Diabetes, Metabolic Syndrome and Obesity

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ORIGINAL RESEARCH

Links Between Adipose Tissue Gene Expression of Gut Leakage Markers, Circulating Levels, Anthropometrics, and Diet in Patients with Coronary Artery Disease

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Background: Recent studies suggest gut-derived lipopolysaccharide (LPS)-translocation to play a role in both systemic inflammation and in inflammatory adipose tissue. We aimed to investigate whether circulating LPS-related inflammatory markers and corresponding genetic expression in adipose tissue were associated with obesity, cardiometabolic risk factors, and dietary habits in patients with coronary artery disease.

Methods: Patients (n=382) suffering a myocardial infarction 2–8 weeks prior to inclusion were enrolled in this cross-sectional study. Subcutaneous adipose tissue (SAT), taken from the gluteal region, and fasting blood samples were collected at inclusion for determination of genetic expression of LPS-binding protein (LBP), CD14, toll-like receptor 2 (TLR2), and TLR4 in SAT, and LPS, LBP, and soluble cluster of differentiation 14 (sCD14) in the circulation. All patients filled out a dietary registration form.

Results: Patients (median age 74 years, 25% women), had a median body mass index (BMI) of 25.9 kg/m^2. Circulating levels of LBP correlated to BMI (p=0.02), were significantly higher in overweight or obese (BMI≥25 kg/m^2) compared to normal- or underweight patients (BMI<25 kg/m^2), and were significantly elevated in patients with T2DM, hypertension, and MetS, compared to patients without (p≤0.04, all). In SAT, gene expression of CD14 and LBP correlated significantly to BMI (p≤0.001, both), and CD14 and TLR2 expressions were significantly higher in patients with T2DM and MetS compared to patients without (p≤0.001, both). Circulating and genetically expressed CD14 associated with use of n-3 PUFAs (p=0.008 and p=0.003, respectively). No other significant associations were found between the measured markers and dietary habits.

Conclusion: In patients with established CAD, circulating levels of LBP and gene expression of CD14 and TLR2 in SAT were related to obesity, MetS, T2DM, and hypertension. This suggests that the LPS–LBP–CD14 inflammatory axis is activated in the chronic low-grade inflammation associated with cardiometabolic abnormalities, whereas no significant associations with dietary habits were observed.

Keywords: endotoxemia, lipopolysaccharide, LPS-binding protein, gut leakage, obesity, metabolic syndrome, coronary artery disease

Introduction

Obesity is one of the five major modifiable risk factors for atherosclerotic cardiovascular disease (CVD).[1] Obesity results from a long-term positive energy balance, now generally accepted as a *disease* of energy homeostasis and body weight regulation.[2] The mechanisms behind this are not fully understood, but seem to include an intricate interplay between genetic, behavioral, environmental, and developmental pathways.[2]

Adipose tissue (AT) mainly consists of adipocytes, but also resident macrophages, T-cells, fibroblasts, and vascular cells.[3] In obesity, the expanding adipocytes produce increased amounts of inflammatory cytokines and metabolites such as free fatty acids and become pro-inflammatory.[4] The anti-inflammatory macrophages (M2) change their phenotype to become pro-inflammatory,

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which promotes further infiltration of immune cells into the AT.[5] The adipocytes themselves lose their normal metabolic function, causing alterations in lipid metabolism, as well as insulin resistance and glucose intolerance.[6,7] Obesity is thus strongly associated with traditional cardiometabolic risk factors such as type 2 diabetes mellitus (T2DM), dyslipidemia, and hypertension (HT).[8–10] The combination of central obesity, HT, and altered lipid and glucose metabolism has been named the metabolic syndrome (MetS),[11] also characterized by low-grade systemic inflammation.[12]

