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Short Communication

The red blood cell proportion of arachidonic acid relates to shorter leukocyte telomeres in Mediterranean elders: A secondary analysis of a randomized controlled trial

Tania-Marisa Freitas-Simoes [a, 1], Montserrat Cofán [a, b, 1], Maria A. Blasco [c], Nora Soberón [c], Miguel Foronda [c], Dolores Corella [b, d], Eva M. Asensio [b, d], Mercè Serra-Mir [a], Irene Roth [a], Carlos Calvo [a, b], Cinta Valls-Pedret [a, b], Ricardo P. Casaroli-Marano [e], Mónica Doménech [a, b], Sujatha Rajaram [f], Joan Sabaté [f], Emilio Ros [a, b], Aleix Sala-Vila [a, b, *]

[a] Lipid Clinic, Department of Endocrinology and Nutrition, Hospital Clínic de Barcelona, Institut d'Investigacions Biomèdiques August Pi Sunyer (IDIBAPS), Barcelona, Spain
[b] Ciber Fisiopatología de la Obesidad y Nutrición (CIBEROBN), Instituto de Salud Carlos III (ISCIII), Madrid, Spain
[c] Telomeres and Telomerase Group, Molecular Oncology Program, Spanish National Cancer Research Centre (CNIO), Madrid, Spain
[d] Department of Preventive Medicine, University of Valencia, Valencia, Spain
[e] Department of Surgery, School of Medicine, University of Barcelona, Barcelona, Spain
[f] Department of Nutrition, School of Public Health, Loma Linda University, Loma Linda, CA, USA

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SUMMARY

Background & aims: Shortening of leukocyte telomere length (LTL) is a biomarker of aging. Epidemiologic studies of LTL in relation to dietary fatty acids have reported conflicting results. The red blood cell (RBC) fatty acid status is a valid objective biomarker of long-term dietary intake of C18:2n-6, C18:3n-3 and long-chain n-3 polyunsaturated fatty acids (C20:5n-3 and C22:6n-3). In healthy older individuals, we investigated whether LTL relates to the RBC proportions of the main dietary polyunsaturated fatty acids (PUFA), and to the RBC proportion of arachidonic acid (C20:4n-6), a fatty acid that can generate pro-inflammatory lipid mediators once released from cell membranes.

Design: Cross-sectional study in 344 subjects (mean age 68.8 y, 68.6% women) who participated in a randomized controlled trial testing whether a diet enriched in walnuts can delay the onset of age-related diseases (https://clinicaltrials.gov/ct2/show/NCT01634841). At baseline, we assessed LTL by high-throughput quantitative fluorescence and determined fatty acids in RBCs by gas chromatography.

Results: In multivariate models adjusted for age and gender, the RBC proportions of dietary PUFA were unrelated to LTL. In contrast, the RBC proportion of arachidonic acid inversely related to LTL (regression coefficient [95% confidence interval], −0.10 (−0.19 to −0.01), P = 0.023).

Conclusion: An increasing proportion of C20:4n-6 in RBCs is associated with shorter telomeres. Further research is needed to investigate the role of this fatty acid and its derived lipid mediators in the aging process.

1. Introduction

Specific dietary interventions can promote healthy aging [1], a topic of utmost public health importance. However, the interaction between diet and the aging process is poorly understood. Attrition of peripheral blood leukocyte telomere length (LTL) is considered a hallmark of aging that has been repeatedly used in epidemiological studies [2]. The issue of whether long-term intake of different dietary fats affects LTL remains controversial. Shorter LTL has been related to the intake of saturated, middle-short chain saturated, and n-6 polyunsaturated fatty acids (PUFA), in particular linoleic acid (C18:2n-6), but only in certain populations [reviewed in Ref. [3]]. This can be explained in part by the fact that most evidence on this topic derives from observational studies, which usually rely on

* Corresponding author. Lipid Clinic, Department of Endocrinology and Nutrition, Villarroel 170, Edifici Helios, despatx 8, 08036 Barcelona, Spain. Fax: +34934537829.
E-mail address: asala@clinic.ub.es (A. Sala-Vila).
[1] These authors contributed to this work equally.

