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ARTICLE

The associations of serum n-6 polyunsaturated fatty acids with serum C-reactive protein in men: the Kuopio Ischaemic Heart Disease Risk Factor Study

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Abstract

Background/objectives There are concerns that high intake of n-6 polyunsaturated fatty acids (PUFA) may promote inflammation, because the end-product of n-6 PUFA metabolism, arachidonic acid, is a precursor for pro-inflammatory eicosanoids. Our aim was to investigate cross-sectional associations of the serum n-6 PUFAs, objective biomarkers for exposure, with serum high-sensitivity C-reactive protein (CRP), a key inflammation marker.

Subjects/methods The study included 1287 generally healthy men aged 42–60 years from the population-based Kuopio Ischaemic Heart Disease Risk Factor Study, examined in 1984–1989. ANCOVA and logistic regression were used for analyses.

Results In the multivariable-adjusted analyses, both serum total n-6 PUFA and linoleic acid, the predominant n-6 PUFA, were associated with lower CRP. The mean CRP concentrations in quartiles of linoleic acid were 1.86, 1.51, 1.53, and 1.37 mg/L (P-trend = 0.001). The odds ratio for elevated CRP (>3 mg/L) in the highest vs. the lowest quartile was 0.47 (95% confidence interval (CI) 0.25–0.87, P-trend = 0.01). Arachidonic acid or the mainly endogenously produced n-6 PUFAs, gamma-linolenic acid and dihomo-gamma-linolenic acid, were not associated with higher CRP, either. Age, body mass index, or serum long-chain n-3 PUFA concentration did not modify the associations (P-interactions > 0.14).

Conclusions Serum n-6 PUFAs were not associated with increased inflammation in men. In contrast, the main n-6 PUFA linoleic acid had a strong inverse association with the key inflammation marker, CRP.

Introduction

Chronic, low-level inflammation is associated with several chronic diseases, such as cardiovascular disease, diabetes, neurodegeneration and cancer [1–3]. The two classes of polyunsaturated fatty acids (PUFA), the n-3 and n-6 PUFA, may have opposing effects on inflammation. The n-6 PUFA have been implicated as pro-inflammatory [4], for example because especially arachidonic acid (AA) is a substrate for several pro-inflammatory eicosanoids or because the n-6 PUFA compete with the potentially anti-inflammatory n-3 PUFA for the same desaturase and elongase enzymes [5, 6].

Despite the potential pro-inflammatory effects, even a relatively high intake of linoleic acid (LA), the predominant n-6 PUFA and a metabolic precursor to AA, has not increased inflammation in clinical trials [7]. However, the generalizability of the findings from these trials to free-living populations may be limited, because the trials have been small and short in duration. Although several epidemiological studies have evaluated the associations of the n-6 PUFAs with inflammation markers, the findings have been heterogeneous [8–22]. Use of circulating PUFAs would remove the bias associated with dietary assessment methods and would also make it possible to investigate the associations of the mainly endogenously produced n-6 PUFAs, gamma-linolenic acid (GLA) and dihomo-gamma-linolenic acid (DGLA). However, only few studies have investigated the associations of GLA or DGLA with inflammation, again with heterogeneous results [12–14, 17, 20, 21]. Therefore, our goal was to investigate the associations of the four serum n-6 PUFAs, LA, GLA, DGLA and AA, with high-

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sensitivity C-reactive protein (CRP), a key inflammation marker, among generally healthy, middle-aged men.