Triggers of inflammation in obesity are poorly understood. One current opinion is that gut dysbiosis and associated increase in intestinal permeability, via metabolic endotoxemia, may contribute to both (1) enhanced systemic inflammation and (2) local inflammation in adipose tissue[13,14] (Figure 1). Endotoxemia is the translocation of bacterial compounds such as lipopolysaccharide (LPS), a potent activator of the innate immune system via the toll-like receptor 4 (TLR4) pathway, across the gut barrier and into the circulation.[15] Gut dysbiosis and endotoxemia are common features in obesity and metabolic dysfunction.[16] However, whether endotoxemia actually contributes to the AT inflammation associated with obesity, is a consequence of excessive calorie intake and poor diet often associated with obesity, or a by-product of visceral AT and gut inflammation is not established yet. LPS has been shown to be able to initiate the transition of the M2 macrophages towards the pro-inflammatory M1 phenotype in obese AT,[17] suggesting a possible link between gut leakage and AT inflammation. LPS has also been reported to activate TLR2.[18] In fact, several of the mediators of the LPS response, including TLR2, TLR4, cluster of differentiation 14 (CD14), and LPS-binding protein (LBP), have been shown to be upregulated in AT in association with obesity and metabolic disturbances,[19–21] indicating a possible LPS-driven inflammation in several conditions of cardiometabolic disease. To our knowledge, there are no reports on the relationship between gut leakage markers and AT inflammation in patients with different features of cardiometabolic disease in populations with established coronary artery disease (CAD). Therefore, to investigate a possible mechanistic link between gut leakage and adipose tissue inflammation, we aimed to explore any associations between circulating gut leakage markers and genes related to the LPS inflammatory pathway in subcutaneous AT (SAT) in patients with established CAD.

Diet is by far the most important modulator of the gut microbiota. As diets high in fat and low in fiber associate with dysbiosis and endotoxemia,[22,23] and intake of n-3 polyunsaturated fatty acids (n-3 PUFAs) has been shown to be protective for the gut microbiota,[24] we also wanted to explore any relationships to dietary habits.

We hypothesize that levels of circulating gut leakage markers and their expression in SAT would be inter-related and elevated in obese patients and patients with concomitant cardiometabolic risk factors, and related to dietary habits.

Methods

Study Population

The current study is part of the Omega-3 fatty acids in Elderly patients with a Myocardial Infarction (OMEMI) trial, which has previously been described in detail.[25] Patients, aged 72–80 years, were recruited between 2012 and 2018, and were

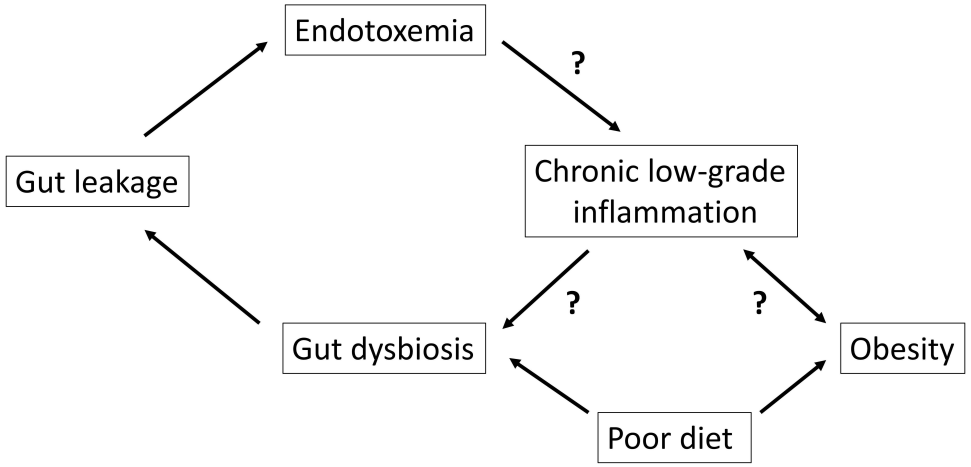

Figure 1 This figure shows the possible interaction between a poor diet, gut dysbiosis, leaky gut and associated endotoxemia, and a state of chronic low-grade inflammation and obesity.

included 2–8 weeks after a myocardial infarction. All patients were clinically stable. The present results are cross-sectionally based on baseline data from patients consecutively enrolled at Oslo University Hospital (OUH) and from whom adipose tissue samples were collected (n=382).

The study was conducted in accordance with the Declaration of Helsinki and approved by the Regional Ethics Committee of the South Eastern Norway Regional Health Authority (2012/1422). All patients gave written informed consent to participate. The study is registered at ClinicalTrials.gov, April 16, 2013, NCT01841944.

