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ORIGINAL ARTICLE

Fatty acids as determinants of *in-vivo* lipid peroxidation: The EFFGE study in Eastern Finnish hypertensive and non-hypertensive subjectsJari E. Kaikkonen¹, Teemu Vilppo², Janne Asikainen³, Sari Voutilainen⁴, Sudhir Kurl⁴ & Jukka T. Salonen^{5,6}

¹The Research Centre of Applied and Preventive Cardiovascular Medicine, University of Turku, Turku, Finland, ²Mekrijärvi Research Station, Ilomantsi, Finland, and the School of Forest Sciences, University of Eastern Finland, Joensuu, Finland, ³National Institute for Health and Welfare, Helsinki, Finland, ⁴Institute of Public Health and Clinical Nutrition, University of Eastern Finland, Kuopio, Finland, ⁵Hjelt Institute, University of Helsinki, Helsinki, Finland, and ⁶MAS-Metabolic Analytical Services Oy, Helsinki, Finland

Background. The degree of fatty acid (FA) unsaturation as a determinant of lipid peroxidation has been inadequately studied.

Methods. We examined associations of plasma free $F_{2\alpha}$ -isoprostanes (F_2 -IsoPs), an indicator of *in-vivo* lipid peroxidation, with the levels/intake of FAs, adjusted for the risk factors of cardiovascular disease (CVD) in 1211 Finnish men and women, of whom 50% were hypertensive, aged 59.3 ± 8.3 years, mean \pm SD.

Results. Elevated age- and sex-adjusted plasma free levels of omega-6 and omega-3 polyunsaturated FAs (PUFAs), saturated FAs (SFAs), and the PUFA/SFA and the omega-6/omega-3 PUFA ratios were all associated with decreased F_2 -IsoPs. High dietary SFA intake was associated with elevated F_2 -IsoP concentrations. In a multivariable regression (with clinical, nutritional, and behavioral CVD risk factors), female gender, body mass index (BMI), serum apolipoprotein A1, and NT-proBNP (natriuretic peptide) were positively associated with the F_2 -IsoPs, whereas the dietary PUFA/SFA ratio, plasma β -carotene, the omega-6/omega-3 PUFA ratio, and protein intake showed inverse associations.

Conclusions. We propose that elevated lipid peroxidation is associated with several risk factors of CVD, such as a low PUFA/SFA ratio, whereas the FA precursors of lipid peroxidation, i.e. omega-3 and omega-6 PUFAs are associated with attenuated F_2 -IsoP levels. These findings provide mechanistic support for earlier observations linking PUFA to improved cardiovascular health.

Key words: Cardiovascular disease, degenerative disease, F_2 -isoprostanes, free radicals, hypertension, lipid peroxidation *in vivo*, metabolic diseases, oxidative stress, type 2 diabetes

Lipid peroxidation and in particular low-density lipoprotein (LDL) oxidation are thought to play important roles in the etiology of atherosclerosis and related cardiovascular diseases (CVD) (1,2). Although there are many body fatty acids (FAs), the polyunsaturated FAs (PUFAs) have a higher susceptibility to become oxidized by free radicals or by non-radical oxygen species,

Key messages

- Elevated polyunsaturated fatty acid intake seems to lower plasma free $F_{2\alpha}$ -isoprostane (F_2 -IsoP) concentration, i.e. reduce lipid peroxidation *in vivo*.
- Plasma free F_2 -IsoP levels do not correlate with the concentration of plasma free arachidonic acid, the precursor molecule for isoprostanes.
- As a disease marker for hypertension, type 2 diabetes, and cardiovascular disease, the predictive value of plasma free F_2 -IsoP levels appears to be low, as compared with other major risk factors, such as the body mass index.

since these are the only FAs to contain at least two double bonds interrupted by methylene groups; these specific methylene groups are prone to oxidative attacks due to their unstable hydrogen-carbon bonds (3). In *in-vitro* studies, PUFA supplementation (linoleate, 18:2) of human subjects has been associated with elevated LDL oxidation susceptibility (4,5).

On the other hand, PUFAs (6) and in particular omega-3 (n3) PUFAs (7) have exerted beneficial effects on cardiovascular health. The current dietary recommendations for omega-6 (n6) PUFAs (8) propose that an intake of between 5% and 10% of the energy as n6 PUFAs (in the context of other lifestyle and dietary recommendations) can exert beneficial cardiovascular effects. Replacement of saturated fatty acids (SFAs) with PUFAs has resulted in a lowered CVD risk (6,9). However, a recent meta-analysis speculated that pure omega-6 (n6) PUFA replacement could even elevate the CVD risk, and instead combined n6 and n3 PUFA replacement was needed to achieve any lowering of the risk (10). In theory, this is possible since it is known that one of the n6 PUFAs, arachidonic acid (20:4), can be enzymatically or non-enzymatically oxidized into prostanoids and isoprostanoids, i.e. compounds which can evoke increased smooth muscle cell proliferation, inflammatory reactions, arterial wall

vasoconstriction, and platelet aggregation (11). It has been proposed that one mechanism behind the beneficial CVD effects of the elevated n3 PUFA intake (as compared with n6 intake) could be the ability of n3 PUFAs to inhibit competitively the harmful 20:4 metabolism, including the production of $F_{2\alpha}$ -isoprostanes (F_2 -IsoPs) (12). It has been reported that eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) supplementation (4 g/day) attenuated urinary and plasma F_2 -IsoP levels in subjects suffering from dyslipidemia, or hypertension and type 2 diabetes (13).

F_2 -IsoPs are considered as one of most reliable markers of free radical-mediated lipid peroxidation *in vivo* (14), even though to some extent they can be produced via cyclo-oxygenase-based processes (15). F_2 -IsoPs are formed as a result of the oxidation of esterified arachidonic acid (20:4) in different membrane bilayers, following enzymatic release to their free form (16,17). Circulating free F_2 -IsoPs are mainly bound to the high-density lipoprotein (HDL) fraction (18), delivered to the liver (19) and excreted in urine where they can be detected partially conjugated as glucuronides (20).

