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Original article

An initial evaluation of newly proposed biomarker of zinc status in humans - linoleic acid: dihomo-γ-linolenic acid (LA:DGLA) ratio

Marija Knez [a, b, *], James C.R. Stangoulis [a], Manja Zec [b], Jasmina Debeljak-Martacic [b], Zoran Pavlovic [c], Mirjana Gurinovic [b], Maria Glibetic [b]

[a] School of Biological Sciences, Flinders University, Bedford Park, South Australia, Australia
[b] Centre of Research Excellence in Nutrition and Metabolism, Institute for Medical Research, University of Belgrade, 11000, Serbia
[c] Institute for Public Health Požarevac, Jovana Šerbanovića 14, 12000 Požarevac, Serbia

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SUMMARY

Background: Zinc is an essential micronutrient for humans with important physiological functions. A sensitive and specific biomarker for assessing Zn status is still needed.
Objective: The major aim of this study was to examine if the changes in the content of plasma phospholipid LA, DGLA and LA: DGLA ratio can be used to efficiently predict the dietary Zn intake and plasma Zn status of humans.
Methods: The study was performed on healthy human volunteers, 25–55 years of age. The dietary Zn intake was assessed using 24 h recall questionnaires. Plasma phospholipid fatty acid analysis was done by gas chromatography, and plasma analysis of minerals by atomic absorption spectrometry. Biochemical, anthropometrical and hematological parameters were assessed.
Results: No significant relationship was found between the dietary and plasma zinc status ($r = 0.07$; $p = 0.6$). There was a statistically significant correlation between DGLA and plasma Zn ($r = 0.39$, $p = 0.00$). No relationship was observed between the linoleic acid and plasma Zn, while there was a significant negative correlation between LA: DGLA ratio and plasma Zn status ($r = -0.35$, $p = 0.01$). Similarly, there were statistically significant difference in DGLA status ($p = 0.004$) and LA: DGLA ratio ($p = 0.042$) between the Zn formed groups.
Conclusions: This study is an initial step in evaluating LA: DGLA ratio as a biomarker of Zn status in humans. The results are encouraging as they show that concentration of DGLA is decreased and LA: DGLA ratio increased in people with lower dietary Zn intake. However, additional studies are needed to fully examine the sensitivity of this biomarker.

1. Introduction

Zinc (Zn) is an essential nutrient for human health with many important biological functions. It plays a significant role in growth and development, cell mediated immunity, protein synthesis, skin and bone metabolism, enzyme function, gene expression, and hormonal excretion [1–3]. Deficiency of Zn may severely affect the homeostasis of a biological system and insufficient Zn intake has profound consequences at all points of the human lifecycle, from the point of conception through to old age [4].

Zn deficiency is very common, with an estimated 2 billion people worldwide being affected by dietary Zn deficiency [5]. Zn inadequacy is identified as a major contributor to the burden of disease in developing countries [6,7].

Nonetheless, a suboptimal Zn status is not easily determined due to the lack of clinical signs and reliable biochemical indicators of Zn status. It is generally accepted that there is currently no specific, reliable biomarker of zinc status [4]. Out of 32 potential biomarkers from 46 publications in humans, serum/plasma zinc concentrations, hair Zn concentration and urinary Zn excretion are the only three biomarkers identified as potentially useful [4]. However, there are still considerable reservations in terms of

* Corresponding author. Bedford Park, Flinders University, Adelaide, SA, Australia. Tel.: +61 8 8201 7997.
E-mail addresses: marijaknez186@gmail.com, knez0004@flinders.edu.au (M. Knez).

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reliability of these biomarkers due to the effect of multiple confounders such as infection, inflammatory conditions and the time of last meal [8]. Similarly, the serum/plasma Zn biomarker is perceived as an unresponsive index of Zn nutritional status due to effective homeostatic regulation which responds to alterations in zinc intake, up-regulating absorption and conserving losses via GI tract and kidneys, when intakes fall [4]. Thus, it clear that there is a need for the development/discovery of a new biological marker of Zn status.

Recent studies by Reed et al., 2014 showed a significant negative correlation between dietary zinc deficiency and the ratio of erythrocyte phospholipid linoleic acid: dihomo-γ-linolenic acid (LA: DGLA) *in-vivo* using the chicken model, *Gallus Gallus*. The authors clearly demonstrated that LA: DGLA is able to differentiate zinc status between zinc adequate and zinc deficient subjects, showing that LA: DGLA ratio can be used as an effective tool to detect an early stage of zinc deficiency before the onset of symptoms [9]. It seems reasonable to postulate that the same relationship may exist in humans and that the LA: DGLA ratio may well prove to be a novel, effective, noninvasive, sensitive and reliable biomarker of Zn status in humans.

