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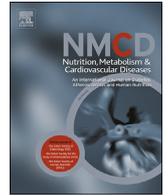
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Omega-3 to omega-6 fatty acid oxidation ratio as a novel inflammation resolution marker for metabolic complications in obesity

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Abstract *Background and aims:* The oxidative metabolism of polyunsaturated fatty acids (PUFAs) leads to bioactive isoprostanooids. The aim was to establish the associations of a complete urinary isoprostanooid profiling in a cohort study of carefully phenotyped obese subjects to determine possible potential differential implications for omega-6 PUFA- and omega-3 PUFA-derived isoprostanooids for obesity, metabolic indicators, and inflammation.

Methods and results: PUFA peroxidation compounds were determined in urine samples from obese human subjects (n = 46) by liquid chromatography coupled to tandem mass spectrometry. Increased omega-6 arachidonic acid (AA) oxidation, mainly represented by 5-F_{2c} isoprostane (5-F_{2c}-IsoP) and metabolites of 15-F_{2t}-IsoP, was associated with body mass index, glycated hemoglobin (HbA1c) and mean arterial blood pressure. In addition, we identified the omega-3 PUFA-derived urinary metabolites 14-F_{4t}-NeuroP from docosahexaenoic acid (DHA) and 5-F_{3t}-IsoP from eicosapentaenoic acid (EPA), which declined with age. The omega-3 to omega-6 oxidation ratio was a significant predictor of inflammation in obesity.

Conclusion: The findings point to full urinary isoprostanooid profiling as a more sensitive measure of PUFA oxidative stress in obesity-induced metabolic complications compared with individual isoprostanooid measures. Furthermore, the results suggest the balance between the omega-3 and omega-6 PUFA oxidation as determinative for the consequences of oxidative stress on inflammation in obesity.

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1. Introduction

Oxidative stress is an important orchestrator of inflammation tightly regulated through alterations in the production and degradation systems for reactive oxygen species (ROS). The net oxidative effects on lipids are extensively used to monitor activity alterations of the oxidative and anti-oxidative systems in different diseases. In particular, F₂-isoprostanol (F₂-IsoPs) are formed by non-enzymatic oxidative metabolism (chemical reactions that occur in parallel to enzyme-catalyzed pathways) of the polyunsaturated fatty acid (PUFA) arachidonic acid (AA). The urinary excretion of 15-F₂t-IsoP and its metabolites 2,3-dinor-15F₂t-IsoP and 2,3-dinor-5,6-dihydro-15F₂t-IsoP is an established oxidative stress marker [1] in several inflammatory diseases, including cardiovascular diseases [2,3]. Urinary F₂-IsoPs also reflect important cardiovascular and metabolic risk factors, e.g., smoking, diabetes, renal disease, and obesity [4]. A recent meta-analysis confirmed a robust significant positive association between urinary F₂-IsoPs and body mass index (BMI) [5].

Importantly, F₂-IsoPs are not only markers but also effectors of inflammation and cardiovascular disease by means of activation and downstream signaling through the thromboxane prostanoid (TP) receptor [6]. Oxidative stress hence emerges as a proinflammatory pathway that escapes the enzymatic inhibition of AA metabolism by cyclooxygenase inhibitors. Previously, TP has been shown to be correlated with obesity markers and has been proposed as a therapeutic target for treating insulin resistance [7]. Thus, the proinflammatory effects of F₂-IsoP may contribute to obesity-induced metabolic consequences. It should also be considered that oxidative AA metabolism leads to the formation of a large spectrum of isoprostanoids. The full characterization of the oxidative AA metabolites hence has the potential to identify novel biomarkers and to gain further insights into the pathophysiological role of bioactive lipid metabolites in obesity-induced inflammation.

In addition to the omega-6 PUFA AA, the omega-3 PUFA eicosapentaenoic acid (EPA) and docosahexaenoic (DHA) are also non-enzymatically metabolized into F₃-isoprostanol (F₃-IsoPs) and F₄-neuroprostanol (F₄-NeuroPs), respectively [8]. Recent data point towards biological activities of these metabolites [9,10], associated with beneficial effects through a promotion of the resolution of inflammation [11–13]. Omega-3 fatty acids may exert beneficial effects in obesity both by affecting lipid homeostasis by lowering triglycerides [14] and by dampening vascular inflammatory circuits through metabolism into specialized proresolving mediators [15]. No previous study has explored F₃-IsoPs and F₄-NeuroPs as biomarkers in obesity.

