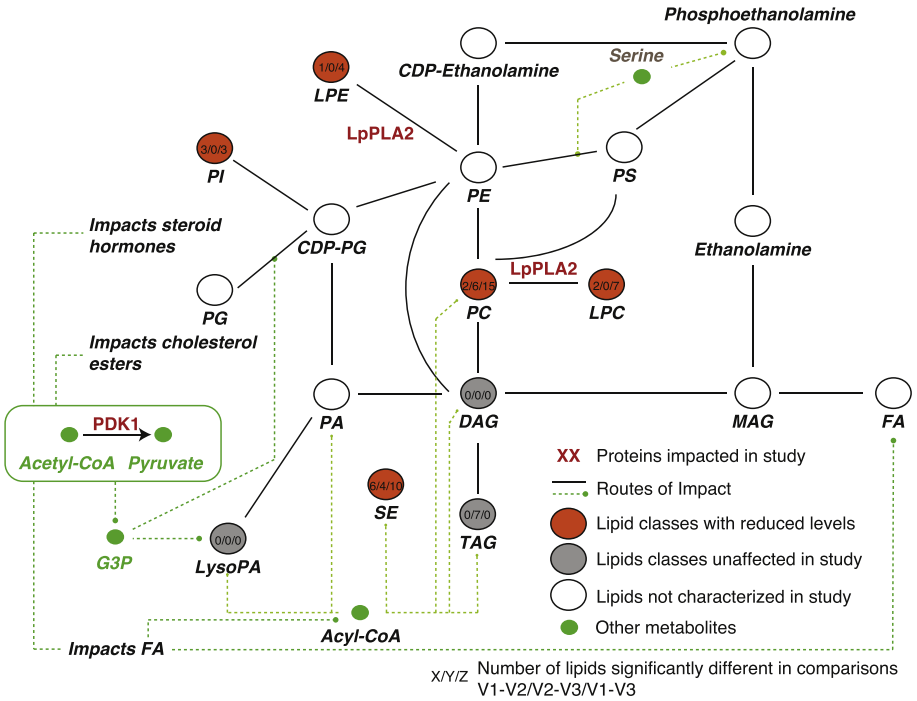

Fig. 4. Schematic overview of the observed alterations in lipids upon supplementation.

hypothesizing potential underlying mechanisms for these observations. The primary findings of the clinical study were previously presented by *Mathias* et al. [29].

Vitamin supplementation led to statistically significant reductions in the levels of several lipid classes, including SEs, PIs, PCs and LPCs (Fig. 1). Impact on some of the lipid species occurred only after the intervention (i.e. changed only between V1–V2), suggesting the presence of additional vitamins and minerals were required to maintain the lipid levels (e.g., free cholesterol). However, some lipid levels changed both between V1–V2 and during washout (V2–V3) in the same direction, indicating a potential long-term impact. In other cases, the levels of some lipid species were maintained throughout the washout period (V1–V3) or in other cases, between V2 and V3. Thus, supplementation had short-term and long-term impacts on selected lipid species. In the current study, we are limited to a 6-week window for both supplementation and washout. A longer chronic cross-over study would further elucidate the plasticity of the impacts on lipidemia.

Analysis of the lipid species suggested that supplementation led to reduction of species with at least one linoleic, oleic, palmitic, stearic and arachidonic acid in the side chains. For example, we found that linoleic acid in the form of PC, SE and TAGs, oleic acid in the form of TAGs, SE and PC, palmitic acid in the form of PC, SE, LPC, arachidonic acid in the form of SE, PI and PC and stearic acid in the form of PI, PC and LPC (both in short and long term) reduced upon supplementation. We also observed reduction in TAGs and SEs enriched in palmitic acid, which is considered to have a positive impact on metabolic health [50,51]. Our findings also agree with our previous observation that cholesteryl linoleate (SE 18:2) is the most abundant lipid species in LDL particles [47]. Elevated LDL-cholesterol and elevated TAGs amongst others are key for cardiovascular risk prediction and management. In that regard, micronutrient based reduction in LDL-cholesterol potentially mediated by the reduction in SEs could be vital in the cardiovascular risks in adulthood. Taken together, these results demonstrate that supplementation had a strong influence in determining the lipid profile and impacting the metabolism of some specific fatty acids namely linoleic, palmitic, oleic and stearic acids.