Smokers were defined as current smokers, hypertension (HT) as previous diagnosis of HT, and diabetes mellitus as known T2DM. MetS was classified by modified NCEP ATP III criteria[26] with body mass index (BMI) ≥30.0 kg/m^2 as a substitute for waist circumference which was not available.[27] MetS was thus diagnosed in patients meeting three or more of the following criteria: Fasting glucose ≥5.6 mmol/L or drug treatment for elevated blood glucose, HDL cholesterol <1.0 mmol/L (men) or <1.3 mmol/L (women), triglycerides ≥1.7 mmol/L, blood pressure ≥130/85 mmHg or drug treatment for HT, or BMI ≥30.0 kg/m^2. Patients were further classified according to the WHO criteria as normal-weight (BMI 18.0–25 kg/m^2) or overweight or obese (BMI >25.0 kg/m^2).[28]

Patients in the OMEMI trial completed the validated food frequency registration form SmartDiet. Details of the SmartDiet have been given previously.[29] The diets were scored as unhealthy ("poor"), intermediate ("intermediate"), and healthy ("healthy").

Blood samples were drawn in fasting state (>10 h) by standard venipuncture between 08AM and 11AM at inclusion. Routine blood samples were determined by conventional methods. Serum and EDTA plasma were prepared by centrifugation at 2500*g* for 10 min within 1 h and at 2500*g* for 20 min at 4 °C, respectively, both kept frozen at −80 °C until analyses. SAT was taken from the gluteal region with a biopsy needle, and immediately frozen at −80 °C until RNA extraction.

Laboratory Methods

LBP and soluble CD14 (sCD14) were measured in EDTA plasma by ELISAs (Hycult Biotech Uden, The Netherlands and R&D Systems Europe, Abingdon, Oxon, UK, respectively). ELISA (DRG Instruments, Marburg/Lahn, Germany) was used to measure high-sensitivity C-reactive protein (hsCRP) in serum. The Kinetic Chromogenic Limulus Amebocyte Lysate (LAL) Assay (Lonza BioScience, Basel, Switzerland) was used to measure LPS in EDTA plasma. The inter-assay coefficients of variation (CV) were 6.8%, 6.9%, 10.0%, and 6.5%, respectively.

Gene Expression in SAT

The RNeasy Lipid Tissue Mini Kit was used to isolate total RNA from SAT, according to the manufacturer's protocol (Qiagen, GmbH, Hilden, Germany). The quantity (ng/µL) and quality of RNA was examined by the NanoDropTM 1000 Spectrophotometer (SaveenWerner, Sweden). Equal amounts of qScriptTM cDNA superMix (Quanta Biosciences, Gaithersburg, MD, USA) and RNA (5 ng/µL) were mixed to make Copy DNA (cDNA). Real-time PCR was performed on a ViiATM7 instrument (Applied Biosystems by Life Technologies, Foster City, CA, USA) to analyze gene expression, using TaqMan® Universal PCR Master Mix (P/N 4324018) with commercially available TaqMan® assays as follows for the selected markers: CD14 (Hs02621496_s1), LBP (Hs01084628_m1), TLR4 (Hs01084628_m1), and TLR2 (Hs01084628_m1) (all Applied Biosystems, Foster City, CA, USA). To determine the mRNA levels, the ΔΔCT method was applied, giving relative quantification (RQ) by using β2-microglobulin (B2M) (HS99999907_m1) (Applied Biosystems by Thermo Fisher Scientific, Life Technologies Corporation, Pleasanton, CA, USA) as an endogenous control related to a reference sample.[30]

Statistical Analyses

Data are given as mean (±SD), median (25th and 75th percentiles), or proportions as appropriate. Correlation analyses were performed by Spearman's Rho, and Bonferroni correction was used to correct for multiple testing. Mann–Whitney *U*-test was used for analyses of differences between two independent groups, while Kruskal–Wallis test was used for differences between three groups. Stata SE version 15 (StataCorp LLC, College Station, Texas, USA) was used to perform statistical calculations. *P*-values <0.05 were considered statistically significant.