We have found one cohort study with more than one thousand subjects ($n = 2828$) in which the associations between clinical risk factors of metabolic diseases and urinary F_2 -IsoPs have been investigated with enzyme-linked immunoassay (ELISA)-based techniques (21). Some smaller studies have focused on common nutritional and clinical CVD risk factors (22). There is some evidence that increased plasma total levels of free FAs could increase plasma and urinary F_2 -IsoPs (23). However, the degree of free fatty acid desaturation (n3 and n6 PUFAs versus monounsaturated fatty acids (MUFAs) versus SFAs) as a determinant of plasma F_2 -IsoPs has been inadequately studied. Therefore, we assessed on a population scale (i.e. among subjects living in Eastern Finland) how the degree of plasma free FA unsaturation or the corresponding daily intake of FAs is associated with plasma free F_2 -IsoP levels, an index of lipid peroxidation *in vivo*. Since we wished to evaluate in a neutral manner, i.e. beneficial or harmful effects of PUFAs and SFAs on lipid peroxidation, we utilized multivariable models which were then adjusted for the well-known clinical, nutritional, and behavioral risk factors of CVD, to determine whether the PUFA/SFA or the n6/n3 PUFA ratios are associated with the F_2 -IsoP levels. In addition, the diluting effects of these common risk factors on the associations of F_2 -IsoPs with hypertension, type 2 diabetes, and CVD (i.e. independency and importance of F_2 -IsoPs as a risk factor) were evaluated. In the present study, we used ethylenediamine tetra-acetic acid (EDTA) plasma samples stored at -80°C , and a gas chromatography/mass spectrometry (GC/MS)-based methodology to assay specimens from over 1200 middle-aged or older subjects. Since under physiological conditions F_2 -IsoPs are released in a free form to the circulation and also for methodological reasons (the analysis method used was sensitive enough), we decided to measure plasma free F_2 -IsoPs.

Methods

Study population

EFFGE Study (East Finland Founder Population Hypertension Genetics Study) sample collections were carried out during the year 2007 in Eastern Finland (five local cities and adjacent rural communities). The Ethics Committee of Kuopio University Hospital approved the study protocol, and the study was carried out according to the Helsinki Declaration (24).

The study population consisted of hypertensive (50%) and non-hypertensive (50%) volunteers who were recruited by

newspaper advertisements or in connection with their routine medical center visits. The hypertensive cases were defined as follows: moderate-to-severe essential hypertension with prescription-proved antihypertensive medication (diagnosed under 60 years of age) and, on the basis of an interview, positive family history of hypertension consisting of at least two affected first-degree relatives among parents, siblings, or offspring. The non-hypertensive control subjects were defined as follows: systolic blood pressure < 140 mmHg and diastolic blood pressure < 90 mmHg (blood pressure was measured during the study visit) and, on the basis of interview, no antihypertensive treatment and no hypertension in the family among parents, siblings, or offspring. In addition, on the basis of an interview, all grandparents of the subjects had to be Finnish, and at least two of them born in Eastern Finland. No first-degree relatives were allowed to participate in the study (checked afterwards by genotype data). Cases and controls were matched for sex. The mean age of controls was 1.0 y older than that of cases. Secondary hypertension (characterized by an interview) caused by 1) pathological conditions, such as renal dysfunction or adrenal malignancy; 2) physiological conditions, such as pregnancy; or 3) nutritional or behavioral factors, such as high salt intake (either additional salt always added to food, and/or daily use of salty licorice) or high alcohol consumption (visual habitus assessment), were exclusion criteria. As the subjects had been advised beforehand, 87% of them reported that they had not used any alcohol within the last 3 days prior to the laboratory visit, with the other 13% having moderate consumption, 0.5 to 10 doses.

A cross-sectional study setting with combined case-control data was used in the present study. The data were split by gender since the sex differences in the clinical CVD risk factors examined were more extensive than those between hypertensives and normotensives. The whole study set consisted of 1353 subjects. Of these, plasma free F_2 -IsoP and plasma FA data were available for 1211 volunteers, 778 women and 433 men (605 hypertensive men and women, aged 58.4 ± 7.9 years, mean \pm SD (range 30–79 years), and 606 non-hypertensive men and women, aged 60.3 ± 8.6 years, mean \pm SD (range 37–85 years), and dietary intake data (food frequency questionnaire data) for 1068 subjects, 689 women and 379 men.

Laboratory analyses

All of the laboratory analyses were carried out by using overnight fasting samples.

Ethylenediamine tetra-acetic acid (EDTA)-containing vacuum tubes were used in the plasma free $F_{2\alpha}$ -isoprostanes (F_2 -IsoPs) sampling; the tubes were kept on an ice bath until centrifuged at $+4^\circ\text{C}$, following plasma deep-freezing to -80°C . F_2 -IsoP levels were analyzed by gas chromatography/mass spectrometry (GC/MS)-based methods (Agilent Technologies, Espoo, Finland) within 1 year from blood drawing. A deuterium(d_4)-labeled $F_{2\alpha}$ -isoprostane (Cayman Chemicals, Ann Arbor, MI) was used as an internal standard. Plasma samples (1 mL) were purified with C18 and silica Sep-Pak columns (Waters Corporation, Milford, MA) following pentafluorobenzyl esterification and thin-layer chromatographic purification. After trimethylsilyl ether derivatization, the F_2 -IsoP concentration was determined by GC/MS with negative-ion chemical ionization (m/z 569 for endogenous F_2 -IsoPs and m/z 573 for (d_4)-labeled F_2 -IsoPs) (25). For plasma free F_2 -IsoPs, day-to-day coefficient of variation (CV) was 3.5% (with a mean concentration of 33 pg/mL, $n = 10$) and within-batch CV 3.7% (with a mean concentration of 29 pg/mL, $n = 10$), and the quantitation limit was 6 pg/mL (with a signal-to-noise ratio = 5).

Plasma free fatty acids (FAs) were extracted into methanol/chloroform/water (4/8/3, v/v/v) and methylated by sulphuric acid/methanol (1/50, v/v), with eicosane (Sigma-Aldrich, Saint Louis, MO) added as an internal standard, as previously described (26). FAs were measured from EDTA plasma by gas chromatograph/mass spectrometer (Agilent Technologies). Individual FAs were subdivided into 1) saturated FAs, i.e. SFAs (myristic acid, 14:0; palmitic acid 16:0; stearic acid, 18:0; behenic acid, 22:0; and lignoceric acid, 24:0); 2) monounsaturated FAs, i.e. MUFAs (palmitoleic acid, 16:1; oleic acid, 18:1; and nervonic acid, 24:1); or 3) polyunsaturated FAs, i.e. PUFAs (linoleic acid, 18:2n6; α -linolenic acid, 18:3n3; gamma-linolenic acid, 18:3n6; dihomogamma-linolenic acid, 20:3n6; arachidonic acid, 20:4n6; eicosapentaenoic acid, 20:5n3; docosapentaenoic acid, 22:5n3; and docosahexaenoic acid, 22:6n3).