Subjects and methods

Study population

The prospective, population-based cohort study Kuopio Ischaemic Heart Disease Risk Factor Study (KIHD) started between 1984 and 1989. KIHD is an on-going study of risk factors for CVD and metabolic conditions in a sample of men from eastern Finland [23]. A total of 2682 men (82.9% of those eligible) aged 42, 48, 54 or 60 years participated in the baseline examinations. The University of Kuopio Research Ethics Committee approved the KIHD study protocol, and all participants provided written informed consent. We excluded from the analyses men who had data missing on serum CRP ($n = 61$) or on serum n-6 PUFAs ($n = 148$), or who had serum CRP > 10 mg/L ($n = 88$) or blood leucocyte count $> 11 \times 10^9$/L ($n = 12$), indicating an acute inflammation. In addition, to explore the associations with serum CRP among generally healthy men and to keep the analyses comparable with our previous analysis with the serum n-3 PUFA [24], we excluded 1017 participants with a disease with inflammatory component: history of rheumatoid arthritis, colitis, diabetes, claudication, ischemic heart disease, cardiac insufficiency, stroke, cancer, or disease of gall bladder, liver or pancreas. Finally, we excluded those men who reported using aspirin ($n = 70$); we did not have information on other anti-inflammatory medications. These exclusions left 1287 men.

Measurements

At the baseline examinations, men provided fasting blood samples at 8–10AM after they had abstained from drinking alcohol for 3 days and had not smoked or eaten for 12 h. Determination of blood pressure, serum lipoproteins and lipids, alcohol intake, smoking, and medical history and medications, have been published in detail previously [25]. Education years were assessed by a self-administered questionnaire. Physical activity at leisure time was evaluated with a 12-month leisure-time physical activity questionnaire and expressed as kcal/day [26]. The most common physical activities at leisure-time were recorded, including the average duration, intensity, and frequency of each activity. Dietary intakes were estimated using a 4-day food record [27]. For measurement of plasma glucose, a glucose dehydrogenase method was used after proteins were precipitated by trichloroacetic acid. Novo Biolabs radioimmunoassay kit (Novo Nordisk, Bagsvaerd, Denmark) was used for determination of serum insulin. Insulin sensitivity was estimated by the homeostatic model assessment computer algorithm [28].

Serum fatty acids

The measurement of serum fatty acids has been previously described in detail [29]. Briefly, esterified and nonesterified fatty acids in serum were determined with GC-FID [Hewlett-Packard 5890 Series II gas chromatograph (Hewlett-Packard Company, Avondale, PA, since 1999 Agilent Technologies Inc.)] after chloroform-methanol extraction and methylation for esterified fatty acids. Each analyte had an individual reference standard, with eicosane as an internal standard. Results are presented as proportion of total serum fatty acids. For repeated serum fatty acid measurements, the coefficient of variation (CV%) was 8.3% for DGLA (20:3n-6), 8.7% for LA (18:2n-6), 9.9% for AA (20:4n-6) and 11.6% for GLA (18:3n-6).

Serum CRP

An immunometric assay (Immulite High Sensitivity C-reactive Protein Assay, DPC, Los Angeles, USA) was used to measure serum high-sensitivity CRP. The assay is standardized against the World Health Organization International Reference Standard for CRP Immunoassay 85/506. The within-run CV of 2.8% and the total CV of 3.1% was observed, at the level of 3.2 mg/L. CRP concentration >3 mg/L was considered as elevated CRP.

Statistical analysis

The univariate associations of serum total n-6 PUFA with population characteristics at baseline were explored by means and linear or logistic regression with continuous and categorical variables, respectively. The mean serum CRP concentrations in n-6 PUFA quartiles were analyzed with ANCOVA. The odd ratios (OR) for elevated CRP (>3.0 mg/L) in n-6 PUFA quartiles were assessed with logistic regression. The analyses were controlled for possible confounders, which were selected based on previously reported relations with CRP or on relations with outcomes or exposures in the current study. The Model 1 included age and the year of examination. The Model 2 included the variables in the Model 1 and pack-years of smoking, serum long-chain n-3 PUFAs (%), serum triglycerides (mmol/L), body mass index (kg/m^2), leisure-time physical activity (kcal/day), and alcohol intake (g/week). Further adjustments for treated hypertension (yes/no), systolic or diastolic blood pressure (mmHg), years of education, homeostatic model assessment of insulin resistance, serum alpha-linolenic acid concentration (%), serum LDL or HDL cholesterol (mmol/L), or intakes of saturated fatty acids (percent of energy) or