Results

Patient Characteristics

Baseline characteristics, use of medication, and biochemical data for the cohort (n=382) at the time of inclusion are given in Table 1. Patients had a median age of 74 years, and 25% were women. More than 40% had previous CVD. Median BMI was 25.9 kg/m^2, about 60% were classified as either overweight or obese, 53% had hypertension, 13.6% had T2DM, and 23% were classified with MetS. More than 90% used aspirin and statins, and 49% used n-3 PUFA supplementation. Levels of the measured circulating gut leakage markers in the total cohort are included in Table 1.

Correlations Between Circulating Levels and SAT Gene Expression of Gut-Related Inflammatory Markers

RNA was not satisfactorily isolated from some SAT samples, mainly due to very high CT-values for the β2-microglobulin gene. After careful consideration of the amplification curves, samples with CT-values over a threshold of 30 were excluded. Gene expression was thus available for CD14 in 377 samples, for LBP in 329 samples, for TLR4 in 376 samples, and for TLR2 in 375 samples (Table 2). Weak correlations between levels of sCD14 and LBP and the genetic expression of CD14 in

Table 1 Baseline Characteristics, Cardiometabolic Risk Factors and Gut-Related Inflammatory Measures

	All (n=382)
Age, years[a]	74 (70, 82)
Sex; female, n (%)	97 (25.4)
Smoker, n (%)	44 (11.5)
Previous CVD, n (%)	157 (41.1)
BMI, kg/m^2	25.9 (23.7, 28.0)
Overweight or obese (BMI ≥25.0 kg/m^2), n (%)	231 (60.5)
T2DM, n (%)	52 (13.6)
Hypertension, n (%)	204 (53.4)
Metabolic syndrome[b]**, n (%)**	89 (23.3)
Total cholesterol, mmol/L	3.8 (3.2, 4.3)
LDL cholesterol, mmol/L	2.0 (1.7, 2.5)
HDL cholesterol, mmol/L	1.3 (1.1, 1.6)
TAG, mmol/L	1.1 (0.8, 1.5)
HbA1c, mmol/L	38.8 (35.5, 44.3)
hsCRP, mg/L	2.1 (1.1, 4.0)
Troponin T_{max}, ng/L	911 (169, 3053)
Circulating gut leakage markers	
LBP, ng/mL	16,570 (14,219, 19,500)
sCD14, ng/mL	1441 (1276, 1653)
LPS, pg/mL	46.1 (40.2, 53.5)

(*Continued*)

Table 1 (Continued).

	All (n=382)
Medication, n (%)	
Aspirin	362 (94.8)
Dual antiplatelet therapy	342 (89.5)
Statin	374 (97.9)
Beta blocker	321 (84.0)
ACEI	126 (33.1)
ARB	101 (26.4)
Prednisolone	23 (6)
NSAIDs	6 (1.6)
n-3 PUFA supplement	185 (48.8)

Notes: Continuous variables are presented as median (25^{th}, 75^{th} percentiles) unless stated otherwise. Categorical data are given as number, n (percent). [a]Min, max. [b]Metabolic syndrome, by modified NCEP ATP III criteria.

Abbreviations: CVD, cardiovascular disease; BMI, body mass index; T2DM, type 2 diabetes mellitus; LDL, low-density lipoprotein; HDL, high-density lipoprotein; TAG, triacylglycerol; hsCRP, high-sensitivity C-reactive protein; LBP, LPS-binding protein; sCD14, soluble cluster of differentiation 14; LPS, lipopolysaccharide; ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin-II receptor blocker; NSAIDs, non-steroidal anti-inflammatory drugs; PUFA, polyunsaturated fatty acid.