The major aim of this study was to assess the correlations of plasma phospholipid content of linoleic (LA, 18:2n-6), dihomo-γ-linolenic acid (DGLA, 20:3n-6) and LA: DGLA ratio, with plasma Zn status and dietary Zn intake in human subjects. This study presents an initial, and at the same time, an essential step, in the process of validating if the newly proposed biomarker of Zn status, LA: DGLA ratio, can be used as a valid, sufficiently sensitive and reliable biomarker of Zn status in human subjects. In addition to this, in this study we examine the correlations of plasma Zn status and dietary Zn intake with the content of ten additional fatty acids in plasma phospholipids. Furthermore, this study looks at the correlations between Zn and other trace elements, comments on the dietary Zn intake of the study population and explains the correlations between the plasma Zn status, dietary Zn intake and plasma LA: DGLA ratio with numerous anthropometrical, biochemical and hematological measures.

2. Materials and methods

2.1. Study participants

The study participants (n = 54) were apparently healthy 28–55 years old males and females volunteers. Eligible subjects included non-smoking volunteers, without any clinical signs of an acute condition or chronic disease, and without the need for medical treatment. Strict inclusion/exclusion criteria has been used (please refer to supplementary material for more information). All subjects went through the informed consent process, both verbal and written. The study protocol was approved by the Clinical Hospital Centre Zemun, Belgrade, Serbia, Ethics Committee Approval, No: 2125, 2013; and by SAC HREC EC00188 (96.15), Adelaide, South Australia. The protocols and procedures of the study were in agreement with the ethical guidelines on biomedical research on human subjects of The Code of Ethics of the World Medical Association's Declaration of Helsinki (1964) and its further amendments.

2.2. Collection of blood samples and anthropometric measurements

Blood samples were collected between 8 and 9 a.m. after an overnight fast (>10hr fasting). Whole-blood samples were collected from participants in seated position at in a trace mineral free tube by venipuncture from an antecubital vein using butterfly needles (Sarstedt, Inc.). All samples were centrifuged (1000 × g for 15 mm). The serum and plasma samples were removed and 1 ml aliquots were stored at − 80 °C until further analysis.

Anthropometric variables height and weight were measured to the nearest 0.1 cm and 0.1 kg, respectively. The weight and percent of fat mass in the body composition of participants were measured using a TANITA UM072 balance (TANITA Health Equipment H.K. LTD). The body mass index (BMI) was computed as the ratio of weight (kg) to height squared (m^2). BMI was used to assess the prevalence of overweight (25–29.9 kg/m^2) and obesity (≥30 kg/m^2) according to WHO criteria [10].

2.3. The assessment of dietary Zn intake

Three 24 h recall questionnaires (interactive, validated) run on three non-consecutive days (two working days and one weekend day) were used for assessing dietary Zn intake of participants. The photographs of different foods and composite dishes were used during the interview to help improve the portion size estimations [11]. The zinc content of foods was determined using the Serbian food composition data base (FCDB) [12,13]. DIETS ASSESS & PLAN, a nutritional tool validated in different national and regional surveys and international projects, evaluated in the EFSA project [14] was used for obtaining comprehensive dietary intake assessments. The dietary intakes from administered questionnaires were calculated by multiplying the frequency of consumption of each food item consumed by composition of that food, using adequate portion sizes. In addition to Zn dietary intake data were obtained for energy, macronutrients, Fe and certain fatty acids. Total zinc intakes were adjusted for total energy intake using the residual method [15,16]. Zinc levels in the participants' diets were verified using the Estimated Average Requirement, as defined in the Dietary Reference Intakes (DRIs) provided by the Institute of Medicine [17].

2.4. Biochemical analysis

The biochemical parameters were measured using a Cobas e411 clinical chemistry analyzer (Roche Diagnostics, Basel, Switzerland) and using Roche Diagnostics Kits according to the manufacturer's instructions.

2.5. Plasma phospholipid fatty acid analysis

Fatty acid concentrations were determined by gas–liquid chromatography (GC). Total lipids were extracted from the plasma according to a method described by Milutinovic et al., 2012 [18].

In short, the phospholipid fraction was isolated from the extracted lipids by one-dimensional neutral lipid solvent using the system of petroleum ether, diethyl ether and glacial acetic acid (87:12:1, by volume) and separated on Silica Gel Chromatography plates (C. Merck, Darmstadt, Germany). The phospholipid fraction was scraped into glass tubes and the phospholipid fatty acid methyl esters were prepared by transmethylation with sodium hydroxide in methanol (heated at 85 °C for 1 h) and after that sulfuric acid in methanol (heated 85 °C for 2 h). After 30 min, samples of esters were centrifuged and the upper phase samples were put into tubes and evaporated with technical grade nitrogen.

Fatty acid methyl ester derivatives were separated by gas chromatography (GC) using a Shimadzu (Kyoto, Japan) GC 2014 equipped with a flame ionization detector and a Chronus GC-CN100 column (60 m x 0.32mmID, film thickness 0.2 μm, SMI-Labhut, Churcham, Gloucester, UK). Adequate separation was obtained over a 50 min period with an initial temperature of 140 °C held for 5 min. The temperature was increased to 220 °C at a rate of

3 °C min^{-1} and held on final temperature for 20 min. Individual peaks were identified by comparison with known standard mixtures (PUFA-2 and/or 37 FAMEs mix, Supelco, Bellefonte, PA), and each peak was quantified by calculating the area under the peak. Finally, the content of individual fatty acids was expressed as a percent of total fatty acids identified.