Information concerning history of diseases, smoking, alcohol use, and duration of the weekly low-intensity physical activity (including exercise exerted in different daily tasks, such as walking to work or to grocery stores) was based on interview with a study nurse. To avoid seasonal variation, the food frequency questionnaire (FFQ) data were collected within two winter months for all of the subjects by evaluating retrospectively the latest 12 month nutrient use. The FFQ comprised a list of 165 food items with nine frequency response options ranging from 'never' to '6 or more times per day'. Each frequency response option included an example of the amount intended (for example, 5 g of margarine, or 2 slices of bread). Study subjects were interviewed to achieve complete data. The Fineli database (27) was used to calculate fatty acid and macronutrient intakes which were proportioned to the used energy and expressed as energy percentages (E%). Validity and reproducibility of the Finnish FFQ, comparable in content with that used in the present study, have been discussed in detail earlier (28).

Anthropometric data, such as height, weight, and waist and hip circumference, were measured during the study visit. Body mass index (BMI) and waist-to-hip circumference ratio (WHR) were calculated from these variables.

Serum lipids, such as low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol and triglycerides, plasma glucose, serum levels of insulin, highly sensitive C-reactive protein (CRP), serum apolipoprotein B (apoB), and apolipoprotein A1 (apoA1), were measured by an auto-analyzer (Konelab 20, Thermo Fisher Scientific, Vantaa, Finland) with commercial kits (Thermo Fisher Scientific).

Concentrations of lipid-soluble vitamins, α -tocopherol and β -carotene, were measured from EDTA plasma by isocratic high-performance liquid chromatograph equipped with a UV detector (Agilent 1100 HPLC System, Agilent Technologies). Briefly, 200 μ L of plasma was extracted with 5 mL of hexane and 1 mL of ethanol. The hexane layer was separated and evaporated to dryness. The residue was dissolved in mobile phase, which consisted of acetonitrile/methanol/chloroform (47/47/6, v/v/v). An exclusively reversed phase C18 column was used, and peaks were detected at wavelengths of 292 nm for α -tocopherol and 454 nm for β -carotene. α -Tocopherol acetate was used as an internal standard (29).

The levels of serum natriuretic peptides, N-terminal fragments of A- and B-type natriuretic peptides (NT-proANP and NT-proBNP) were determined by RIA (30) by Biotop Ltd (Turku, Finland).

Statistical analyses

IBM SPSS Statistics software (version 19, SPSS, Inc.) was used to perform the statistical analyses, and a two-tailed *P* value

below 0.05 was considered statistically significant. Variables with skewed distribution were log-transformed prior to the statistical analyses (see Tables I–IV for details). Similarly, serum CRP (from 1 to 5), combined fruit, vegetable, and berry consumption (from 1 to 5), and alcohol consumption (from 1 to 3) were categorized prior to their statistical use. The *t* test for independent samples was used to assess differences in FA levels and in the CVD risk factors between men and women. Age- and sex-adjusted partial correlation coefficients (*r*) were calculated for the combined men + women data to test for associations between F_2 -IsoPs and its determinants.

The relationships between plasma F_2 -IsoPs or the plasma F_2 -IsoP/20:4 ratio and the cardiovascular risk factors, including the degrees of FA unsaturation, were also examined with linear regression models. These models with mean substitution were constructed in two steps. Firstly, age and sex (or age alone) were modeled as independent variables. Secondly, the CVD risk factors were allowed to enter into the models using a stepwise technique ($P < 0.05$ for entry and $P > 0.1$ for removal). These variables included energy intake, FA variables (the plasma or dietary PUFA/SFA ratio and the n6/n3 PUFA ratio, dietary SFA + *trans*-FA (E%), the total fat/n3 PUFA intake ratio), dietary use of fiber and macronutrients proportioned to energy intake, sodium intake, combined intake of fruits, vegetables, and berries, smoking (no versus yes), alcohol use, duration of the weekly low-intensity physical activity, BMI, WHR, essential hypertension (no versus yes), systolic blood pressure, serum NT-proANP and NT-proBNP, type 2 diabetes (no versus yes in interview), plasma glucose, serum insulin, triglycerides, LDL cholesterol, HDL cholesterol, apoB and A1, CRP, and plasma α -tocopherol and β -carotene. In addition, logistic binary regression with the equal phenomena (as compared with linear regression) was used to assess the links between F_2 -IsoPs and hypertension, type 2 diabetes, and CVD, and the diluting effect of the above-mentioned risk factors on the associations between F_2 -IsoPs and these diseases. Even though hypertension status was incorporated into all of the models, a fixed model (enter method in IBM SPSS Statistics software) was also used to study whether the risk factor associations found in the entire population were similar in both hypertensive and non-hypertensive subjects.

In the multivariable models, the purpose was to identify the 'overall determinants' of the F_2 -IsoP levels. For this reason, also variables which were causally dependent upon each other were offered for the same multivariable regression models. However, parallel variables or variables correlating strongly with each other, such as plasma and dietary PUFA/SFA ratio, or LDL cholesterol and apoB, or BMI and WHR, or insulin and BMI, or β -carotene and α -tocopherol, were not allowed to enter into the same model. In such a situation, the variable having the strongest age- and sex-adjusted association with the dependent variable was selected for incorporation into the final model.

Results

Study characteristics

Study characteristics are presented separately for men (aged 60.6 ± 8.5 years; range 30–85 years) and women (aged 58.6 ± 8.1 years; range 31–79 years) in Table I and Table II. Women had 16.1% higher F_2 -IsoP levels, as compared with men (Table I). Plasma free FA levels and daily intake of FAs were similar between the sexes. However, in women, plasma arachidonic acid (20:4) levels were 4.4% higher and the dietary PUFA/SFA ratio 4.5% higher than in men. Correspondingly in men, dietary SFA intake (E%) was 6.3% and SFA + *trans*-FA intake (E%) 5.2%

Table I. Fatty acid characteristics, and age- and sex-adjusted correlations between the different degrees of fatty acid unsaturation and plasma free F_{2α}-isoprostanes.