Table 2 Gut-Related Inflammation as Related to Cardiometabolic Risk Factors

	sCD14 (ng/mL)	LBP (ng/mL)	LPS (pg/mL)	CD14 RQ (n=377)	LBP RQ (n=332)	TLR4 RQ (n=379)	TLR2 RQ (n=379)
T2DM							
+	1464 (1297, 1691)	18,410 (14,966, 21,465)	49 (41, 58)	1.0 (0.8, 1.4)	0.7 (0.4, 1.3)	0.7 (0.6, 0.9)	0.8 (0.4, 1.8)
–	1434 (1268, 1645)	16,517 (14,174, 19,288)	46 (40, 53)	0.9 (0.7, 1.1)	0.7 (0.3, 1.1)	0.7 (0.5, 0.9)	1.2 (0.6, 2.3)
p-value	0.30	0.03	0.15	0.02	0.28	0.15	0.04
HT							
+	1460 (1311, 1668)	17,044 (14,392, 20,439)	45 (39, 53)	0.9 (0.7, 1.3)	0.7 (0.3, 1.2)	0.7 (0.5, 0.9)	0.8 (0.4, 1.9)
-	1411 (1252, 1629)	16,369 (13,882, 18,908)	47 (41, 53)	0.8 (0.6, 1.1)	0.6 (0.3, 0.9)	0.7 (0.6, 0.9)	0.8 (0.4, 1.8)
p-value	0.06	0.02	0.13	0.005	0.24	0.66	0.35
MetS							
+	1485 (1295, 1680)	17,943 (15,234, 20,118)	44 (41, 53)	1.0 (0.8, 1.5)	0.8 (0.3, 1.4)	0.7 (0.6, 0.9)	1.2 (0.6, 2.3)
-	1432 (1265, 1649)	16,319 (13,952, 19,212)	47 (40, 53)		0.6 (0.3, 1.0)	0.7 (0.5, 0.9)	0.8 (0.4, 1.8)
p-value	0.33	0.01	0.40	<0.001	0.19	0.5	0.001

Notes: Variables are presented as median (interquartile range). "+" and "-" indicates having or not having the specific cariometabolic risk factor.

Abbreviations: T2DM, type 2 diabetes mellitus; HT, hypertension; sCD14, soluble cluster of differentiation 14; LBP, LPS-binding protein; LPS, lipopolysaccharide; TLR4, toll-like receptor 4; TLR2, toll-like receptor 2; MetS, metabolic syndrome; RQ, relative quantification.

SAT were observed (rho=0.13 and 0.12, p=0.01 and 0.02, respectively); however, this was not significant when correcting for multiple testing (12 comparisons). The corresponding scatter plots demonstrated a cluster of observations with some outliers and no apparent correlation (Supplementary Figure 1a and 1b). Circulating levels of LPS were negatively correlated to gene expression of CD14 and LBP (rho= −0.14 and −0.13, p=0.007 and 0.02, respectively); this was not significant after correction (12 comparisons). As the scatter plots illustrate, two outliers with extreme values of LPS (>200 pg/mL) seem to drive the negative correlation (Supplementary Figure 1c and 1d). Neither of the circulating gut leakage markers correlated significantly to gene expression of TLR2 or 4 in SAT (p>0.05, all).

Gut-Related Inflammation as Related to Anthropometry and Cardiometabolic Risk Factors

Circulating levels of LBP correlated positively to BMI (rho=0.12, p=0.02), and were significantly higher in patients considered overweight or obese (BMI≥25 kg/m^2) compared to those considered normal- or underweight (p=0.04) (Figure 2a). When dividing patients into *high* or *low* LBP (by median), we found that patients with LBP above median had significantly higher BMI (p=0.015). Gene expression of both LBP and CD14 in SAT correlated positively to BMI (rho=0.20 and 0.15, p<0.001 and p=0.005, respectively). The expressions of both were also significantly higher in SAT of obese and overweight patients compared to those considered normal-weight (Figure 3a and b). When classifying patients as normal-weight (BMI<25 kg/m^2, n=151), overweight (BMI=25–30 kg/m^2, n=178), or obese (BMI≥30 kg/m^2, n=53), we found significantly higher expression of LBP with increasing BMI (Figure 4); however, this was not significantly different between overweight and obese patients (Figure 4).