2.6. Determination of plasma Zn concentrations

The analysis of plasma Zn was conducted at The Institute of Public Health, Pozarevac, Serbia, using flame atomic absorption spectrometry (AAS) on a Varian SpectrAA-10 instrument with instrument parameters: wavelength 213.9 nm, slit width 1.0 nm and air-acetylene flame according to the method described by Jian Xin, 1990 [19]. The concentration of Zn was measured after dilution 1:10 with MilliQ water. To verify the accuracy of the method, the control serum ClinChek-Control (Recipe, Chemical + Instruments Gmbh, Germany) with a zinc content of 889 ± 178 μg/l (Level I) and 1738 ± 261 μg/l (Level II) was analyzed. Method performances were monitored by the analysis of the same control serum within the each series. The average obtained results for Zn content were 914 ± 23 μg/l (Level I) and 1801 ± 35 μg/l (Level II) which is in accordance with the certified values. In order to avoid zinc contamination, all tubes and utensils were either soaked in HNO3 (25%, w/w) for 16 h, or were known from previous studies to be zinc-free.

2.7. Statistical analysis

Data analysis was done using the statistical package SPSS 22 for Windows. All models were checked as to their appropriateness to the data. Normality of the distributions was assessed by a Shapiro–Wilk test. Spearman's rank-correlation coefficients (r) adjusted for sex, age, BMI, weight, height and energy intake were calculated to determine correlations between fatty acid composition, plasma Zn status and dietary Zn intake. Between-group differences in variables were compared with unpaired t-tests or by Mann–Whitney U tests. Where variables were not normally distributed, logarithmic transformation was undertaken to normalize the distribution. Data are presented as means ± standard errors. *p* values less than 0.05 were considered significant.

3. Results

3.1. General characteristics of study participants

The average age of participants (n = 54) was 40.4 ± 7 years, with average height of 173 cm ± 7.8 and weight of 83 ± 2.4 kg. The average BMI of this group was 27.7 ± 4.7 kg/m^2, there were no underweight individuals, 50% of participants were in the healthy weight range and ~25% were obese, according to WHO criteria [10].

3.2. Plasma Zn is not correlated with plasma concentrations of other trace elements

Plasma Zn concentrations of our study participants ranged from 0.72 mg/l to 1.37 mg/l. The overall mean plasma Zn concentration was 1.04 ± 0.16 mg/l (15.9 μmol/l).

All subjects had adequate plasma Zn concentrations (reference range 0.7–1.6 mg/l) and no deficiencies were observed (no plasma Zn values <0.7 mg/l). Generally, the trace element concentrations in this study (Table 1) were within the expected normal ranges for healthy humans and similar to reported levels of trace element concentrations in healthy individuals elsewhere [20–25].

Overall, trace element concentrations were not gender dependent, there were no differences in the concentration of measured nutrients between the male and female participants. No statistically significant difference in plasma Zn concentrations was seen between the genders ($p = 0.14$) which is consistent with results of several other studies [26,27]. The only statistically significant difference was observed for Cu, with females having higher mean levels of Cu compared to males (1.0 ± 0.03 and 0.78 ± 0.02 correspondingly; $p < 0.00$).

The observed difference in Cu levels is most likely due to the higher estrogen levels in females. The average Zn: Cu ratio of subjects in this study was 0.91 ± 0.22 which demonstrates the absence of Zn deficiency and inflammatory conditions in our study population, since the increment of this ratio above 1.5 reflects an inflammatory response or a decreased nutritional Zn status [28]. There were no correlations between the plasma Zn and other elements (Table 1). Similarly, the LA: DGLA ratio was correlated with plasma Zn ($r = -0.35$, $p = 0.01$), without any association with Fe, Ca, Cu and Mg, $r = -0.12$, $p = 0.39$; $r = 0.16$, $p = 0.26$; $r = -0.21$, $p = 0.12$; $r = 0.78$, $p = 0.95$, respectively.

3.3. Correlations of plasma Zn with anthropometric, hematological and biochemical measures

Similarly to previous findings [26,29,30] in this study we have not found a relationship between the zinc status and age (Table 2, $p = 0.87$). Alike, no relationship ($p = 0.2$) was found between the plasma zinc and BMI (Table 2). However, there was statistically significant link between the weight ($r = 0.27$; $p = 0.04$), waist circumference ($r = 0.35$, $p = 0.01$) and free-fat mass ($r = 0.29$; $p = 0.03$) with plasma Zn (Table 2), as was also shown by others [31–33].

Comparable to plasma Zn, the LA: DGLA ratio was correlated with weight related parameters. The observed correlations were stronger, waist circumference ($r = -0.43$; $p = 0.001$), hip circumference ($r = -0.36$, $p = 0.008$), weight ($r = -0.35$, p-0.008) and BMI ($r = 0.36$, $p = 0.007$). We found a correlation between the plasma Zn and glucose ($r = 0.28$, $p = 0.04$). There was no such correlation between glucose and any other mineral measured (data not shown).

In summary, except for weak correlations between Zn and glucose and Zn and weight related parameters, we found no correlation between the plasma Zn status and any other biochemical, anthropometrical or hematological indicator measured (Table 2).