Since obesity is tightly linked to oxidative stress and inflammation, the aim of the present study was to establish the associations of a complete urinary isoprostanoid profiling in a carefully phenotyped cohort of obese subjects to determine possible potential differential implications for the complete spectrum of omega-6 and omega-3-

derived isoprostanoids for obesity, metabolic indicators, and inflammation.

2. Methods

2.1. Study population

Patients admitted to the University Hospital of Montpellier (France) between January 2011 and February 2012 for assessment of obesity (BMI ≥ 30 kg/m²) were considered for participation. Exclusion criteria were treatment with anti-inflammatory medications, asthma, known diabetes mellitus (hemoglobin A1c (HbA1c) $> 6.5\%$; fasting plasma glucose > 7 mmol/l and/or treatment with any anti-diabetic medications), and hs-CRP above 10 mg/l^{16, 17}. Height and weight were measured wearing light clothing and no shoes. BMI was calculated as weight in kg divided by the square of height in meters (kg/m²). Waist circumference was recorded to the nearest 0.1 cm midway between the last rib and the iliac crest using a non-stretch tape measure on lightly clad participants.

All clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki and in compliance with International Conference on Harmonization/Good Clinical Practice regulations. After approval by the Montpellier University Hospital's ethics committee (Comité de Protection des Personnes Sud Méditerranée IV), the study was registered at the Ministère de la Santé et de l'Enseignement Supérieur according to French law; study number DC-2009-1052. All patients gave their written informed consent. For minor participants, written informed consent was obtained from the legal guardian.

2.2. Blood sample collection and analysis

Blood samples were collected after an 8 h overnight fasting period. Plasma glucose and glycated hemoglobin (HbA1c) were analyzed by routine high-performance liquid chromatography (HPLC)-based ion-exchange procedure (HA-8140; Menarini, Rungis Cedex, France). Total cholesterol, triglycerides (TG), high-density lipoprotein (HDL) were measured using routine enzymatic methods (KonePro; Konelab, Epoo, Finland). LDL cholesterol was calculated according to the Friedwald formula. Hs-CRP was assessed by immunoturbidimetry (Randox reagents on Olympus apparatus, Rungis, France). The Homeostatic Model Assessment Index-insulin Resistance (HOMA-IR) was calculated as follows: fasting insulin \times fasting glucose/22.5 (insulin-resistance was defined by a HOMA-IR ≥ 3) [18].

2.3. Urine collection and measurements

Urine was collected in the morning and frozen in aliquots at -80 °C until analysis. Urinary isoprostanoid profiling was performed using micro-LC-MS/MS equipment after performing extractive steps of the biological matrix, the highlights of which are presented here. First, 1 mL of urine is added to 1 mL of formic acid 40 mM (pH 4.5) and to 4 μ L of internal standard mixture (1 ng/ μ L of each IS) before

being vortexed for 30s. Then, mixtures are extracted by Solid Phase Extraction (SPE) after a suitable conditioning of Oasis Max cartridge (30 μ m, 60 mg, Waters, Milford, MA, U.S.A.): 2 mL of MeOH followed by 2 mL of formic acid 20 mM. This extraction consists of a wash with 2×1 mL of four successive solvent mixtures: respectively, NH₃ 2%, methanol/formic acid 20 mM (30:70, v/v), hexane, and hexane/ethyl acetate (70:30, v/v). Then metabolites of interest are eluted with hexane/ethanol/acetic acid (70:29.4:0.6, v/v/v), concentrated to dryness under a nitrogen stream and then reconstituted by adding 100 μ L of mobile phase (water/acetonitrile, 83:17, v/v, with each containing 0.1% formic acid). Therewith metabolites were analyzed by microLC-MS/MS on an Eksigent chromatographic system (Sciex Applied Biosystems, Framingham, MA, USA) coupled to a QTRAP 5500 mass system (Sciex, Concord, ON, Canada) as described in previous work [19,20]. The MS/MS was operated in electrospray ionization (ESI) negative mode. Detection of the fragmentation ion products from each deprotonated molecule $[M-H]^-$ was performed in the multiple reaction monitoring mode (MRM). Concentration of the analytes was obtained by calibration curves calculated by the area ratio of analytes and IS. Data processing was achieved using the MultiQuant 3.0 software (Sciex Applied Biosystems). The obtained isoprostanoids levels were normalized to urinary creatinine concentrations, which were determined using a standard colorimetric assay (Cayman Chemicals, Ann Arbor, MI, USA).