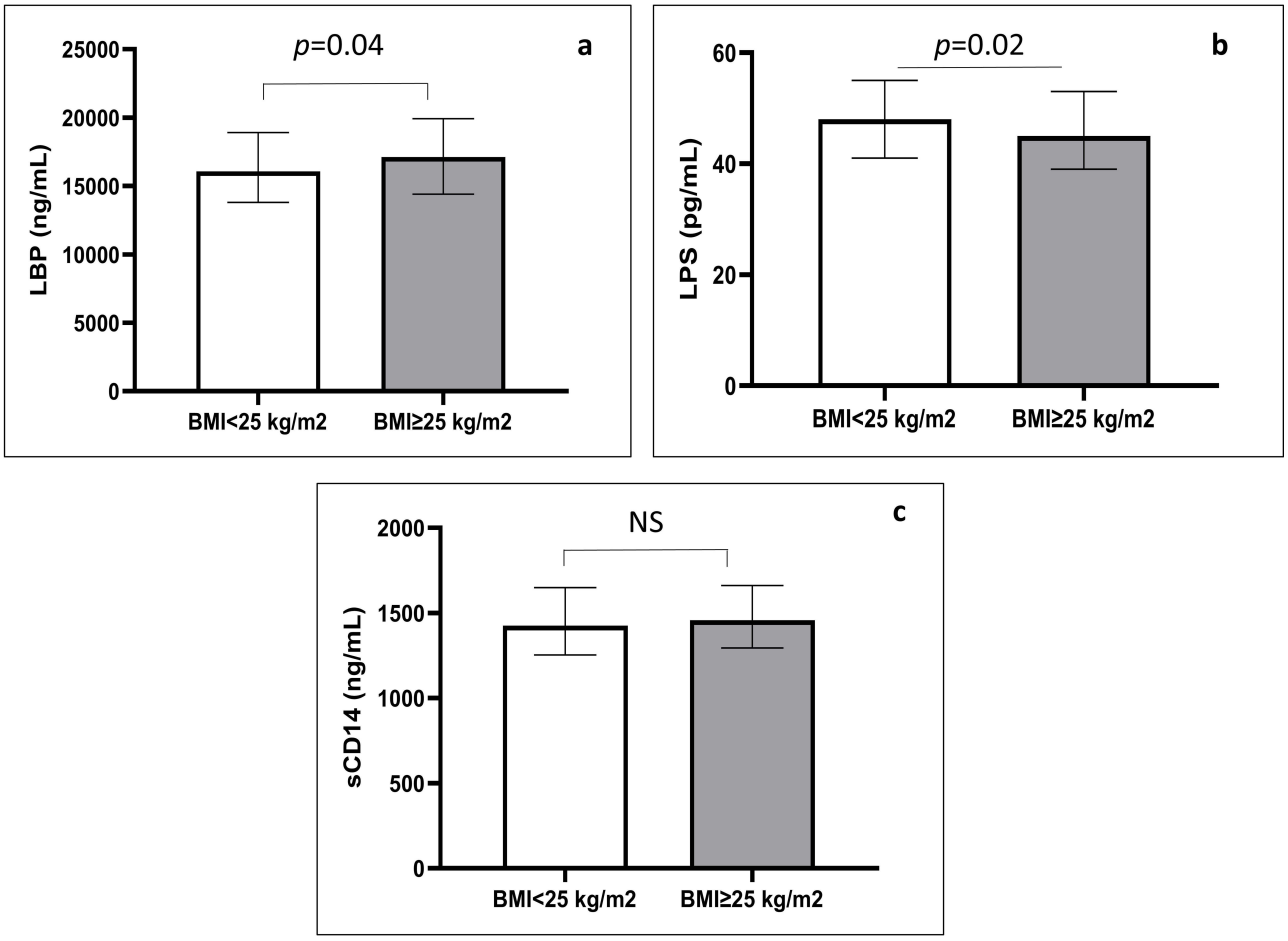

Figure 2 (**a**–**c**) Circulating gut leakage markers as related to BMI.
Abbreviations: BMI, body mass index; sCD14, soluble cluster of differentiation 14; LBP, LPS-binding protein; LPS, lipopolysaccharide; NS, not significant.