Table 1 Population characteristics according to serum total n-6 polyunsaturated fatty acids of men in the Kuopio Ischaemic Heart Disease Risk Factor Study (KIHD)

	Serum total n-6 polyunsaturated fatty acid quartile (%)				
	1 (<30.2)	2 (30.2–33.3)	3 (33.4–36.2)	4 (>36.2)	P for trend
Number of subjects	321	322	322	322	
Age, y	52.6 ± 5.1	52.4 ± 5.2	52.0 ± 5.5	51.5 ± 5.9	0.01
Serum long-chain n-3 polyunsaturated fatty acids, %	4.71 ± 1.95	4.74 ± 1.52	4.75 ± 1.43	4.40 ± 1.25	0.02
Serum alpha-linolenic acid, %	0.73 ± 0.24	0.73 ± 0.22	0.76 ± 0.24	0.76 ± 0.21	0.07
Body mass index, kg/m^2	27.8 ± 3.7	27.1 ± 3.3	26.0 ± 2.9	25.3 ± 2.7	<0.001
Leisure time physical activity, kcal/d	116 ± 163	135 ± 158	131 ± 153	158 ± 159	0.002
Income, euro	13,128 ± 7780	14,468 ± 10,284	14,815 ± 9462	15,924 ± 10,639	0.001
Education, y	8.6 ± 3.4	8.5 ± 3.4	9.2 ± 3.9	9.7 ± 3.9	<0.001
Serum LDL cholesterol, mmol/L	3.96 ± 1.00	4.09 ± 0.92	4.02 ± 0.94	4.00 ± 1.01	0.78
Serum HDL cholesterol, mmol/L	1.20 ± 0.27	1.29 ± 0.27	1.35 ± 0.30	1.41 ± 0.31	<0.001
Serum triglycerides, mmol/L	1.84 ± 1.09	1.23 ± 0.52	1.04 ± 0.43	0.86 ± 0.35	<0.001
HOMA-IR	1.66 ± 0.80	1.41 ± 0.66	1.27 ± 0.59	1.12 ± 0.40	<0.001
Blood leucocyte count, ×10^9/L	5.74 ± 1.46	5.54 ± 1.36	5.37 ± 1.30	5.13 ± 1.34	<0.001
Smoking, %	37.1	27.6	29.2	21.1	<0.001
Hypertension, %	64.5	58.1	48.1	42.9	<0.001
Alcohol, g/week	94.1 ± 149.4	79.2 ± 111.5	60.7 ± 87.5	54.5 ± 90.7	<0.001
Dietary intakes					
Energy, kcal/d	2418 ± 600	2507 ± 650	2470 ± 625	2524 ± 617	0.06
Saturated fatty acids, E%	18.4 ± 4.1	18.6 ± 3.8	18.0 ± 3.7	17.3 ± 3.5	<0.001
Monounsaturated fatty acids, E%	11.1 ± 2.0	11.4 ± 2.1	11.9 ± 2.0	12.2 ± 2.2	<0.001
Fish, g/d	49 ± 66	44 ± 48	47 ± 54	39 ± 42	0.03
Red meat, g/d	134 ± 71	140 ± 70	147 ± 81	138 ± 78	0.30
Dairy, g/d	744 ± 370	747 ± 376	695 ± 361	651 ± 325	<0.001
Whole grains, g/d	153 ± 74	151 ± 70	160 ± 77	174 ± 80	<0.001
Fruits, berries and vegetables, g/d[a]	245 ± 173	253 ± 146	265 ± 160	282 ± 157	0.002
Vegetable oils, g/d	2.2 ± 4.1	2.3 ± 3.4	2.5 ± 3.5	3.0 ± 3.7	0.01
Vegetable margarine, g/d	12.1 ± 11.5	13.5 ± 13.7	18.1 ± 16.3	26.7 ± 20.0	<0.001