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inherently limited, subjective data from food questionnaires and diet records. The fatty acid composition of red blood cells (RBC) has long been used as an objective surrogate of long-term intake of dietary fatty acids with absent or marginal endogenous synthesis, namely polyunsaturated fatty acids (PUFA) such as linoleic acid, alpha-linolenic acid (C18:3n-3), and the long-chain n-3 fatty acids eicosapentaenoic acid (C20:5n-3) and docosahexaenoic acid (C22:6n-3) [4]. Here we searched for associations between LTL and the RBC proportion of these dietary PUFA in 344 healthy older Spanish men and women. We also explored the RBC proportion of arachidonic acid (C20:4n-6), a fatty acid which is a substrate for the synthesis of pro-inflammatory lipid mediators once released from cell membranes.

2. Methods

2.1. Study population

This is sub-study of the Walnuts and Healthy Aging (WAHA) trial (https://clinicaltrials.gov/ct2/show/NCT01634841), aimed at investigating whether a walnut-enriched diet for 2 y delays the onset of age-related diseases such as cognitive decline and macular degeneration. WAHA was conducted in older subjects (63–79 y) in two settings (Loma Linda, CA, US, and Barcelona, Spain). The details of the protocol have been published [5]. For this cross-sectional sub-study we selected baseline data of the participants recruited in the Barcelona site. The protocol was conducted according to the guidelines of the Declaration of Helsinki and was approved by the ethics committee of Hospital Clínic of Barcelona. Written informed consent was obtained from all subjects. From April 2012 to December 2013, we screened 642 potential candidates, of whom 198 were excluded for not meeting eligibility criteria [5], and 92 were further excluded after clinical visit and physical examination. For this study we excluded participants with missing data on RBC fatty acids (n = 2) or LTL (n = 6), hence data from 344 study participants remained for final analyses.

2.2. Data acquisition

Risk factors were assessed as described [5]. Blood samples were drawn after overnight fasting. Serum lipid and glucose concentrations were determined by standard enzymatic methods in the hospital clinical laboratory. Peripheral blood mononuclear cells were obtained from 5 mL of EDTA-collected blood by Ficoll density gradient centrifugation (Histopaque 1077, Sigma–Aldrich, St Louis, MO, US). The resulting pellet was re-suspended in fetal bovine serum (Sigma–Aldrich, St Louis, MO, US) supplemented with 10% dimethylsulfoxide and stored at −80 °C. An aliquot of whole blood was stored at −80 °C until fatty acids analysis.

The RBC fatty acid profile was determined as described [6]. In brief, cells contained in 100 μl aliquot of EDTA-collected blood were hemolyzed and spun. The pellet, almost entirely composed of RBC membranes, was dried and dissolved in BF_3 methanol solution and transferred into screw-cap test tubes, which were heated for 10 min at 100 °C. The fatty acid methyl esters were isolated in n-hexane and were separated by gas chromatography using an Agilent 7890A Gas Chromatograph (Agilent España, Madrid, Spain) equipped with a 30 m × 0.25 μm × 0.25 mm SupraWAX-280 capillary column (Teknokroma, Barcelona, Spain), an autosampler, and a flame ionization detector. The amount of each fatty acid is expressed as a percentage of the total identified fatty acids in the sample.

LTL was determined by high-throughput quantitative fluorescence *in situ* hybridization with automated fluorescence microscopy, as described [7]. Briefly, cells were counted and plated (80,000 to 100,000 cells/well) in clear-bottomed black-walled, 96-well plates. The 4′,6-diamidino phenylindole channel was used for nucleus staining and the Cy3 for telomere detection. LTL was analyzed using individual telomere spots. Fluorescence intensities were converted into kb using L5178-R, L5178-S, and CEM cells as calibration standards, which have respective stable telomere lengths of 79.7 kb, 10.2 kb, and 7.5 kb. Samples were analyzed in duplicate, and in the case of calibration standards, in triplicate.