	Men (n = 443)		Women (n = 778)		P ^b	Combined men + women F ₂ -IsoPs ^a	
	Mean	SD	Mean	SD		r	P ^c
Plasma free F ₂ -IsoPs, pg/mL ^a	21.1	5.8	24.5	8.4	< 0.001		
Age, years	60.6	8.5	58.6	8.1	< 0.001		
Age range, years	30–85		31–79				
Plasma fatty acids							
Plasma SFA, mmol/L ^a	4.12	2.61	4.21	2.51	0.32	−0.109	< 0.001
Plasma MUFA, mmol/L ^a	3.30	1.85	3.27	1.74	0.97	−0.079	0.006
Plasma PUFA, mmol/L ^a	4.65	2.72	4.63	2.44	0.72	−0.175	< 0.001
Plasma n6 PUFA, mmol/L ^a	4.13	2.58	4.09	2.32	0.86	−0.166	< 0.001
Plasma n3 PUFA, mmol/L ^a	0.52	0.25	0.54	0.24	0.08	−0.146	< 0.001
Plasma arachidonic acid (20:4), mmol/L ^a	0.45	0.17	0.47	0.17	0.033	0.00	1.00
Plasma PUFA/SFA ratio ^a	1.19	0.30	1.16	0.33	0.19	−0.115	< 0.001
Plasma n6/n3 PUFA ratio ^a	8.18	4.01	7.91	3.99	0.21	−0.072	0.013
Intake of fatty acids	(n = 379)		(n = 689)				
Dietary SFA, E%	9.9	2.6	9.5	2.6	0.009	0.062	0.043
Dietary MUFA, E%	9.2	1.8	9.0	2.1	0.11	−0.03	0.36
Dietary PUFA, E%	4.1	0.9	4.1	1.1	0.64	−0.094	0.002
Dietary n6 PUFA, E% ^a	3.1	0.7	3.1	1.0	0.60	−0.079	0.010
Dietary n3 PUFA, E% ^a	1.0	0.3	1.0	0.3	0.88	−0.094	0.002
Dietary PUFA/SFA ratio	0.44	0.13	0.46	0.15	0.014	−0.124	< 0.001
Dietary n6/n3 PUFA ratio ^a	3.3	0.9	3.3	1.3	0.52	0.02	0.52
Dietary <i>trans</i> -fatty acids, E%	0.25	0.11	0.25	0.11	0.92	0.080	0.009
Dietary SFA + <i>trans</i> -fatty acids, E%	10.2	2.7	9.7	2.7	0.011	0.063	0.041
Dietary SFA + <i>trans</i> -fatty acid intake per total fat intake, %	42.0	5.4	41.3	5.6	0.042	0.117	< 0.001
Dietary total fat intake/n3 PUFA intake ratio ^a	30.7	10.5	30.1	9.3	0.36	0.096	0.002

F₂-IsoPs = F_{2α}-isoprostanes; MUFA = monounsaturated fatty acid; n3 = omega-3; n6 = omega-6; PUFA = polyunsaturated fatty acid; SFA = saturated fatty acid. Characteristics data are mean ± SD.

^aDue to skewed distribution, the variable was log-transformed prior to the statistical analysis.

^bP for sex difference (*t* test for independent samples).

^cP for correlation (partial correlation adjusted for age and sex).

higher than in women. The CVD prevalence and almost all of the CVD risk factors were higher in men (Table II). These risk factors included alcohol use, WHR, systolic blood pressure, NT-proBNP, serum insulin, triglycerides, plasma glucose, and energy intake. In women, the protective factors, such as serum HDL cholesterol, apoA1, plasma α-tocopherol and β-carotene, and dietary protein, fiber, and fruit + vegetable use, were all higher than in men. Of the divergent variables, serum LDL cholesterol was highest in women, whereas men took more physical activity (borderline significant).

Associations between F₂-IsoPs and fatty acid variables

All of the age- and sex-adjusted plasma FA classes, i.e. SFAs, MUFAs, PUFAs, n6 PUFAs, and n3 PUFAs were associated inversely (negative correlation coefficient) with the F₂-IsoPs, a measure of lipid peroxidation *in vivo* (Table I). In addition, the plasma PUFA/SFA and the n6/n3 PUFA ratios correlated inversely with the F₂-IsoPs. Of the individual FAs, 20:4 was not correlated with the F₂-IsoPs (*r* = 0.00). With respect to dietary FAs, intake of SFAs (E%), *trans*-FAs (E%), SFAs + *trans*-FAs (E%), the SFA + *trans*-FA/total fat intake ratio, and the total fat/n3 PUFA intake ratio associated positively (positive correlation coefficient) with the F₂-IsoPs, whereas dietary PUFAs (E%), n6 PUFAs (E%), n3 PUFAs (E%), and the PUFA/SFA ratio exhibited inverse associations.

Associations between F₂-IsoPs and CVD risk factors

We evaluated age- and sex-adjusted correlations between plasma free F₂-IsoPs and different CVD risk factors in Table II. Since it was unclear whether pure plasma values or the values adjusted for plasma free 20:4 should be used for the F₂-IsoPs, we evaluated both of these options. BMI, female sex, serum insulin, serum

triglycerides, WHR, plasma glucose, serum CRP, systolic blood pressure, and essential hypertension were the factors which were positively associated with the elevated F₂-IsoP levels (in this order of importance), whereas plasma β-carotene and duration of the weekly low-intensity physical activity were inversely associated. On the other hand, BMI, female sex, dietary intake of carbohydrates (E%), and type 2 diabetes were positively associated with the F₂-IsoP/20:4 ratio (in order of importance), whereas plasma levels of α-tocopherol, serum apoB, apoA1, dietary protein intake (E%), serum LDL cholesterol, sodium intake (mg/kcal), and HDL cholesterol were inversely associated.

Plasma F₂-IsoPs and fatty acid variables adjusted for CVD risk factors in multivariable models

BMI was the most important determinant of the elevated F₂-IsoP levels, but it did not dilute the contributions of the fatty acid variables in the model. In the combined men + women data (*n* = 1211, Table III), BMI, female sex, and serum apoA1 were the most important CVD risk factors which were positively associated with the F₂-IsoPs (in order of importance), whereas the dietary PUFA/SFA ratio, plasma β-carotene, dietary protein intake (E%), and the plasma n6/n3 PUFA ratio were inversely associated (Table III). The whole model accounted for 15.7% of the variation in F₂-IsoPs. When hypertensive (*n* = 605) and non-hypertensive (*n* = 606) subjects were evaluated separately by the fixed model, which included the variables presented above, the characterized associations were rather similar in both of these groups with no conflicting associations, i.e. even though all of the above-mentioned associations were not statistically significant, no conflicting associations were observed (data not presented).

In men (*n* = 433), BMI, the dietary total fat/n3 PUFA intake ratio, plasma glucose, and serum NT-proBNP were positively

Table II. Subject characteristics, and age- and sex-adjusted correlations between the clinical and nutritional risk factors of CVD and plasma free $F_{2\alpha}$ -isoprostane levels, the plasma free $F_{2\alpha}$ -isoprostane/20:4 ratio, or plasma free 20:4 concentration.