Values are means ± SD or percentages

E% percent of energy, *HOMA-IR* homeostatic model assessment-insulin resistance

[a]Excluding potatoes

monounsaturated fatty acids (percent of energy), or whole grains, red meat, dairy, or vegetables, fruits and berries (g/day), did not significantly affect the associations (<10% change in estimates). Missing values (<2.5%) in covariates were replaced with the cohort mean. Statistical significance of the potential interactions by age, body mass index and serum long-chain n-3 PUFA concentration was assessed by stratified analysis and likelihood ratio tests using a multiplicative interaction term. Linear trends across n-6 PUFA quartiles were assessed after assigning the median value for each fatty acid quartile and then treating that as a continuous variable in the statistical models. Correlation coefficients were estimated by Spearman correlations. All P-values were 2-tailed ($\alpha = 0.05$). SPSS 21.0 (Armonk, NY: IBM Corp.) was used for analyzing the data.

Results

Those with higher serum total n-6 PUFA concentration were younger, had lower blood pressure, BMI and serum long-chain n-3 PUFA and triglyceride concentrations, and lower intakes of fish, dairy and alcohol (Table 1). They also had higher serum HDL cholesterol concentration, physical activity, income and education, and higher intakes of whole

Table 2 Mean values of serum C-reactive protein and odds for elevated serum C-reactive protein in quartiles of serum n-6 polyunsaturated fatty acids in men from the Kuopio Ischaemic Heart Disease Risk Factor Study (KIHD)

	Mean C-reactive protein, mg/L			Odds for C-reactive protein >3 mg/L	
	Model 1	Model 2	No. of cases	Model 1	Model 2
Total n-6 polyunsaturated fatty acids (%)					
Q1 (<30.2)	2.03 (1.87–2.19)[a]	1.81 (1.63–1.99)	59	1	1
Q2 (30.2–33.3)	1.67 (1.51–1.83)	1.59 (1.43–1.75)	43	0.68 (0.45–1.05)[b]	0.81 (0.51–1.30)
Q3 (33.4–36.2)	1.37 (1.21–1.53)	1.46 (1.30–1.62)	20	0.29 (0.17–0.50)	0.41 (0.23–0.74)
Q4 (>36.2)	1.20 (1.04–1.36)	1.41 (1.24–1.58)	20	0.29 (0.17–0.49)	0.49 (0.27–0.91)
P for trend	<0.001	0.002		<0.001	0.004
Linoleic acid (%)					
Q1 (<24.0)	2.07 (1.91–2.24)	1.86 (1.68–2.03)	61	1	1
Q2 (24.0–26.7)	1.55 (1.39–1.72)	1.51 (1.35–1.67)	36	0.54 (0.34–0.84)	0.66 (0.41–1.07)
Q3 (26.8–29.7)	1.47 (1.30–1.63)	1.53 (1.37–1.69)	25	0.36 (0.22–0.59)	0.51 (0.29–0.87)
Q4 (>29.7)	1.18 (1.02–1.34)	1.37 (1.21–1.54)	20	0.28 (0.16–0.47)	0.47 (0.25–0.87)
P for trend	<0.001	0.001		<0.001	0.01
Gamma-linolenic acid (%)					
Q1 (<0.2)	1.45 (1.28–1.62)	1.48 (1.32–1.64)	31	1	1
Q2 (0.2–0.3)	1.70 (1.53–1.87)	1.73 (1.57–1.88)	43	1.45 (0.89–2.37)	1.47 (0.89–2.45)
Q3 (0.3–0.4)	1.53 (1.36–1.69)	1.50 (1.35–1.66)	36	1.17 (0.70–1.94)	1.09 (0.64–1.85)
Q4 (>0.4)	1.59 (1.43–1.76)	1.56 (1.41–1.72)	32	1.01 (0.60–1.70)	0.96 (0.56–1.65)
P for trend	0.69	0.91		0.72	0.54
Dihomo-gamma-linolenic acid (%)					
Q1 (<1.1)	1.38 (1.22–1.55)	1.48 (1.32–1.64)	28	1	1
Q2 (1.1–1.3)	1.65 (1.49–1.82)	1.64 (1.48–1.79)	39	1.43 (0.86–2.39)	1.23 (0.72–2.10)
Q3 (1.3–1.5)	1.55 (1.39–1.72)	1.55 (1.39–1.70)	36	1.32 (0.78–2.22)	1.18 (0.67–2.06)
Q4 (>1.5)	1.68 (1.52–1.85)	1.61 (1.45–1.77)	39	1.43 (0.86–2.39)	1.17 (0.67–2.06)
P for trend	0.03	0.39		0.23	0.67
Arachidonic acid (%)					
Q1 (<4.1)	1.68 (1.51–1.85)	1.58 (1.41–1.76)	44	1	1
Q2 (4.1–4.7)	1.64 (1.47–1.81)	1.61 (1.45–1.77)	38	0.85 (0.53–1.35)	0.90 (0.54–1.49)
Q3 (4.8–5.4)	1.46 (1.29–1.62)	1.48 (1.32–1.64)	27	0.57 (0.35–0.95)	0.62 (0.35–1.09)
Q4 (>5.4)	1.50 (1.33–1.66)	1.59 (1.42–1.77)	33	0.72 (0.44–1.16)	0.80 (0.44–1.45)
P for trend	0.06	0.87		0.09	0.31