2.3. Statistical analyses

We assessed normal distribution of data using graphical methods and the Shapiro–Wilk test. We used multivariate linear regression analysis to search for independent associations between LTL and selected RBC fatty acids. The model included C18:2n-6, C18:3n-3, the sum of C20:5n-3 and C22:6n-3 (also known as the omega-3 index), and C20:4n-6. Age and gender were included into the model as potential confounders. The RBC proportion of C18:3n-3 had a skewed distribution, a reason why this variable was transformed to its natural logarithm in regression analyses. Statistical significance was set at the $P < 0.05$ level. Analyses were performed using SPSS software, release 19.0 (IBM Corp., Armonk, NY, US).

3. Results

Clinical characteristics, demographic data and treatment regimes are shown in Table 1. The mean age of the study subjects was 68.8 years and 85% of them had at least one classical cardiovascular risk factor (dyslipidemia, diabetes, hypertension, smoking, obesity, or family history of premature cardiovascular disease). RBC membrane fatty-acid composition is presented in Table 2. The omega-3 index (sum of EPA and DHA) was above 8% (the proposed low-risk cutoff for cardiovascular risk) in 1.8% of the study group, and below 4% (highest-risk cutoff) in 4.5%. Multivariate associations of RBC unsaturated fatty acids with LTL are shown in Table 3. The RBC

Table 1
Participants' clinical and laboratory characteristics and treatment regimes (n = 344).

Variable	Values
Male sex, n (%)	108 (31.4)
Age, years	68.8 (3.3)
Family history of premature CVD, n (%)	20 (5.8)
Body mass index, kg/m^2	27.1 (3.8)
Waist circumference, cm	98.5 (10.9)
Physical activity, METs-min per week	2510 (1606–3888)
Smoking status, n (%)	
Current	17 (4.9)
Former	81 (23.5)
Never	246 (71.5)
Smoking, pack-years	0 (0–4)
Educational level, years	10 (8–15)
Dyslipidemia, n (%)	186 (54.1)
Total cholesterol, mg/dL	206 (34)
HDL cholesterol, mg/dL	58 (14)
LDL cholesterol, mg/dL	127 (29)
Triglycerides, mg/dL	90 (71–122)
Treated with statins, n (%)	119 (35)
Hypertension, n (%)	189 (54.9)
Type 2 diabetes, n (%)	38 (11)
Glucose, mg/dL	92.0 (86.0–101.0)
Hemoglobin A1c, %	5.8 (0.5)
Treated with metformin, n (%)	21 (6)

Values are means (SD) except for quantitative variables (expressed as n and %), and physical activity, smoking in pack-years, educational level, fasting triglycerides, and fasting glucose, expressed as medians (interquartile ranges). CVD, cardiovascular disease; METs-min, minutes at a given metabolic equivalent level (units of energy expenditure in physical activity; 1 MET-min is roughly equivalent to 1 kcal).

Table 2
Red blood cell fatty acid composition of study participants (n = 344).

Fatty acid (% of total fatty acids)	Median (interquartile range)
C14:0	1.19 (0.56–2.91)
C16:0	20.35 (19.82–20.85)
C18:0	14.79 (13.99–15.62)
C20:0	0.22 (0.19–0.25)
C22:0	0.07 (0.05–0.08)
C24:0	0.61 (0.50–0.73)
Sum of saturated fatty acids	37.67 (36.74–39.05)
C16:1n-7 *cis*	0.56 (0.47–0.68)
C16:1n-7 *trans*	0.27 (0.23–0.34)
C18:1n-9 *cis*	18.53 (17.39–20.04)
C18:1n-9 *trans*	1.32 (1.23–1.41)
C20:1n-9	0.36 (0.32–0.40)
C24:1n-9	0.81 (0.69–0.97)
Sum of monounsaturated fatty acids	22.07 (20.88–23.36)
C18:2n-6	12.55 (11.44–13.83)
C18:3n-6	0.18 (0.15–0.22)
C20:2n-6	0.27 (0.24–0.32)
C20:3n-6	1.62 (1.46–1.83)
C20:4n-6	15.05 (13.71–16.09)
C22:4n-6	2.14 (1.85–2.52)
C22:5n-6	0.37 (0.31–0.45)
Sum of n-6 polyunsaturated fatty acids	32.60 (30.40–34.24)
C18:3n-3	0.12 (0.10–0.14)
C20:5n-3	0.71 (0.55–0.94)
C22:5n-3	1.57 (1.40–1.75)
C22:6n-3	4.96 (4.30–5.55)
Omega-3 index (C20:5n-3 + C22:6n-3)	5.64 (4.90–6.48)