	Men				Women				Correlations for combined men + women data					
	n	Mean	SD	n	Mean	SD	P^d	r	F_2 -IsoPs ^a		F_2 -IsoPs/20:4 ratio ^a		Plasma 20:4 ^a	
									r	P^e	r	P^e	r	P^e
Age, years	433	60.6	8.5	778	58.6	8.1	<0.001	-0.02	0.61	0.03	0.39	0.001	-0.04	0.13
Sex (1 = male, 2 = female)	433	1	0	778	2	0	<0.001	0.210	<0.001	0.089	0.002	0.001	0.057	0.048
Plasma free F_2 -IsoPs, pg/mL ^a	433	21.1	5.8	778	24.5	8.4	<0.001	0.622	<0.001	-0.783	<0.001	<0.001	0.00	0.94
Plasma free $F_{2\alpha}$ -IsoPs, ng/ μ mol 20:4 ^a	433	0.054	0.032	778	0.060	0.037	0.002	0.00	0.16	0.02	0.41	0.001	0.00	0.94
Plasma arachidonic acid (20:4), μ mol/L ^a	433	450	169	778	470	169	0.033	0.04	0.16	0.02	0.41	0.001	0.00	0.94
Smoker (0 = no, 1 = yes)	433	0.10	0.30	778	0.07	0.26	0.09	0.01	0.75	-0.04	0.19	0.001	0.06	0.05
Alcohol use, g/day ^b	433	7.38	9.42	778	3.05	5.29	<0.001	-0.096	0.001	-0.01	0.70	0.001	-0.062	0.035
Duration of weekly physical activity, h ^a	433	7.7	7.3	776	6.6	5.5	0.05	0.283	<0.001	0.105	<0.001	0.001	0.091	0.002
Body mass index, kg/m ^{2a}	433	27.1	3.9	778	26.9	4.8	0.19	0.153	<0.001	-0.01	0.81	0.001	0.130	<0.001
Waist-to-hip circumference ratio	433	0.95	0.06	778	0.84	0.07	<0.001	0.066	0.021	-0.02	0.46	0.001	0.080	0.005
Essential hypertension (1 = no, 2 = yes)	433	1.50	0.50	778	1.50	0.50	0.97	0.072	0.012	0.01	0.67	0.001	0.04	0.15
Systolic blood pressure, mmHg ^a	433	135	17	778	132	19	<0.001	0.05	0.08	0.02	0.55	0.001	0.02	0.53
Cardiovascular disease (0 = no, 1 = yes)	433	0.12	0.33	778	0.06	0.25	0.001	0.05	0.08	0.02	0.55	0.001	0.02	0.53
Myocardial infarction (0 = no, 1 = yes)	433	0.05	0.22	778	0.01	0.10	0.001	0.071	0.013	0.03	0.27	0.001	0.02	0.59
Angina pectoris (0 = no, 1 = yes)	433	0.10	0.31	778	0.06	0.24	0.008	0.05	0.10	0.01	0.05	0.001	0.03	0.35
Serum NT-proANP, nmol/L ^a	429	1.023	0.745	763	0.998	0.482	0.10	0.01	0.70	0.05	0.07	0.001	-0.058	0.044
Serum NT-proBNP, nmol/L ^a	429	0.093	0.127	763	0.091	0.076	0.013	0.06	0.06	0.06	0.05	0.001	-0.03	0.31
Type 2 diabetes (0 = no, 1 = yes in interview)	433	0.07	0.26	778	0.05	0.21	0.06	0.05	0.08	0.058	0.045	0.001	-0.03	0.24
Serum insulin, mU/L ^a	433	9.3	9.1	775	7.3	5.8	<0.001	0.168	<0.001	0.03	0.35	0.001	0.099	0.001
Serum triglycerides, mmol/L ^a	433	1.32	0.79	778	1.15	0.60	<0.001	0.162	<0.001	-0.04	0.22	0.001	0.174	<0.001
Serum LDL cholesterol, mmol/L	433	3.24	0.89	778	3.35	0.87	0.037	0.02	0.47	-0.101	<0.001	0.146	<0.001	<0.001
Serum HDL cholesterol, mmol/L	433	1.45	0.42	778	1.79	0.47	<0.001	-0.05	0.10	-0.067	0.019	0.05	0.10	0.10
Apolipoprotein A1, g/L	432	1.5	0.3	771	1.8	0.3	<0.001	0.02	0.56	-0.119	<0.001	0.165	<0.001	<0.001
Apolipoprotein B, g/L	432	1.1	0.3	771	1.1	0.3	0.61	0.05	0.08	-0.133	<0.001	0.209	<0.001	<0.001
Serum CRP, mg/L ^c	433	1.9	3.5	778	2.1	3.1	0.09	0.119	<0.001	0.02	0.41	0.065	0.025	0.025
Plasma glucose, mmol/L ^a	433	5.95	0.96	778	5.56	0.93	<0.001	0.135	<0.001	0.05	0.09	0.04	0.13	0.13
Plasma alpha-tocopherol, μ mol/L ^a	433	30.3	6.9	776	31.6	6.4	0.001	-0.04	0.14	-0.190	<0.001	0.209	<0.001	<0.001
Plasma beta-carotene, μ mol/L ^a	433	0.30	0.20	776	0.47	0.32	<0.001	-0.201	<0.001	-0.05	0.11	-0.100	0.001	0.001
Energy intake, kcal/day ^a	379	2120	760	689	1930	780	<0.001	-0.03	0.42	0.05	0.11	-0.082	0.007	0.007
Dietary protein intake, E%	379	18.2	2.7	689	18.8	2.7	<0.001	-0.05	0.10	-0.118	<0.001	0.110	<0.001	<0.001
Dietary fat intake, E%	379	28.3	4.8	689	28.0	5.3	0.36	-0.00	0.93	-0.02	0.60	0.02	0.55	0.55
Dietary carbohydrates, E%	379	49.9	6.1	689	50.3	6.5	0.40	-0.01	0.72	0.061	0.047	-0.086	0.005	0.005
Dietary fiber, g/MJ	379	3.4	0.9	689	3.9	1.0	<0.001	-0.03	0.28	0.02	0.49	-0.05	0.09	0.09
Dietary sodium, mg/kcal ^a	379	1.64	0.32	689	1.61	0.33	0.17	-0.03	0.28	-0.073	0.017	0.067	0.030	0.030
Dietary fruit, vegetable and berry intake, g/day ^c	379	581	496	689	701	583	<0.001	-0.03	0.35	0.01	0.87	-0.03	0.35	0.35

CRP = highly sensitive C-reactive protein; F_2 -IsoPs = $F_{2\alpha}$ -isoprostanes; HDL = high-density lipoprotein; LDL = low-density lipoprotein; NT-proANP and NT-proBNP = N-terminal fragments of A and B-type natriuretic peptides; 20:4 = arachidonic acid.Characteristics data are mean \pm SD.^{a-c}Due to skewed distribution, the variable was either ^alog-transformed, or categorized ^b1 to 3, or ^c1 to 5 prior to the statistical analysis.^d P for sex difference (t test for independent samples).^e P for correlation (partial correlation adjusted for age and sex). Due to zero values, the log transformation was carried out for 1191 subjects in NT-proANP and for 1089 subjects in NT-proBNP.