3.4. Dietary Zn intake. Plasma Zn is not correlated with dietary Zn intake

Mean intake of zinc obtained from the 24 h dietary recalls was 9.98 ± 0.86 mg/day (mean ± SE). Around 30% of participants consumed zinc at levels below the EAR (6.8 mg/day for women and 9.4 mg/day for men; [17]) and none of the study participants reported taking Zn supplements. The meat, nuts and grain products were the main sources of the dietary Zn intake of our study population. Additionally, good sources of dietary Zn presented cheese products, yeast, sesame and poppy seeds, beans and cocoa powder.

Intakes of energy and protein in this study population were 1927 ± 112 kcal/day and 75 g/day of protein. The ranked data show significant correlations between the meat consumption ($r = 0.49$; $p = 0.00$) and energy ($r = 0.39$, $p = 0.003$) with zinc intake. Among the iron biochemical indices, hemoglobin and RBC were correlated with dietary zinc intake ($r = 0.36$, $p = 0.007$; $r = 0.35$, $p = 0.01$ respectively). Similarly to other studies [16,34–37] this study shows no significant relationship between the dietary and plasma zinc status ($r = 0.07$; $p = 0.6$).

Table 1
Plasma concentrations of Zn, Fe, Cu, Mg and Ca and their correlations.

Chemical Element	Zinc (Zn)	Iron (Fe)	Copper (Cu)	Magnesium (Mg)	Calcium (Ca)
Concentration (mg/l)	1.04 ± 0.22	1.25 ± 0.11	0.94 ± 0.28	21.28 ± 0.26	101.7 ± 2.92
Correlation with Zn (r)	–	0.14 (0.33)	0.92 (0.51)	0.78 (0.58)	0.03 (0.85)

The values presented are means ± standard errors (SE); n = 54, two replicates; r = correlation coefficient with p values in brackets. $p < 0.05$ is considered statistically significant.

Table 2
Correlations of plasma Zn with anthropometrical, biochemical and hematological indicators.

Indicator	**Age**	**Height (cm)**	**Weight (kg)**	**BMI (kg/m^2)**	**Hip Circ. (cm)**	**Waist Circ. (cm)**
Value	40.41 ± 0.95	173.15 ± 1.19	83.62 ± 2.42	27.68 ± 0.64	108.79 ± 1.53	91.83 ± 1.73
Correlation with Zn	0.23 (0.87)	0.17 (0.21)	0.27 (0.04)*	0.21 (0.13)	0.18 (0.19)	0.35 (0.01)*
Indicator	**FF Mass (kg)**	**HCT (L/L)**	**Glu (mmol/L)**	**Se (mm)**	**Cho (mmol/L)**	**TAG (mmol/L)**
Value	52.26 ± 1.47	0.41 ± 0.01	4.93 ± 0.07	7.85 ± 0.91	5.29 ± 0.14	1.02 ± 0.05
Correlation with Zn	0.29 (0.03)*	0.02 (0.87)	0.28 (0.04)*	0.02 (0.86)	−0.54 (0.69)	0.26 (0.05)
Indicator	**HDL (mmol/L)**	**LDL (mmol/L)**	**Cre (μmol/L)**	**Urea (mmol/L)**	**BILT (μmol/L)**	**BILD (μmol/L)**
Value	1.74 ± 0.06	3.57 ± 0.13	6.96 ± 1.73	3.43 ± 0.13	9.39 ± 0.52	2.92 ± 0.14
Correlation with Zn	−0.11 (0.45)	−0.03 (0.81)	0.19 (0.16)	0.68 (0.63)	0.20 (0.15)	0.19 (0.16)
Indicator	**UA (μmol/L)**	**ALT (U/L)**	**AST (U/L)**	**Gamma GT (U/L)**	**LDH (U/L)**	**Hgb (g/L)**
Value	263.16 ± 10.07	26.25 ± 2.19	22.49 ± 1.20	15.43 ± 1.18	153.19 ± 4.64	135.50 ± 1.93
Correlation with Zn	0.24 (0.08)	0.15 (0.27)	0.19 (0.16)	0.22 (0.11)	0.26 (0.06)	−0.02 (0.98)
Indicator	**WBC × 10^9/L**	**RBC × 10^{12}/L**	**PLT × 10^9/L**	**Lym (%)**	**Mon (%)**	**Gra (%)**
Value	5.78 ± 0.18	4.69 ± 0.06	253.05 ± 7.26	34.26 ± 0.80	6.40 ± 0.31	59.34 ± 0.84
Correlation with Zn	0.19 (0.16)	0.12 (0.39)	0.11 (0.45)	−0.17 (0.21)	0.06 (0.65)	0.15 (0.27)

BMI – body mass index; Circ. – circumference; FF – free fat; HCT – hematocrit; Glu – glucose; Se – sedimentation; Cho – cholesterol; TAG – triglyceride; HDL – high density lipoprotein; LDL – low density lipoprotein; Cre – creatinine; BILT – bilirubin total; BILD – bilirubin direct; UA -urine analysis; ALT – alanine aminotransferase; AST – aspartate aminotransferase; Gamma GT – Gamma-glutamyl transpeptidase; LDH – lactate dehydrogenase; Hgb-hemoglobin; WBC – white blood cells; RBC – red blood cells; HCT – hematocrit; PLT – platelet count; Lym – lymphocytes; Mon – Monocytes; Gra – Granulocytes. Values presented are means ± SE (standard errors). r- Correlation coefficient with p values in brackets. *$p < 0.05$ is considered statistically significant.