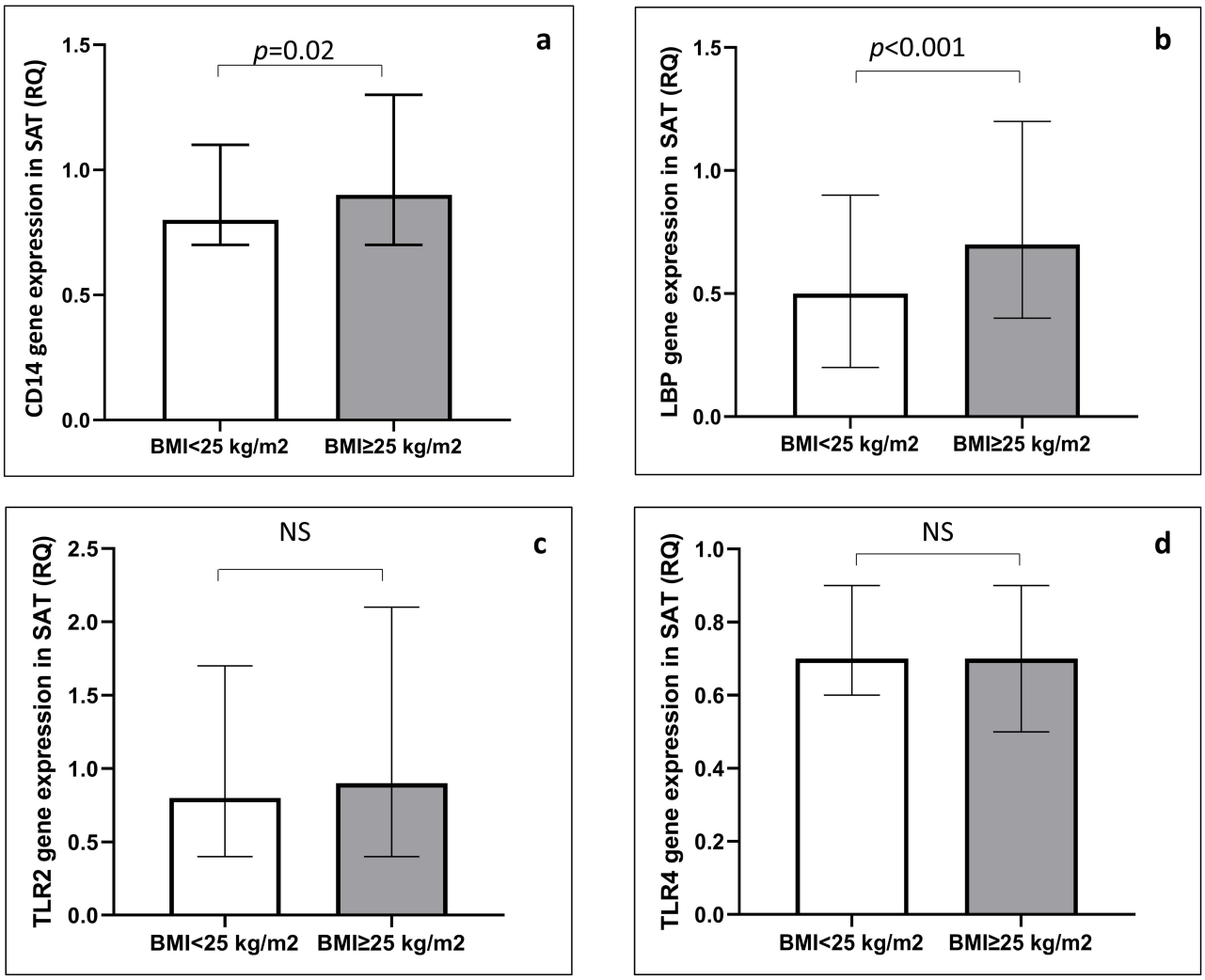

Figure 3 (a–d) Gene expression of gut leakage markers in SAT as related to BMI.
Abbreviations: BMI, body mass index; CD14, cluster of differentiation 14; LBP, LPS-binding protein; TLR2/4, toll-like receptor 2/4; RQ, relative quantification; SAT, subcutaneous adipose tissue; NS, not significant.

Circulating levels of sCD14 did not correlate significantly to BMI (rho=0.07, p=0.16), and was not different between normal-weight, obese, and overweight patients (Figure 2c), whereas circulating LPS levels correlated negatively to BMI (rho=−0.12, p=0.02), and was significantly lower in patients with obesity or overweight compared to those considered normal-weight (p=0.02) (Figure 2b).

The expressions of TLR2 and TLR4 did not associate significantly with BMI, nor were they differently expressed in groups of BMI (Figure 3c and d).