Number of subjects in quartiles: 321, 322, 322, and 322, Model 1 adjusted for age and examination year, Model 2 adjusted for Model 1 and pack-years of smoking, body mass index (kg/m^2), leisure-time physical activity (kcal/day), serum long-chain n-3 polyunsaturated fatty acids (%), serum triglycerides (mmol/L), and alcohol intake (g/week)

[a]Values are mean (95% confidence interval)

[b]Values are odds ratio (95% confidence interval)

grains, vegetables, fruits and berries, vegetable oils and margarines (Table 1). They were also less likely to have hypertension and to smoke. The mean (SD) concentrations in serum were 26.8% (4.4) for LA, 0.3% (0.1) for GLA, 1.3% (0.3) for DGLA and 4.8% (1.0) for AA.

The mean concentration of serum CRP was 1.57 mg/L (SD 1.52, range 0.10–9.97 mg/L). CRP > 3 mg/L was observed in 142 men (11.0%). In the models adjusted for age and year of examination, serum total n-6 PUFA and LA were related to lower CRP (Model 1, Table 2). The inverse associations remained highly statistically significant, but were attenuated after further adjustments for potential confounders (Model 2). The extreme-quartile difference in serum CRP concentration was 0.40 mg/L (95% CI 0.13–0.66 mg/L) for total n-6 PUFA and 0.48 mg/L (95% CI 0.22–0.74 mg/L) for LA. Serum DGLA was associated with higher CRP in the basic model, but not after multivariable adjustments (Table 2). Serum total n-6 PUFA and LA were related also to markedly lower odds for CRP > 3 mg/L (Table 2). Those in the top serum total n-6 PUFA

quartile had 51% (95% CI 9–73%) lower odds for CRP > 3 mg/L and those in the top LA quartile had 53% (95% CI 13–75%) lower odds, when compared to the bottom quartiles. Serum GLA or AA were not associated with CRP.

Age (< or ≥ median 54.3 years, P-interactions > 0.14), body mass index (< or ≥ median 26.2 kg/m ^{2}, P-interactions > 0.47) or serum long-chain n-3 PUFA concentration (< or ≥ median 4.32%, P-interactions > 0.19) did not modify the associations.

Discussion

Our results do not suggest that higher n-6 PUFA exposure would be associated with increased inflammation among middle-aged and older men. Instead, especially the main n-6 PUFA, LA, was associated with lower CRP. AA or the mainly endogenously produced n-6 PUFAs, GLA and DGLA, were not associated with higher CRP, either.