2.4. Statistics

The strength and direction of associations between clinical and laboratory parameters were assessed through a Spearman correlogram. Significant associations between parameter pairs are marked with circles, where the radii are proportional to the magnitude of association strength. Cold colors represent a positive association, while warm colors indicate an inverse relationship. Adjusted multi-variable analyses were carried out by implementing linear regression models. A p-value of less than 0.05 was considered statistically significant and all tests were two-tailed. The statistical analyses were performed in R version

4.2 (R Core Team [2021]. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL <https://www.R-project.org/>).

3. Results

3.1. Study cohort

After the initial screening, 59 subjects were included in the study. Of these, 11 subjects were excluded because their CRP-levels exceeded 10 mg/L (defined as exclusion criteria in Ref. [17]). Another two subjects were excluded because of non-useable urine samples, resulting in a study cohort of 46 subjects. The characteristics of $n = 46$ individuals included in the study cohort have been previously reported, with stratification on sex [17] and high and low urinary prostaglandin levels [16]. The baseline characteristics stratified for smoking are shown in Table 1. Given the few smokers in this cohort and that the active smokers ($n = 6$) were younger with lower BMI compared with former and non-smoking subjects, the smoking stratification was not statistically tested and not used for group comparisons in biomarker analyses.

3.2. Lipidomics

The full urinary isoprostanoid profile is shown in Table 2. The prevailing detected urinary isoprostanoids were 5-F_{2c}-IsoP and metabolites of 15-F_{2t}-IsoP. Importantly, the analysis also revealed detectable levels of 5-F_{2t}-IsoP and the two omega-3-derived metabolites 5-F_{3t}-IsoP and 14-F_{4t}-NeuroP (Table 1).

3.3. Urinary markers of AA, DHA, and EPA oxidation

The concentrations of all F₂-IsoP metabolites and 15-A₂-IsoP were summarized to obtain a total measure of the omega-6 PUFA AA oxidation ($M = 26\ 508.5$; $SD = 10\ 728.7$). The sum of the EPA oxidation product 5(RS)-5-F_{3t}-IsoP and the DHA oxidation product 14(RS)-14-F_{4t}-NeuroP was used to estimate the total omega-3 PUFA oxidation ($M = 4060.7$; $SD = 3233.8$). Finally, the

Table 1 Characteristics of subjects in the study cohort. Continuous variables are presented as mean \pm SD.

	All (n = 46)		Non-smokers (n = 35)		Previous smokers (n = 5)		Active smokers (n = 6)	
Age (years)	44.0	± 12.9	43.3	± 14.2	43.6	± 7.8	28.6	± 17.4
Males (n, %)	14	(30%)	13	(40%)	1	(20%)	0	(0%)
BMI (kg/m ²)	37.9	± 5.9	37.6	± 5.8	36.5	± 5.5	21.8	± 18.4
Waist to Hip Ratio	0.9	± 0.1	0.9	± 0.1	0.9	± 0.1	0.5	± 0.5
MAP (mmHg)	90.1	± 8.4	89.7	± 8.3	89.0	± 6.2	49.1	± 47.1
CRP (mg/dL)	4.0	± 2.4	3.7	± 2.3	4.1	± 2.5	3.1	± 0.9
Creatinine (μ mol/l)	66.6	± 14.1	68.8	± 14.9	60.4	± 9.0	41.1	± 30.7
Cholesterol (g/L)	2.0	± 0.4	2.0	± 0.4	1.9	± 0.5	1.2	± 0.9
LDL (g/L)	1.2	± 0.3	1.2	± 0.3	1.2	± 0.1	0.8	± 0.5
HDL (g/L)	0.5	± 0.1	0.4	± 0.1	0.5	± 0.0	0.3	± 0.2
TG (g/L)	1.5	± 0.6	1.5	± 0.7	1.6	± 0.4	1.1	± 0.5
HbA1c (%)	5.8	± 0.5	5.8	± 0.5	5.5	± 0.5	3.2	± 3.0
HOMA-IR	3.4	± 2.3	3.3	± 2.5	2.8	± 0.7	2.9	± 0.5