Table III. Linear multivariable regression for the associations between plasma free $F_{2\alpha}$ -isoprostane levels (pg/mL) and the CVD risk factors.

	<i>B</i>	<i>Beta</i>	<i>P</i>
All of the subjects (<i>n</i> = 1211)			
(Constant)	0.583		
Body mass index, kg/m ^{2a}	0.517	0.268	< 0.001
Sex (1 = male, 2 = female)	0.062	0.225	< 0.001
Dietary PUFA/SFA ratio	-0.105	-0.105	< 0.001
Serum apolipoprotein A1, g/L	0.044	0.098	0.001
Plasma β -carotene, μ mol/L ^a	-0.043	-0.097	0.001
Dietary protein intake, E%	-0.003	-0.060	0.026
Plasma n6/n3 PUFA ratio ^a	-0.036	-0.058	0.030
Age, years	-0.001	-0.035	0.195
Men (<i>n</i> = 433)			
(Constant)	0.479		
Body mass index, kg/m ^{2a}	0.313	0.165	0.001
Dietary total fat intake/n3 PUFA intake ratio ^a	0.159	0.164	< 0.001
Plasma glucose, mmol/L ^a	0.280	0.153	0.003
Serum NT-proBNP, nmol/L ^a	0.040	0.139	0.003
Age, years	0.000	-0.023	0.63
Women (<i>n</i> = 778)			
(Constant)	0.691		
Body mass index, kg/m ^{2a}	0.534	0.284	< 0.001
Dietary PUFA/SFA ratio	-0.105	-0.106	0.002
Plasma n6/n3 PUFA ratio ^a	-0.060	-0.094	0.005
Serum apolipoprotein A1, g/L	0.040	0.087	0.013
Plasma β -carotene, μ mol/L ^a	-0.040	-0.083	0.023
Duration of the weekly physical activity, h	-0.037	-0.072	0.036
Age, years	-0.001	-0.046	0.19

n3 = omega-3; n6 = omega-6; NT-proBNP = N-terminal fragments of B-type natriuretic peptides; PUFA = polyunsaturated fatty acid; SFA = saturated fatty acid.

Values are regression coefficients and standardized regression coefficients indicating the change in $F_{2\alpha}$ -isoprostanes in pg/mL in response to one unit change in explanatory factor.

^aDue to skewed distribution, the variable was log-transformed prior to the statistical analysis. Regression models contained two steps: Step 1: age and sex, or age alone, were included in the model. Step 2: Step 1 + stepwise regression (*P* for entry 0.05 and *P* for removal 0.10) for different CVD risk factors.

associated with the F_2 -IsoPs. The whole model accounted for 10.7% of the variation in F_2 -IsoPs. In women (*n* = 778), BMI and serum apoA1 were positively associated with the F_2 -IsoPs, whereas the dietary PUFA/SFA ratio, the plasma n6/n3 PUFA ratio, plasma β -carotene, and duration of the weekly physical activity were inversely associated. The whole model accounted for 13.8% of the variation in F_2 -IsoPs.

The plasma F_2 -IsoP/20:4 ratio and fatty acid variables adjusted for CVD risk factors in multivariable models

Once again, fatty acid variables were found to be important determinants of the F_2 -IsoP levels. In the combined men + women data (*n* = 1211), female sex, BMI, dietary SFA + *trans*-FA (E%), and serum NT-proBNP were positively associated with the F_2 -IsoP/20:4 ratio (in this order of importance), whereas serum apoB, the plasma PUFA/SFA ratio, the plasma n6/n3 PUFA ratio, dietary protein intake (E%), serum apoA1, and hypertension were inversely associated (Table IV). The whole model accounted for 12.9% of the variation in the F_2 -IsoP/20:4 ratio. When hypertensive (*n* = 605) and non-hypertensive (*n* = 606) subjects were studied separately by the fixed model, which included the variables presented above, the characterized associations were very similar in both of these groups with no conflicting links (data not presented).

In men (*n* = 433), dietary SFAs + *trans*-FAs (E%), energy intake, and plasma glucose were positively associated with the F_2 -IsoP/20:4 ratio (in order of importance), whereas serum apoB, dietary protein intake (E%), the plasma PUFA/SFA ratio, serum apoA1, and the plasma n6/n3 PUFA ratio were inversely associated. The whole model accounted for 19.9% of the variation in the F_2 -IsoP/20:4 ratio. In women (*n* = 778), BMI and

smoking were positively associated with the F_2 -IsoP/20:4 ratio. Correspondingly, the plasma n6/n3 PUFA ratio, serum apoB, the plasma PUFA/SFA ratio, hypertension, dietary protein intake (E%), and serum apoA1 were inversely associated. The whole model accounted for 10.4% of the variation in the F_2 -IsoP/20:4 ratio.

Plasma F_2 -IsoP levels and hypertension, type 2 diabetes, and CVD

With regard to hypertension (combined men + women data), there was a significant age- and sex-adjusted association between F_2 -IsoPs and hypertension prevalence (605 hypertensive cases, 606 controls, OR = 2.8, *P* = 0.021). Further adjustment for BMI (the strongest covariate in a stepwise logistic multivariable regression) diluted the association to non-significance, and it became inverse (OR = 0.55, *P* = 0.23) (data not shown in tables). With regard to type 2 diabetes (case: diagnosed T2D based on interview, or plasma glucose \geq 7.0 mmol/L; control: non-diabetic based on interview, and plasma glucose < 5.0 mmol/L), age- and sex-adjusted F_2 -IsoP association was statistically significant (99 cases, 139 controls, OR = 18.0, *P* = 0.010). Further adjustment for plasma glucose or BMI, the strongest covariates, diluted the association to non-significance (OR = 0.0, *P* = 0.99, when both of these covariates were incorporated into the model) (data not shown in tables). Similarly, age- and sex-adjusted F_2 -IsoPs tended to associate with CVD (103 cases with either suffered myocardial infarction or angina pectoris or coronary artery bypass surgery, 1108 controls, OR = 4.4, *P* = 0.07). Further adjustment for the strongest covariate, hypertension, converted this borderline significant association into statistical non-significance (OR = 3.3, *P* = 0.17).

Table IV. Linear multivariable regression for the associations between plasma free F₂_o-isoprostane levels (ng/μmol 20:4) and the CVD risk factors.