Our findings of the inverse associations of the serum total n-6 PUFA or LA with CRP are supported by several previous epidemiological observations [11, 13–20], although some epidemiological studies have not found associations [8, 9, 21] or inverse associations have been found only in the subgroup analyses [10, 22]. There is also evidence from randomized trials that even very large changes in LA intake do not substantially affect circulating AA concentrations [30] and do not increase inflammation markers [7]. Similarly, AA supplementation has not had an impact on inflammation markers [31, 32], not even with several fold higher doses (1.5 g/d) compared to typical dietary intakes [31]. Although two population studies found a positive correlation between erythrocyte membrane AA and increased CRP [17, 21], other epidemiological studies, including ours, have found little evidence to support the pro-inflammatory effects of AA [9, 12, 14, 15, 20].

AA is indeed a precursor to eicosanoids with pro-inflammatory properties, but it is also a precursor to compounds that have anti-inflammatory and pro-resolving (turning off inflammation) effects, such as lipoxins and epoxy fatty acids [5, 33, 34] Furthermore, in addition to AA, LA is a precursor for several other metabolites, some of which, such as nitrated LA, have potent anti-inflammatory and pro-resolving properties [6, 35]. Therefore, the concept that LA is a precursor to AA, which in turn is a precursor to pro-inflammatory eicosanoids that would increase systemic inflammation, seems to be too simplistic.

The role of the mainly endogenously produced n-6 PUFAs, GLA and DGLA, in chronic inflammation has not been extensively examined in population studies. In most previous studies, as in our study, higher circulating GLA has not been associated with CRP [14, 21]. DGLA is a precursor to predominantly anti-inflammatory eicosanoids [36], so in this regard the previous study findings considering the association of DGLA with CRP have been somewhat unexpected. Several studies have found a direct association with CRP [12, 14, 20, 21], whereas one study [17], like ours, found no association. Clearly, more research is needed to elucidate the impact of these minor n-6 PUFAs on inflammation.

A major strength of our study is the use of objective biomarkers for exposure, the serum PUFAs, which also enabled to investigate the associations with GLA and DGLA. The tissue levels of fatty acids are especially good biomarkers for fatty acids that cannot be produced in the human body but must be obtained from diet, such as LA. The erythrocyte membranes rather than total serum could be a more preferable tissue for measurement of longer-term exposure, because of the long turnover of erythrocytes (120 days), and because the fatty acid profile of cell membranes has an important role in inflammatory processes [5]. However, at least in the case of LA, both total serum and erythrocyte membranes have been regarded as equally representative compartments for measuring the LA content of the tissues [37]. Both reflect a similar time period of exposure, about 1–2 weeks, and change in the LA content in one blood compartment reflects changes in other fractions [37, 38]. The transfer of fatty acids from plasma to erythrocytes is suggested as the main determinant of the membrane LA content in circulating erythrocytes [37]. There is less such research data available about the other n-6 PUFAs to indicate whether a certain tissue would be a preferable choice as a biomarker. Other strengths include the population-based recruitment and extensive examination of potential confounders. For example, we were able to exclude participants based on several diseases with an inflammatory component that could have potentially had an impact on the associations between the n-6 PUFAs and CRP, or they could have used medications that could affect systemic inflammation, significantly confounding the associations. However, higher serum total n-6 PUFA concentration was favorably associated with several risk factors for inflammation (Table 1), and although the inverse associations with total n-6 PUFA and LA remained after multivariable adjustments, we cannot exclude the possibility of residual confounding. A limitation is the cross-sectional and observational nature, which prevents any conclusions of causality. Besides CRP, we did not have data on other inflammation markers [8, 9, 14, 17]. Also, our study included only middle-aged men, and studies that have evaluated the associations separately in men and women have generally found stronger inverse associations with LA in women [10, 13, 19, 20].

In conclusion, our results do not support the suggested pro-inflammatory effects of the n-6 PUFA. In contrast, especially LA, the predominant n-6 PUFA, appears to have

an inverse association with the key inflammation marker, serum CRP.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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