Table 2 Results of the isoprostanoid profiling for the n = 46 subjects in the study cohort. Results (mean \pm SD) are expressed as pg/mg of creatinine.

Arachidonic acid (AA) oxidation		
15-F _{2t} -IsoP	195.9	\pm 122.0
15- <i>epi</i> -15-F _{2t} -IsoP	907.9	\pm 670.1
15(RS)-F _{2t} -IsoP	1103.1	\pm 769.9
15-A ₂ -IsoP	226.7	\pm 222.0
2,3-dinor-15-F _{2t} -IsoP	4201.9	\pm 1781.3
2,3-dinor-15- <i>epi</i> -15-F _{2t} -IsoP	2982.5	\pm 1381.2
<i>ent</i> -2,3-dinor-5,6-dihydro-15-F _{2t} -IsoP	2454.7	\pm 1194.2
Metabolites of 15(RS)-F _{2t} -IsoP	9639.1	\pm 4056.8
5(RS)-5-F _{2t} -IsoP	3932.5	\pm 1930.3
5-F _{2c} -IsoP	11616.4	\pm 4934.2
15-F _{2t} -IsoP + Metabolites	10732.9	\pm 4493.3
Eicosapentaenoic acid (EPA) oxidation		
5(RS)-5-F _{3t} -IsoP	2313.7	\pm 1429.1
Docosahexaenoic acid (DHA) oxidation		
14(RS)-14-F _{4t} -isoP	1692.1	\pm 2388.0
Oxidation sums		
Total omega-6 F _{2t}	14665.4	\pm 6144.7
Total omega-6 oxidation	26508.5	\pm 10728.7
Total omega-3 F _{3t} F _{4t}	3881.9	\pm 3204.5
Oxidation ratios		
F _{4t} /F _{3t}	2.7	\pm 11.7
omega-6 F _{2t} /(omega-3 F _{4t} + F _{3t})	0.3	\pm 0.2
omega-3/omega-6	0.16	\pm 0.11

ratio between omega-3 and omega-6 PUFA oxidation was calculated (M = 0.16; SD = 0.11). Between these grouped products, the urinary fatty acid oxidation marker inter-correlations included a significant positive correlation between AA and EPA oxidation, whereas DHA oxidation did not exhibit any significant inter-correlations in univariate analysis (Fig. 1). In the age-adjusted analysis, DHA was significantly associated with EPA (B = 0.54; P = 0.03) but not AA oxidation, and the association between AA oxidation remained significant with EPA (B = 3.36; P = 0.003).

3.4. Urinary PUFA oxidation markers in relation to age, sex, and smoking

Urinary oxidation products were the most abundant from AA, followed by EPA, and DHA (Fig. 1). Total omega-3 oxidation products represented 12.9% of the total PUFA oxidation products.

Comparing oxidation products after stratification according to sex did not reveal any differences between males and females for AA (P = 0.24; Fig. 1A), EPA (P = 0.73; Fig. 1B), DHA (P = 0.57; Fig. 1C), total omega-3 oxidation (P = 0.69; Fig. 1D) or omega-3/omega-6 ratio (P = 0.32; Fig. 1E). There was a trend towards increased levels of AA oxidation and decreased omega-3 oxidation with aging. In a sex-adjusted analysis, DHA (B = -57.7; P = 0.04) and total omega-3 (P = 0.02) were significantly decreased with age. The urinary IsoP profile according to stratification for smoking status (non-smoking, former smoking, or active smoking) did not exhibit any significant differences between the groups (data not shown).