Changes in several lipid species (specifically DAGs, TAGs and PCs) were consistently positively correlated to changes in composition of specific lipids in triglyceride and VLDL cholesterol (Fig. 2). These results are relevant to reducing metabolic/cardiovascular risks. Additionally, changes in PCs, PIs, TAGs, SEs and DAGs were strongly correlated to the plasma levels of fat-soluble vitamin E (α-tocopherol). We previously reported that supplementation led to statistically significant increase in α-tocopherol levels, which reduced to below baseline levels upon washout (*Mathias* et al. [29]). The correlations between α-tocopherol and TAGs became even more pronounced during the washout phase. Vitamin A (retinol), which did not change significantly during supplementation but reduced during washout, was strongly correlated to several lipid classes including SEs, TAGs, LPCs, LPEs, PIs and PCs. A vitamin A deficient diet has a prooxidative effect and influences cellular and molecular regulation of phospholipid metabolism and function [52]. Deficiency of vitamin A has been shown to decrease liver phospholipid content in rats [53] and has been attributed to lower synthesis of phosphatidylcholine and lower availability of fatty acids [54], decrease of linoleic acid and a high level of saturated long-chain fatty acids, 16:0 and 18:0, in the heart mitochondrial phospholipids [55]. Mechanistically, vitamin E impacts phospholipase activity and directly impacts phospholipid metabolism [49]. One report found that hydrolysis of acyl groups of PC was inhibited by increasing dietary vitamin E [49]. Our observations of reduced levels of C16:0, C18:0 components in lipids and the correlations between the lipids and the vitamins (A and E) are consistent with these studies mentioned above.

To further explore the potential connections between vitamins supplementation and the lipids, we used proteomic analysis to characterize the plasma protein levels. Phospholipases A1 and A2 are the major enzymes responsible for generation of LPC from PC. Increase in vitamin E supplementation has been shown earlier to reduce phospholipases A1 and A2 activity [49]. We observed an

impact upon supplementation on the phospholipases, specially PLA2G2A protein levels increased and PLA2G10 and PLA2G5 levels decreased. Additionally, pyruvate kinase activity (indicator of myopathy) is reported to be reduced upon increase in vitamin E feeding [49]. Consistent with this, we noticed that the micronutrient supplementation led to reduction in the protein levels of PDK1.

Our analysis suggests that multi-micronutrient supplementation has a combination of a short-term and long-term impact on lipid metabolism with a significant impact on the circulating levels of phospholipids, lysophospholipids and cholesterol esters (Fig. 4). Combining these observations with the changes in observed protein levels of phospholipases and PDK1, we suggest a potential mechanism explaining these observations (Fig. 4). The levels of phospholipids changed and the incorporation of different fatty acids such as stearic, palmitic, oleic and arachidonic acids was also altered following the intervention. Correlation based analysis highlighted the potential importance of α-tocopherol (vitamin E) and retinol (vitamin A) in comparison to the other components in the supplement. Finally, the associations between the observed changes in lipid species and VLDL-cholesterol levels further elucidated the impact of micronutrient intervention on lipid metabolism. The changes in levels of these lipid species suggest that micronutrients may play a role in reducing the risk of cardiovascular diseases. However, this conclusion requires further investigation. While we assume that our study population is healthy (based on the exclusion criterion described in Mathias et al. [29]), we cannot control the presence of any unknown condition or disease that could influence the metabolism [56,57].

Authors' contributions

MM designed and performed the lipidomics study.
DMR analyzed the samples.
AC and MM analyzed and interpreted the data.
AC and MM wrote the manuscript.
JPM and JK performed the clinical study. All the authors read and contributed to the manuscript.

Conflict of Interest

AC, DMR, NC and MM were employees of Nestle SA. MM currently works at University Hospital Bern. ME works for Eidea Bioscience Ltd., JK works for Vydiant Inc. The study was funded by Nestle Institute of Health Sciences.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clnu.2019.09.010.

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Please cite this article as: Chakrabarti A et al., Impact of multi-micronutrient supplementation on lipidemia of children and adolescents, Clinical Nutrition, https://doi.org/10.1016/j.clnu.2019.09.010

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Please cite this article as: Chakrabarti A et al., Impact of multi-micronutrient supplementation on lipidemia of children and adolescents, Clinical Nutrition, https://doi.org/10.1016/j.clnu.2019.09.010