Table 3
Multivariate associations of selected red blood cell (RBC) unsaturated fatty acids with leukocyte telomere length.

Independent variable	B (95% CI)	β	P
Sex (male)	−0.29 (−0.62 to 0.04)	−0.09	0.088
Age (1 year)	−0.09 (−0.14 to −0.05)	−0.21	<0.001
RBC C18:2n-6 (1%)	−0.04 (−0.14 to 0.06)	−0.05	0.423
RBC C18:3n-3 (1%)	−0.76 (−2.05 to 0.53)	−0.07	0.249
RBC C20:5n-3 + C22:6n-3 (1%)	−0.09 (−0.22 to 0.05)	−0.07	0.203
RBC C20:4n-6 (1%)	−0.10 (−0.19 to −0.01)	−0.12	0.023

Data obtained by multiple regression analysis are presented as B (non-standardized regression coefficient) with 95% confidence interval for the stated increase in independent variables. β, standardized regression coefficient.

proportion of arachidonic acid was inversely associated with LTL, while no significant associations were found for other exposures.

4. Discussion

In this cross-sectional study conducted in healthy Mediterranean elders we found that the RBC proportions of main dietary PUFA were unrelated to LTL. Interestingly, we uncovered an inverse association between LTL and the RBC content of C20:4n-6, a fatty acid that generates pro-inflammatory lipid mediators once released from cell membranes. This novel finding is consistent with experimental data from Kilpinen and co-workers [8], who reported that long-term cultivated (so-called “aging”) bone marrow mesenchymal stromal cells showed an increased content of C20:4n-6 in cellular lipids at the expense of n-3 PUFA. These overall reinforces the concept of an interplay between C20:4n-6, inflammation and aging, as suggested by Das [9]. C20:4n-6 has a marginal presence in dietary fats, hence, rather than reducing consumption of its parent foods (poultry and eggs), an optimal strategy to displace it from plasma cell membranes with an ensuing reduction of the inflammatory milieu is intake of marine-derived C20:5n-3 and C22:6n-3 [9]. This concurs with results from a randomized controlled trial in overweight subjects supplemented with gram doses of marine n-3 PUFA for 4 months, wherein a decreasing blood C20:4n-6:(C20:5n-3 + C22:6n-3) ratio was associated with longer LTL [10].

The study has limitations. The first one is its cross-sectional nature. Second, the number of participants is relatively small. The reason for a lack of a pre-specified sample size is that this is a secondary study using baseline data of a randomized controlled trial examining whether consumption of walnuts for 2 years retards the development of age-related diseases. Third, because the study was conducted in an older population, results cannot be easily extrapolated to younger individuals. The study also has strengths, such as the use of state-of-the-art technology to determine LTL and of objective and stable biomarkers of long-term intake of dietary PUFA. In conclusion, the associations found for C20:4n-6 warrant further research to elucidate the role of this fatty acid and its derived lipid mediators on the aging process.

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Statement of authorship

The authors' responsibilities were as follows: ER and AS-V designed research; T-MF-S, MC, NS, MF, E-MA, MS-M, IR, CC, CV-P, MD, and R-PC-M conducted research; T-MF-S, M-AB, DC, SR, JS, ER and AS-V wrote the paper; ER had primary responsibility for final content. All authors read and approved the final manuscript. Each author certifies that this material or similar material has not previously been published elsewhere.

Conflict of interest

JS, ER and AS-V have received research funding from the California Walnut Commission, Sacramento, CA. JS and ER are nonpaid members of its Scientific Advisory Committee. The other authors report no conflicts.

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