3.5. Urinary oxidation marker association with obesity and metabolic phenotype

AA oxidation was significantly associated with obesity and metabolic phenotype, being significant for BMI, Hb1Ac, HOMA-IR, and blood pressure (Fig. 2), of which BMI (B = 829.7; P = 0.0014), Hb1Ac (B = 6068.1; P = 0.047), and mean arterial pressure (MAP; B = 2449.1; P = 0.0016) remained significant in age-adjusted analyses. Urinary omega-3 fatty acid oxidation markers did not exhibit any significant associations in the univariate (Fig. 2) or age-adjusted (data not shown) analysis. The ratio between omega-3 and omega-6 fatty acids exhibited a significant inverse correlation with Hb1Ac, which was significant in univariate (Fig. 2) but not in age-adjusted (B = -0.037; P = 0.22) analyses. None of the oxidation markers were significantly associated with HDL, LDL, and TG levels (Fig. 2). In the age-adjusted analyses, there was a trend towards an inverse association for LDL concentration with EPA (B = -933.7; P = 0.17), DHA (B = -1891.2; P = 0.09), and total omega-3 oxidation (B = -2753.0; P = 0.06).

3.6. Urinary oxidation marker association with inflammation

Using CRP as a marker of low-grade systemic chronic inflammation confirmed a significant positive association with BMI, Hb1Ac, HOMA, and blood pressure (Fig. 2). In contrast, CRP was not significantly associated with AA oxidation. However, the omega-3/omega-6 oxidation ratio was significantly and inversely associated with CRP. In an age-adjusted analysis, only the CRP association with the omega-3/omega-6 oxidation ratio (Fig. 3) remained significant (B = -0.014; P = 0.03).

4. Discussion

This is the first complete urinary isoprostanoid profiling in a clinically relevant obesity cohort, reporting for the first time the detection of a broad spectrum of metabolites, of which the decline in omega-3 isoprostanoids with age is a novel observation. In addition to confirming the association of AA oxidation products with BMI, a full isoprostanoid profiling allowed to extend AA oxidation to associations with metabolic control and blood pressure, indicating a major role of AA oxidation in metabolic complications of obesity. Finally, we evoke the omega-3 to omega-6 oxidation ratio as a novel predictor of inflammation in obesity.

The broad spectrum of urinary isoprostanoids detected in obese subjects in the present study opens up for distinguishing specific pathways in obese subjects. The lack of complete intercorrelation between the classes of oxidative metabolites according to PUFA substrates support the differential PUFA oxidizability, being preferential for AA followed by EPA and then DHA [21]. A preferential AA oxidation is supported by the predominant AA oxidation in this cohort. The significant association between AA