3.5. Correlations of plasma zinc with fatty acids. LA: DGLA is correlated with plasma Zn status in healthy subjects

The mean plasma fatty acid values of our study population are similar to the average serum/plasma fatty acid composition described by others [38–41].

Dietary Zn intake was mainly not correlated with different fatty acids measured in this study, the exception is the 22:5n-3, the end product of metabolic pathway of alpha linolenic acid. Plasma Zn status was not related to the status of SFA or MUFA and most of the PUFA. However, as presented in Table 3, there is statistically significant correlation between the DGLA and plasma Zn (r = 0.39, p = 0.00). No relation was observed between the linoleic acid and plasma Zn status while there was a significant negative correlation between LA: DGLA ratio and plasma Zn status (r = − 0.35, p = 0.01). Out of all fatty acids measured in this study DGLA was the only one that shows significant association with plasma Zn status. Multiple regression analysis (controlled for confounders) revealed that dietary and plasma Zn can predict changes in the LA/DGLA ratio ($R^2 = 0.23$ F [6,47] = 2.39, p = 0.042).

3.6. LA: DGLA ratio responds to the changes in dietary Zn intake while plasma Zn does not

In order to test the proposition that LA: DGLA is responsive to dietary Zn manipulations we divided samples into two groups with statistically significant differences in dietary Zn intake (Table 4).

We examined the extent the differences in the plasma Zn status, dietary intake of fatty acids and alterations in the concentrations of

Table 3
Correlations of major plasma phospholipid fatty acids content with plasma Zn status and dietary Zn intake.

Fatty acids	Common name	Content (%)	Correlation with plasma Zn status	Correlation with dietary Zn intake
SFA	Palmitic acid (16:0)	30.13 ± 0.26	−0.81 (0.56)	−0.04 (0.77)
	Stearic acid (18:0)	16.69 ± 0.18	−0.12 (0.43)	−0.23 (0.09)
MUFA	Palmitoleic acid (16:1n-7)	0.58 ± 0.03	0.12 (0.42)	0.68 (0.63)
	Cis-vaccenic acid (18:1n-7)	2.51 ± 0.07	−2.38 (0.08)	0.12 (0.40)
	Oleic acid (18:1n-9)	7.83 ± 0.14	0.23 (0.97)	0.06 (0.66)
PUFA	Linoleic acid (LA; 18:2n-6)	24.07 ± 0.37	0.01 (0.93)	0.08 (0.55)
	Dihomo-gamma-linolenic acid (DGLA, 20:3n-6)	2.88 ± 0.09	0.39 (0.00)*	0.04 (0.75)
	Arachidonic acid (AA, 20:4n-6)	11.17 ± 0.27	−0.05 (0.69)	0.06 (0.67)
	Adrenic acid (22:4n-6)	0.43 ± 0.02	0.06 (0.67)	0.05 (0.69)
	Docosapentaenoic acid (22:5n-3)	0.56 ± 0.03	−0.15 (0.26)	0.30 (0.03)*
	Docosahexaenoic acid (22:6n-3)	2.82 ± 0.12	0.01 (0.98)	0.05 (0.73)
	Eicosapentaenoic acid (20:5n-3)	0.32 ± 0.02	−0.98 (0.48)	0.17 (0.20)
	n-6: n-3	11.39 ± 0.49	0.01 (0.98)	−0.11 (0.45)
	LA: DGLA	8.87 ± 0.33	−0.35 (0.01)*,[a]	−0.01 (0.94)

FA – fatty acid; SFA – saturated fatty acid; MUFA – mono-unsaturated fatty acid; PUFA – poly-unsaturated fatty acid. Values presented are means ± SE (standard errors) of the % of total fatty acids. r- Correlation coefficient with p values in brackets.
*$p < 0.05$ is considered statistically significant.
[a] Correlations are calculated controlling for age, sex, BMI, energy intake and dietary Zn intake.

LA and DGLA (individually), as well as, LA: DGLA ratio follow the pattern of statistically significant differences in the dietary Zn intake among the groups.

Except for the statistically significant difference in intake of meat ($p = 0.015$) there were no statistically important dissimilarities in the consumption of any other food items that contributes to Zn intake (i.e. seafood, grain, vegetables, fruits, nuts). Similarly, no statistically significant variance was seen in dietary Fe intake ($p = 0.85$). Furthermore, there were no statistically significant differences between the zinc groups for any of the trace elements measured (data not presented). Finally, no dissimilarities were observed for any of the biochemical parameters between the groups.