	B	Beta	P
All of the subjects (n = 1211)			
(Constant)	-1.313		
Serum apolipoprotein B, g/L	-0.138	-0.170	< 0.001
Plasma PUFA/SFA ratio ^a	-0.264	-0.152	< 0.001
Plasma n6/n3 PUFA ratio ^a	-0.142	-0.146	< 0.001
Sex (1 = male, 2 = female)	0.057	0.130	< 0.001
Body mass index, kg/m ^{2a}	0.387	0.127	< 0.001
Dietary protein intake, E%	-0.009	-0.114	< 0.001
Serum apolipoprotein A1, g/L	-0.077	-0.110	< 0.001
Essential hypertension (1 = no, 2 = yes)	-0.039	-0.093	0.002
Dietary SFA + <i>trans</i> -fatty acids, E%	0.006	0.070	0.011
Serum NT-proBNP, nmol/L ^a	0.033	0.056	0.047
Age, years	0.000	0.004	0.90
Men (n = 433)			
(Constant)	-1.451		
Serum apolipoprotein B, g/L	-0.183	-0.246	< 0.001
Dietary protein intake, E%	-0.013	-0.166	< 0.001
Plasma PUFA/SFA ratio ^a	-0.269	-0.159	0.001
Serum apolipoprotein A1, g/L	-0.113	-0.141	0.002
Plasma n6/n3 PUFA ratio ^a	-0.121	-0.130	0.005
Dietary SFA + <i>trans</i> -fatty acids, E%	0.008	0.106	0.017
Energy intake, kcal/day ^a	0.140	0.097	0.028
Plasma glucose, mmol/L ^a	0.295	0.093	0.044
Age, years	0.002	0.065	0.15
Women (n = 778)			
(Constant)	-1.403		
Body mass index, kg/m ^{2a}	0.492	0.168	< 0.001
Plasma n6/n3 PUFA ratio ^a	-0.149	-0.150	< 0.001
Serum apolipoprotein B, g/L	-0.120	-0.142	< 0.001
Plasma PUFA/SFA ratio ^a	-0.222	-0.126	0.001
Essential hypertension (1 = no, 2 = yes)	-0.044	-0.102	0.005
Dietary protein intake, E%	-0.007	-0.083	0.017
Serum apolipoprotein A1, g/L	-0.053	-0.072	0.043
Smoker (0 = no, 1 = yes)	0.058	0.070	0.047
Age, years	0.000	-0.014	0.69

n3 = omega-3; n6 = omega-6; NT-proBNP = N-terminal fragments of B-type natriuretic peptides; PUFA = polyunsaturated fatty acid; SFA = saturated fatty acid; 20:4 = arachidonic acid.

Values are regression coefficients and standardized regression coefficients indicating the change in F₂_o-isoprostanes in ng/μmol 20:4 in response to one unit change in explanatory factor.

^aDue to the skewed distribution, the variable was log-transformed prior to the statistical analysis. Regression models contained two steps: Step 1: age and sex, or age alone, were included in the model. Step 2: Step 1 + stepwise regression (*P* for entry 0.05 and *P* for removal 0.10) for different CVD risk factors.

Discussion

There are recent findings that an elevated n6 PUFA intake could elevate rather than attenuate the risk of CVD and related mortality (10,31,32). Lipid peroxidation (PUFA oxidation) might be one potential mechanism leading to this kind of causality (4,5,33). In this respect, our observations support the long-term evidence which has accumulated over several decades from epidemiological research and clinical trials, i.e. that both elevated n6 and n3 PUFA levels can have beneficial health effects; against expectations, they lower *in-vivo* indices of lipid peroxidation. Our results also suggest that elevated F₂-IsoP levels are associated with several clinical risk factors of CVD, such as high body mass index and the low plasma PUFA/SFA ratio, and that these clinical factors dilute the disease links with F₂-IsoPs down to statistical non-significance. In addition, our findings show that F₂-IsoP levels are not dependent on the amount of plasma free 20:4, the precursor molecule for the isoprostanes.

The current dietary recommendations for n6 PUFAs (8) are based on the assumption that the risk of CVD becomes reduced

when SFAs or refined carbohydrates in the diet are replaced with n6 PUFAs. In the present study, low PUFA (either n3 or n6 PUFA) and a low PUFA/SFA ratio were associated with the elevated F₂-IsoPs, whereas a high dietary intake of SFA and high consumption of *trans*-FAs were associated with the elevated F₂-IsoPs (Tables I, III, and IV). These findings support the view that PUFA intake has beneficial health effects, as compared with SFA or *trans*-FA intake (even though plasma SFAs were inversely associated with the F₂-IsoPs), i.e. a high intake may attenuate lipid peroxidation in the circulation. In addition, we found that the plasma n6/n3 PUFA ratio was inversely associated with the F₂-IsoPs (Tables I, III, and IV). This finding provides evidence against the proposal that n6 PUFA could increase and n3 PUFA decrease the formation of F₂-IsoPs (12), or alternatively that n6 PUFA could itself exert lipid peroxidation-mediated adverse cardiovascular effects. However, in our study, high levels of both plasma n3 PUFA levels and dietary n3 PUFA intake were associated with decreased F₂-IsoPs (Table I). Dietary sources of FAs may also explain the link of the n6/n3 PUFA ratio with the lowered F₂-IsoPs since in Finland, with its Western diet, circulating n3 PUFA levels may reflect the animal fats present in the diet (34). Recently, findings from small supplementation studies have suggested that a fish-rich diet could lower urinary F₂-IsoPs (35) and that n3 PUFA supplementation (purified eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) ethyl esters, 4 g daily for 6 weeks) was able to attenuate urinary and plasma F₂-IsoP concentrations in subjects suffering from dyslipidemia, or hypertension and type 2 diabetes (13,35,36). The inverse association noted here between the plasma n3 PUFA and the plasma F₂-IsoPs (Table I) confirms these earlier observations on the population level.