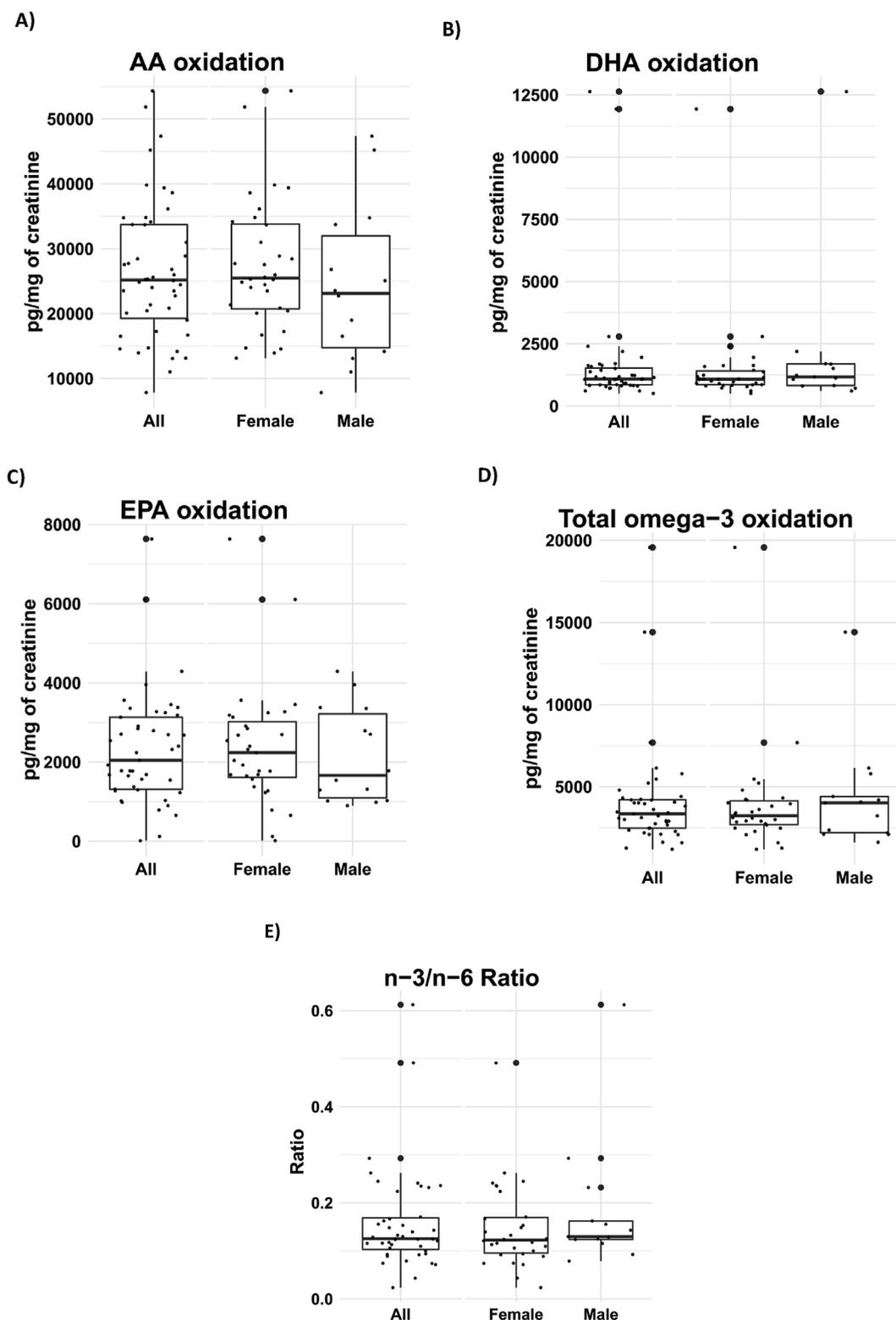


Figure 1 Urinary isoprostanoid concentration representing products generated from A) arachidonic acid (AA) oxidation, B) docosahexaenoic acid (DHA) oxidation, and C) eicosapentaenoic acid (EPA) oxidation. D) The total isoprostanoids from omega-3 fatty acids. E) The ratio between isoprostanoids derived from omega-3 (n-3) and omega-6 (n-3) oxidation.

oxidation and BMI is in line with previous studies in mixed populations [5], and extends the observation to an obese cohort, suggesting that an increased degree of obesity

further enhances AA oxidation. In addition to BMI, HbA1c was an independent predictor of AA oxidation. Previous studies have established increased urinary levels of 15-F_{2t}-