For anthropometrical measures there were variations in % fat ($p = 0.03$) and fat free mass ($p = 0.001$) between participants in two groups, but no differences were seen for BMI ($p = 0.71$). Hemoglobin ($p = 0.03$) and red blood cells ($p = 0.01$) were the only two hematological parameters with statistically significant differences. No dissimilarities were seen in the dietary intake of LA or PUFA between the groups. Plasma Zn did not reflect changes in the dietary Zn intake, so no statistically significant variances were seen in plasma Zn concentrations among the groups.

On the contrary, there was statistically significant difference in DGLA status ($p = 0.004$) and LA: DGLA ratio ($p = 0.042$) between the groups (Table 4). The effect size, Cohen's d for LA: DGLA ratio was 0.58, which demonstrates the medium size effect. Considering other fatty acids, generally there were no variations observed between the Zn groups. The exceptions are the 22:4 n-6 (adrenic acid) and 18:1n-9 (oleic acid) with statistically significant differences 0.39 ± 0.02 and 0.47 ± 0.03, mean ± SE, $p = 0.014$ and 7.5 ± 0.16 and 8.12 ± 0.21, $p = 0.03$, respectively.

4. Discussion

This study is an initial step in the evaluation of LA: DGLA ratio as a biomarker of Zn status in humans. Our findings demonstrate that while plasma Zn concentrations remain stable the LA: DGLA ratio responds to dietary Zn intake; the concentration of DGLA is decreased and LA: DGLA ratio is increased in people with lower dietary Zn intake.

It is generally accepted that plasma Zn concentration is a valid indicator of whole-body Zn status in the absence of confounding factors, such as infection or stress [4,8]. However, as shown by many [34,35,37,42] plasma Zn level is not reflecting the nutritional state and dietary Zn intake of an individual faithfully. This clearly means that we need a new biomarker that responds more effectively to alterations in dietary Zn intake. The aim of this study was to test if the changes in recently proposed biomarker of Zn status (LA: DGLA ratio) can successfully predict dietary Zn intake and Zn status of an individual.

In this study plasma Zn, rather than serum was measured, in order to avoid contamination of zinc from the erythrocytes. Blood was collected in accordance with the standard protocol suggested by the International Zinc Nutrition Consultative Group [43]. Fatty acids in plasma/serum phospholipids stored at −80 °C for 7–12 years showed minimal degradation over time [44]. In order to avoid variations in Zn concentrations caused by the time of the day when the blood was taken and the time since last meal, in this study the blood was taken in the morning (between 8 and 9 a.m.) and after an overnight fast (>10hr).

The trace element concentrations in this study were within the anticipated normal ranges for healthy humans reported elsewhere [20–25]. There were no correlation of plasma Zn with any other trace elements measured in this study, which is consistent with previous findings [45,46].

Strict inclusion/exclusion criteria was used in order to exclude participants with infections, inflammatory conditions and allergies. In addition, we looked at the Cu: Zn to show the overall health status of participants. The ratio of copper to zinc (Cu: Zn) is believed to be clinically more important than the individual concentrations of either of these trace metals. Cu: Zn ratio is often used to show the general health state of an individual, as it is easily affected by inflammatory parameters [28]. Numerous studies have shown that the serum Cu: Zn ratio is a sensitive indicator for the identification of various diseases [22,47,48]. It has also been proposed that the ratio of these metals can be used as reference information for diagnosing zinc deficiency [42,49]. The optimal plasma or serum Cu: Zn ratio is 0.70–1.00 [28]. The increment of this ratio above 1.5 reflects an inflammatory response or a decreased nutritional Zn status [28]. The average Cu: Zn ratio of subjects in this study was 0.91 (reference range 0.7–1) which indicates the absence of Zn deficiency and inflammatory conditions in our study population.

All values obtained for various biochemical and hematological parameters measured in this study fall within the reference ranges for healthy population subjects [50–52]. The correlation of plasma Zn with various biochemical, anthropometrical and hematological parameters in healthy population subjects has not been investigated widely. The researchers were mainly interested in following the changes of these parameters and Zn status as a consequence of certain diseases (liver disease, diabetes, cardiovascular disease, various tumors). In general, no correlations were seen between the Zn status and biochemical, anthropometrical or hematological indicators measured in this study. The exemption is the weak correlation between the plasma Zn and glucose and Zn and weight related parameters. Zinc is known to have an insulin like effect and is required for the synthesis and release of insulin from pancreatic β cells [53,54]. Zinc ions have also been shown to suppress protein tyrosine phosphatases associated with the insulin signaling cascade [55] thus activating the insulin signaling cascade resulting in glucose uptake, which explains the observed correlation.

Our results are in agreement with previous findings, no relationship was found between the lipid profile (TAG, CHO, HDL, LDL) and plasma Zn concentrations [32,33].

The assessment of dietary Zn intake confirmed that zinc is obtained from a wide range of foods, the richest sources include red

Table 4
Differences in the dietary content and plasma concentrations of LA, DGLA and Zn between the two Zn groups.