A recent review has summarized the impacts of elevated F₂-IsoP levels in different metabolic states which are known to be linked with elevated CVD risk (33). One of the most convincing findings is that F₂-IsoP levels are 2.3- to 4.1-fold higher in smokers and 1.4- to 3.2-fold higher in subjects with high LDL, as compared with control subjects. The Framingham Study with ELISA-based creatinine-corrected urinary F₂-IsoPs (*n* = 2828) (21) seems to be the largest F₂-IsoP study reported so far. Our associations (either with F₂-IsoPs or the F₂-IsoP/20:4 ratio) were similar to the findings of that American study: following age and sex adjustment, F₂-IsoPs were positively associated with female sex, hypertension treatment, diabetes, blood glucose, BMI, and the history of CVD (with myocardial infarction in the present study, Table II) in both of these studies, whereas total cholesterol (the correlations between LDL cholesterol and apoB with the F₂-IsoP/20:4 ratio in the present study, Table II) revealed an inverse association. In addition, following adjustment for several covariates, decreasing age (no age association was observed in the present study), female sex, smoking (with the F₂-IsoP/20:4 ratio in women in the present study, Table IV), BMI, and blood/plasma glucose (in men in the present study, Table III) were associated with the elevated F₂-IsoP levels. The above findings suggest that elevated F₂-IsoP levels are associated with several risk markers of CVD, i.e. their plasma and urinary levels seem to reflect the general CVD risk factor status of the subjects. However, the finding that age was not positively associated with the F₂-IsoPs does not support the above conclusion, or the free radical theory (37) which claims that the levels of oxidative stress and lipid peroxidation are elevated with increasing age. Furthermore, women had higher F₂-IsoP levels, as compared with men (before and after adjusting for the common CVD risk factors), even though men are—perhaps due to hormonal

(38) and dietary (39) reasons—prone to suffer symptomatic CVD 10 years earlier than women (38). In the literature, increased severity of the CVD has been shown to associate with the elevated F_2 -IsoP levels (33). We can propose one possible mechanism; after the menopause there might be an increase in the F_2 -IsoP levels in women since hormone replacement therapy has been shown to attenuate the F_2 -IsoP levels (40). In our study, most women were post-menopausal. In addition, higher LDL cholesterol levels and the borderline lower duration of the weekly low-intensity physical activity might also lead to higher F_2 -IsoP levels in women. Block and co-workers (22) have also noted this phenomenon that plasma F_2 -IsoPs are higher in female subjects but could not provide any mechanistic explanation. Our study also confirms the previous results that urinary or plasma levels of F_2 -IsoPs correlate with both the elevated plasma CRP (Table II) (22,41) and BNP levels (Tables III and IV) (42).

With respect to the behavioral and dietary factors, duration of the weekly low-intensity physical activity and plasma β -carotene (with F_2 -IsoPs), and plasma α -tocopherol and dietary protein intake (with the F_2 -IsoP/20:4 ratio) all showed inverse F_2 -IsoP associations. Plasma α/β -carotene and ascorbate have also previously been associated with the lowered F_2 -IsoPs (22). It is known that strenuous exercise increases oxidative stress and lipid peroxidation (43), but according to the present study it seems that low-intensity physical activity may have an attenuating effect on these factors. Physical activity may also be a marker of a healthier lifestyle in general. High protein intake and circulating β -carotene or α -tocopherol levels may be indicators of the healthier than average dietary pattern, or the above antioxidants may simply prevent the F_2 -IsoP formation via their antioxidative properties. If one consumes more protein, the amounts of easily oxidized FAs are probably lower, which may result in a lowered status of lipid peroxidation (even though we did not find any association between lipid peroxidation precursors and F_2 -IsoP levels).

We found that, after adjusting for sex and age, F_2 -IsoP levels were significantly higher in subjects with hypertension (and there was also an association with the elevated systolic blood pressure) and diabetes (and there was also an association with the elevated plasma glucose and serum insulin levels), as compared with corresponding non-diseased subjects. With respect to CVD, the association was borderline significant. In the present study, plasma F_2 -IsoP levels were not an independent risk factor for the above-mentioned metabolic diseases since the clinical CVD risk factors, such as plasma glucose or BMI (in the case of type 2 diabetes) or BMI (in the case of hypertension), attenuated the observed associations to statistical non-significance. Studies in which F_2 -IsoP levels have been higher in cases with several metabolic diseases than in their controls have been recently reviewed (33). Diabetic subjects have had 2.0- to 5.9-fold, obese subjects 3.2- to 4.2-fold, and hypertensive subjects 2.0- to 2.5-fold higher F_2 -IsoP levels. In addition, a recent review (44) has summarized all of the most important studies in which the associations between elevated plasma or urinary F_2 -isoprostanes and the CVD have been examined. Of the 22 eligible studies reviewed, 20 studies showed a statistically significant association with the isoprostane level, four of these studies being population-based, and two being prospective. However, also inverse type 2 diabetes (45) and non-existing hypertension (46) associations have been reported. In addition, there is at least one large study (227 cases, 420 controls) with no evidence for any association between GC/MS-based plasma F_2 -IsoPs and the risk of fatal or non-fatal coronary heart

disease (47). Overall, a small number of subjects and the lack of multivariable analyses, resulting in an inappropriate control for confounding factors, are shortcomings in many of these studies.

Should we adjust the plasma free F_2 -IsoP levels for arachidonic acid (20:4) prior to their statistical analyses? Since we and others (16) had no clear answer to this question, we have estimated the CVD risk factor associations for both F_2 -IsoPs and for the F_2 -IsoP/20:4 ratio. Even though plasma total n6 PUFA level was linked with F_2 -IsoPs, there was no association between 20:4 and F_2 -IsoPs. One has to remember that 20:4 accounts for only about 10% of the plasma total free n6 PUFA, and desaturase activity can affect the ratios between different individual circulating FAs (48). In fact, a very small portion of the available 20:4 is oxidized to isoprostanes. For this reason, it would seem that the 20:4 concentration is not the rate-limiting step in the formation of F_2 -IsoPs. Overall, our data do not support the argument that there should be adjustment for 20:4. However, the plasma free 20:4 concentration is not a direct marker of the 20:4 status in different membrane bilayers. For this reason, the possible non-existence of an association between 20:4 and plasma F_2 -IsoPs needs to be confirmed by measuring the plasma total 20:4 concentration.

The present study findings are not necessarily fully generalizable to the wider population since 50% of the subjects suffered from hypertension. For this reason, the associations found in the entire population were also separately tested in both hypertensive and non-hypertensive subjects. There were no conflicting associations observed between the groups. One shortcoming was that this cross-sectional, retrospective study could not assess causality. In addition, we did not analyze other lipid peroxidation products, such as 4-hydroxy-2-nonenal, which originates from the n6 PUFAs including linoleic acid (18:2), the major PUFA in the circulation (49). In food frequency questionnaires, a reporter bias is always present since intakes of unhealthy food items are underestimated and vice versa. F_2 -IsoPs were determined from overnight fasting plasma spot samples, which are believed to represent a reliable approach in all subjects, i.e. those who are healthy as well as those suffering from a chronic metabolic disease (16). The strength of this study is that we used GC/MS-based F_2 -IsoP assays, the 'gold standard' measure for lipid peroxidation, as our index for *in-vivo* lipid peroxidation.

In summary, our findings indicate that elevated circulating n6 and n3 PUFA levels are linked to lowered lipid peroxidation *in vivo*, which is in line with the long-term epidemiological and clinical observations according to which an elevated intake of PUFA confers beneficial effects on cardiovascular health.

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Declaration of interest: The authors declare that there is no conflict of interest.

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