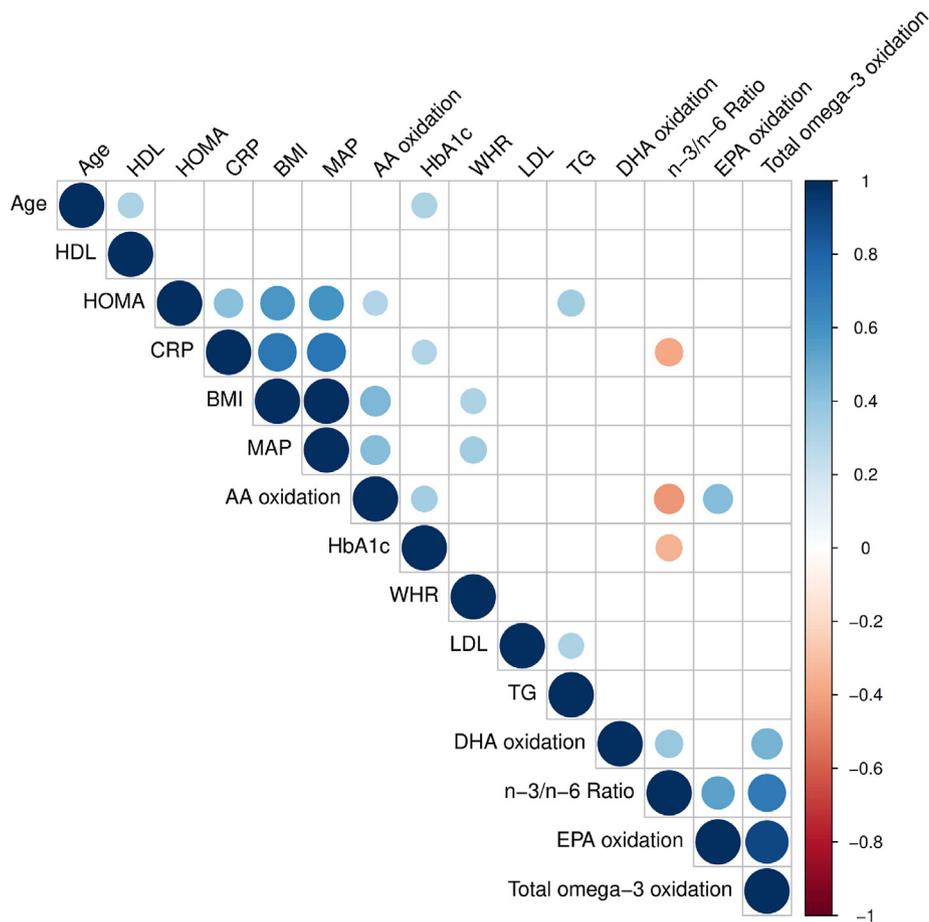


Figure 2 Spearman correlogram for univariate associations in the cohort of n = 45 obese subjects in the study cohort. Abbreviations: HDL: High-density lipoprotein, HOMA: Homeostatic Model Assessment of Insulin Resistance, CRP: C-reactive protein, BMI: body mass index, MAP: mean arterial blood pressure, AA oxidation: arachidonic acid oxidation products, HbA1c: hemoglobin H1c, WHR: waist-to-hip ratio, LDL: Low-density lipoprotein, TG: triglycerides, DHA oxidation: docosahexaenoic acid oxidation products, n-3/n-6 Ratio: calculated from (DHA oxidation + EPA oxidation)/AA oxidation, EPA oxidation: eicosapentaenoic acid oxidation products, Total omega-3 oxidation: calculated from DHA oxidation + EPA oxidation.

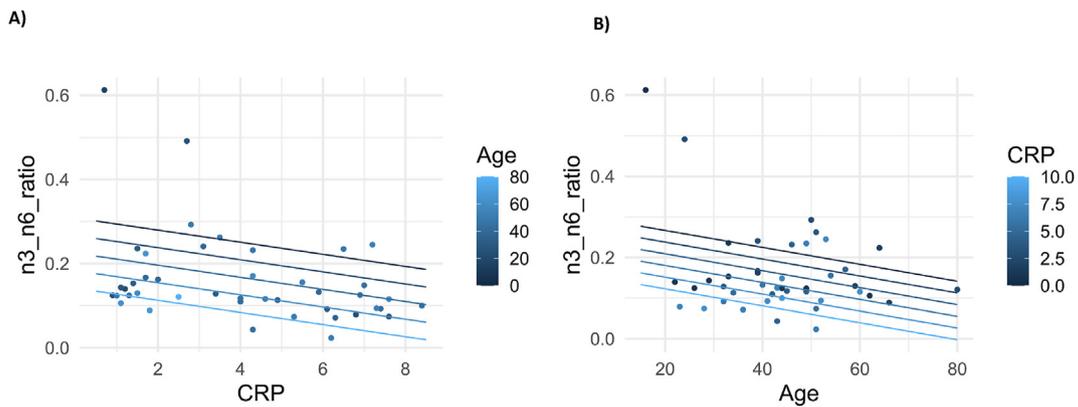


Figure 3 The association of the omega-3 to omega-6 fatty acid oxidation ratio (n3/n6 ratio) with (A) C-reactive protein (CRP) adjusted for age, and (B) age adjusted for CRP.