	Group 1 (n = 27)	Group 2 (n = 27)	Significance (2-tailed)
Dietary Zn (mg)	7.01 ± 0.52	12.78 ± 1.54	0.001*
Dietary LA (g)	17.54 ± 3.54	19.75 ± 2.78	0.650
Dietary PUFA (g)	12.61 ± 2.86	12.15 ± 2.17	0.755
Plasma Zn (mg/L)	1.02 ± 0.03	1.07 ± 0.03	0.283
LA (%)	24.01 ± 0.49	24.15 ± 0.54	0.528
DGLA (%)	2.61 ± 0.12	3.14 ± 0.14	0.004*
LA/DGLA	9.53 ± 0.43	8.21 ± 0.47	0.040*

Dietary Zn–Zn content of the diets based on three 24 h recall questionnaires n = 54; 27 in each of the group. LA – linoleic acid; DGLA – dihomo-gamma-linolenic acid; PUFA – poly-unsaturated fatty acid. Values presented are means ± SE (standard errors). *$p < 0.05$ is considered statistically significant.

meats and liver, nuts, seeds and grains. The red meat and grain products were the main sources of the dietary Zn intake of our study population. Moderate sources included whole grain cereals, and legumes, with lower quantities being taken from other vegetables, fruits, and refined cereals. Zinc in animal products is more readily absorbed than from plant foods. Cereal grains, legumes, and nuts are rich in phytate, which binds zinc in the intestine and reduces its absorption [6]. The molar ratio of phytate: zinc in the diet has been proposed as a predictor of zinc bioavailability, and ratios >15 have been associated with suboptimal zinc status [6,56]. One of the limitations of the present study is the lack of information on the intake of phytate. However, in an omnivorous population, the impact of phytate is likely to be less significant than expected in vegetarians and those whose diets are mainly based on plant foods. Similarly, according to recently developed calculator for inadequate micronutrient intake, Zn bioavailability from the diets consumed by our study population belong to the group of 'high Zn bioavailability', where ≤50% of total energy intake is accounted for from rice, other grains, other starchy staples, and pulses and nuts (38% in our population) and >5% (15% in our study population) of total energy intake is accounted for by protein from fish, eggs, dairy and meat [57].

Dietary Zn intake has not been correlated with plasma zinc status which has also previously been demonstrated by others [34,35,37]. Due to the effective homeostatic regulation, plasma Zn does not reflect realistically dietary Zn intake and nutritional state of an individual. Unchanged plasma/serum Zn concentrations were observed with the intakes as low as 2.8 mg/kg to as much as 40 mg/kg, showing the limitation of plasma Zn status to reliably present the dietary Zn intake [16,58].

In addition, it is not unusual that the plasma zinc level falls at the lower end of the normal range even in the presence of zinc deficiency [42]. In this study we have participants with Zn intakes below the estimated average requirements for Zn [17] (around 30%) and still their plasma Zn levels remain stable (within the reference ranges). This finding, once again proves that plasma Zn is able to show relatively large variations in zinc status, but is not sensitive enough to reveal the early changes in Zn status or the changes in and between the deficient states. Mild to moderate Zn deficiency is not usually presented with specific organ pathologies [59], and basing the determination of Zn status solely on plasma Zn concentrations, early Zn deficiency states easily remain undiagnosed. The better biomarker of Zn status is undoubtedly needed.

The role of Zn in fatty acid metabolism has been demonstrated in several ways [60,61]. Zinc modulates cyclooxy-genase activity [62] and it is a co-enzyme for delta desaturase [63,64]. As desaturase enzymes require zinc and have a relatively low binding constant their activity is quite sensitive to early stage zinc deficiency. Zn deficiency leads to inconsistencies in the ratio of desaturase substrates and products, in this case linoleic acid (LA) and dihomo-γ-linolenic acid (DGLA) respectively [63]. The delta 6-catalyzed step required for conversion of LA to DGLA is usually the highest flux pathway, so an elevation in the LA: DGLA ratio may be a sensitive marker for Zn deficiency [9].

This study is an initial step in examining the LA: DGLA ratio as a biomarker of Zn status in humans. In addition to this, we looked at the correlation of plasma Zn and dietary Zn intake with other polyunsaturated, as well as some, saturated and monounsaturated fatty acids.

Out of the twelve fatty acids examined plasma Zn was correlated with only one polyunsaturated acid, DGLA.

Comparison of the differences in fatty acid content among the groups with different dietary Zn intake demonstrate that the concentration of oleic acid (18:1n-9; delta 9 desaturase product) and adrenic acid (22:4n-6; delta 5 desaturase) was lower in the group of people with lower Zn intake. Similar findings were provided by others [60,65,66]. The lower concentrations of oleic and adrenic acid in the group with lower dietary Zn intake indicate that Zn may have a role to play in desaturase activity.

We found no statistically important dissimilarities in the consumption of any other food items that contributes to Zn intake (i.e. seafood, grain, vegetables, fruits, nuts) among the investigated groups. The changes in fatty acid composition that are caused by food restrictions are different from the changes caused by Zn deficiency [67,68]. For example Kudo et al., 1990, demonstrated that during Zn deficiency oleic acid is reduced but increased during food restrictions. Analogous findings were provided by Cunnane et al., 2005. The authors state that the inhibition of the desaturases by zinc deficiency is so strong that it causes a more rapid decline in tissue arachidonic acid and docosahexaenoic acid than does the direct dietary deficiency of all the omega 6 or omega 3 polyunsaturated fatty acids.