IsoP in diabetic vs non-diabetic subjects [22,23], but a non-significant association for 15-F_{2t}-IsoP with HbA1c has been reported in non-stratified analyses [23]. The significant association between HbA1c and total AA oxidation in the present obese cohort underlines the importance of

considering the whole oxidative urinary metabolome rather than individual markers to reflect the PUFA oxidation status. In addition, the importance of the oxidative serum metabolome has also been implicated previously in a Japanese study involving 142 overweight type 2 diabetes

patients, where an elevated ratio between EPA and AA was indicative of better ability to reduce the bodyweight under a calory-restricted diet [24]. Furthermore, we show that AA oxidation was also independently associated with MAP, which extends the known implications of 15-F_{2t}-IsoP for hypertension [22], to replication in obese subjects and to the total urinary oxidative AA metabolome. Taken together, the present findings support that a full AA oxidation profiling allows to detect a close relation to obesity-induced metabolic consequences in terms of diabetes and hypertension.

In addition to AA oxidation, the present study identified the oxidative omega-3 PUFA metabolites 5-F_{3t}-IsoP from EPA and 14-F_{4t}-NeuroP from DHA in urinary samples from obese subjects. Importantly, sex-adjusted analyses detected a decreased DHA and total omega-3 oxidation with age in obesity. Given the association of circulating omega-3 with healthy ageing [25], the age-dependent effects should also potentially consider the oxidative omega-3 PUFA metabolism into biologically active lipid mediators.

Inflammation is a key feature of obesity and a possible link to its metabolic consequences. Oxidative stress is a potent inducer of inflammation, for example through isoprostanoinduced proinflammatory signaling. Unexpectedly, AA oxidation was in the present study not significantly associated with the degree of chronic inflammation in obese subjects, as determined by CRP. The AA oxidation may, however, be balanced by anti-inflammatory/pro-resolving effects of omega-3 PUFA oxidative metabolites. Importantly, our results show that the omega-3/omega-6 oxidation ratio was significantly and inversely associated with chronic inflammation, independently of ageing. These findings implicate that the balance between the omega-3 and omega-6 PUFA oxidation may be determinative for the consequences of oxidative stress on inflammation in obesity.

The present study provided the first analyses of full urinary isoprostanoind profiling in obesity, pointing to a more important predictive value for the complete measures of AA oxidation in terms of metabolic complications, and identifying the omega-3 PUFA oxidative metabolites as balancers of inflammation measures. There are, however, some limitations. First, the low number of subjects should be acknowledged, and development of high throughput isoprostanoind profiling will allow to extend the study to larger populations. Second, the distribution of smokers (76% non-smokers) in the cohort did not allow us to fully evaluate the influence of smoking-induced PUFA oxidation in the present study. Third, the lack of food frequency questionnaires cannot exclude that dietary choices of the participants had an effect on the outcomes of the study. Fourth, further studies are needed to elucidate the mechanistic implications of omega-3 PUFA-derived oxidative products as pro-resolving mediators of obesity-induced inflammation.

In summary, AA-oxidation captured by a complete urinary isoprostanoind profiling allowed to extend a significant isoprostanoind association with obesity to its metabolic complications since total AA oxidation was

significantly associated with HbA1c and MAP in obesity. We also report, for the first time, urinary omega-3 PUFA-derived isoprostanoinds in obesity with declined omega-3 PUFA oxidation with age. Finally, obesity-related chronic inflammation can potentially be determined by the omega-3 to omega-6 oxidation ratio. In conclusion, the full isoprostanoind profiling in urine is demonstrated as a more complete measure of PUFA oxidation compared with isolated urinary metabolites. The full urinary isoprostanoind profiling, in addition, identified the ratio for urinary non-enzymatic metabolites derived from omega-3 and omega-6 PUFA oxidation as a novel inflammatory marker in obesity, which may reflect the balance between proinflammatory and pro-resolving isoprostanoinds. Given the findings pertaining to the urinary omega-3/omega-6 oxidation ratio, it would be beneficial to further investigate, in future studies, its clinical utility, predictive value, and extension of its relation to other medical conditions.

Author contribution statement

All authors contributed to the design, analyses, and writing of the manuscript.

Declaration of competing interest

None declared.

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