Our results suggest that the activities of delta 6 and delta 9 desaturase are reduced when lower intake of dietary Zn is present, which confirms the sensitivity of desaturases to Zn intake. Desaturase enzymes are coupled to the NAD (P) H-cytochrome b5 electron transferrin chain and lower dietary Zn intake most likely affects the electron transferring chain and subsequently changes the activities of desaturases [68].

In addition to zinc, iron has also been shown to inhibit delta 9 desaturase activity [68]. Iron is a structural component of the desaturase enzymes, which are required to add double bonds to long chain fatty acids. Besides the changes in the concentrations of oleic acid between the Zn groups, there were also statistically significant differences in hemoglobin and RBC status. The new findings propose that dietary Zn intake is having a role to play in Fe deficiency [69,70], so the observed changes in the production of oleic acid may mean that Zn is also indirectly controlling the activity of iron ions. In further support to this argument, it is interesting to note that while there was no relationship between the dietary Zn intake and plasma Zn status, dietary Zn intake correlated with iron indices, hemoglobin and red blood cell count. Zn is shown to be a strong predictor of hemoglobin concentrations [59,71]. Additionally, a number of data sets over the years, have clearly shown a positive correlation between anemia and signs of the risk of Zn deficiency in adult males, children, and pregnant women [72,73]. The negative interaction between iron and zinc for absorption has been forgotten. There is more and more evidence showing the positive link between Fe and Zn and a strong positive influence of Zn on Fe absorption and Fe status. The information on the precise mechanisms of Zn involvement in the Fe absorption processes is accumulating [69,70,74].

There is a possibility that other confounding variables may not have been controlled for in our analysis and this suggests that our estimate of the effect of dietary zinc on changes in the LA: DGLA may be subject to some residual confounding. Nevertheless, given that the most important dietary (energy, fat and protein intake) and non-dietary confounders (sex, age, BMI) have been controlled for this outstanding confounding is likely to be very small.

In summary, our study results show that LA: DGLA ratio changes in accordance to dietary Zn intake. Similar findings were provided by Reed et al. scientists who were first to propose that LA: DGLA can potentially be used as a new biomarker of Zn status. With their chicken model the authors illustrate that LA: DGLA is sensitive to changes in dietary Zn intake, and that the biomarker can be used to assess the outcomes of changing levels of dietary Zn rapidly [9].

While plasma Zn concentrations of our study population remained unchanged (most likely due to the good homeostatic regulation) there was a statistically significant difference in DGLA production and the LA: DGLA ratio between the groups of subjects

with statistically different dietary Zn intake. The effect size of this difference shows that the observed variation is of moderate effect size. Finally, the percentages of adrenic acid (22:4n-6) the main end product of linoleic acid and oleic acid (18:1n-9) the end product of stearic acid were clearly different between the corresponding dietary Zn groups, which supports the idea that dietary Zn deficiency can affect the chain elongation/desaturation pathway of essential fatty acids [9,63].

This study is an initial step in evaluating LA: DGLA ratio as a biomarker of Zn status in humans. Indeed, further studies and dietary intervention trials are needed to entirely describe the effectiveness of this biomarker in relation to zinc status and zinc bioavailability over time. The initial results are encouraging as they show that LA: DGLA ratio changes in accordance to dietary Zn intake in humans. However, additional studies are needed to examine the sensitivity of this biomarker in different setting: in larger study populations, in Zn deficient populations, as well as in the treatment groups with various levels of zinc deficiency. Additional work is needed to clarify any potential limitations of this biomarker, i.e. the effect of inflammatory conditions and infections states on this biomarker.

The usefulness of LA: DGLA ratio in reflecting the Zn status of an individual should further be examined by looking at the changes of this biomarker during different time frames (long vs. short low/high Zn intake). The kinetics of desaturase enzymes in humans should also be examined. Similarly, the changes in LA: DGLA ratio may be investigated in relation to the alterations of Zn depended proteins and genes in various tissues (i.e. ZnT1, Zip4).

5. Conclusion

This study investigated the correlation of the newly proposed biomarker of Zn status, LA:DGLA ratio, with plasma Zn status and dietary Zn intake in healthy human subjects.

In addition, the correlations of Zn related indices with fatty acids and various biochemical, anthropometrical and hematological parameters were investigated. This initial study confirms that LA: DGLA ratio responds to dietary Zn manipulations. The study provided new information related to the link between plasma Zn, fatty acid status and dietary Zn intake. In conclusion, additional dietary intervention trials are needed to investigate the efficacy of newfangled biomarker of Zn status fully.

Author contributions

MK- designed and conducted part of the research, analyzed data and wrote the manuscript and had primary responsibility for the final content. JS- designed research and corrected the paper. MZ- helped with fatty acid analysis, revised the first and the final draft of the paper critically for important intellectual content. JM- conducted fatty acid analysis. ZP- performed mineral analysis. MG- proofread the paper and provided constructive advice. MG- provided essential materials, checked the paper. All authors have proofread the manuscript and approved the final version of the paper.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.clnesp.2016.06.013.

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