Circulating LBP was significantly higher in patients with T2DM, in patients with HT, and in patients with MetS compared to patients without (p=0.03, p=0.02, p=0.01, respectively) (Table 2); however, this was not differently expressed in SAT in patients with these cardiometabolic comorbidities, as compared to patients without (Table 2). Circulating sCD14 and LPS did not differ between any group of cardiometabolic risk factors (Table 2). In SAT, however, CD14 expression was significantly higher in patients with T2DM (p=0.02), HT (p=0.005), and MetS (p<0.001) compared to patients without (Table 2). Also, TLR2 expression was higher in patients with T2DM (p=0.04) and MetS (p<0.001) compared to those without (Table 2).

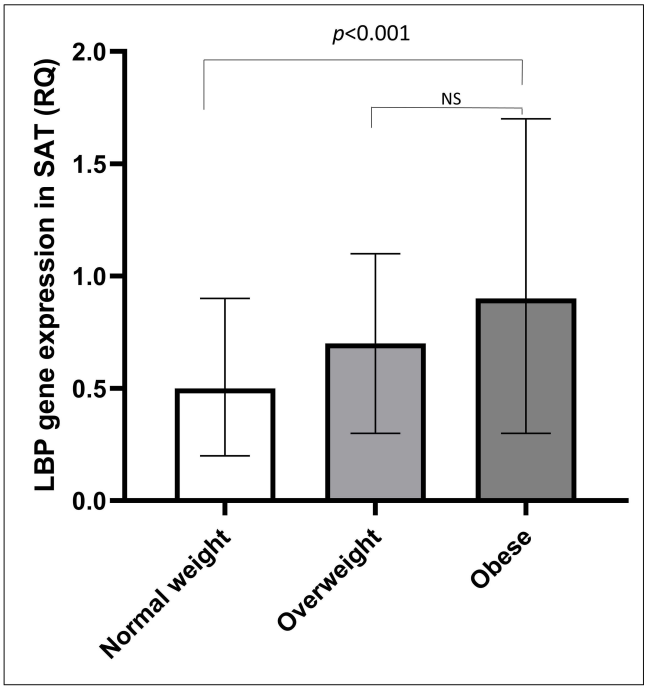

Figure 4 Gene expression of LBP in SAT of normal-weight, overweight, and obese patients.
Abbreviations: LBP, LPS-binding protein; RQ, relative quantification; SAT, subcutaneous adipose tissue; NS, not significant.

Gut-Related Inflammation as Related to Dietary Habits

The dietary questionnaire was filled out by 306 patients. Of these, 27% had a diet that was defined as "poor", 65% had a diet defined as "intermediate", and only 8% of the patients adhered to a diet that was defined as "healthy". We observed no significant correlations between the diet score and any of the circulating gut leakage markers, nor were there any correlations between diet score and SAT gene expression of the markers (data not shown). There were also no significant differences in the circulating gut leakage markers or SAT gene expression when categorizing diet into "poor", "intermediate", or "healthy" (Supplementary Figure 2a-g).

Patients using n-3 PUFA supplement, compared to those who did not, had significantly higher circulating levels of LPS (p=0.04) (Figure 5a), lower circulating levels of sCD14 (Figure 5b), and lower expression levels of CD14 (p=0.003) and TLR2 (p<0.001) in SAT (Figure 6a and c). No significant differences in circulating levels of LBP (Figure 5c) or gene expression in SAT of LBP or TLR4 were observed (Figure 6b and d).

Discussion

In this paper we investigated the gut-related LPS inflammatory pathway in the circulation and in adipose tissue in a cohort of elderly patients with established CAD. We found that circulating levels of LBP increased with increasing BMI, and that gene expression of both CD14 and LBP was increased in patients considered overweight or obese. Circulating LBP was also significantly elevated in patients with concomitant cardiometabolic disease such as T2DM and MetS. Additionally, gene expression of CD14 and TLR2 was increased in SAT of patients with T2DM and MetS, indicating a role for gut-related inflammation in cardiometabolic disease. There were, however, no convincing correlations between circulating gut leakage markers and corresponding genetic expression in SAT. Lastly, diet quality did not seem to affect levels of gut-related inflammatory markers in our population, neither circulating nor as expressed in SAT, whereas use of n-3 supplements associated to some degree.

The positive correlation observed between LBP in the circulation and BMI, and the higher levels in overweight and obese patients than in those considered normal-weight, is in line with what we previously have shown in patients with symptoms of coronary artery disease.[31] We have, however, reported LBP to be even more strongly correlated to increased

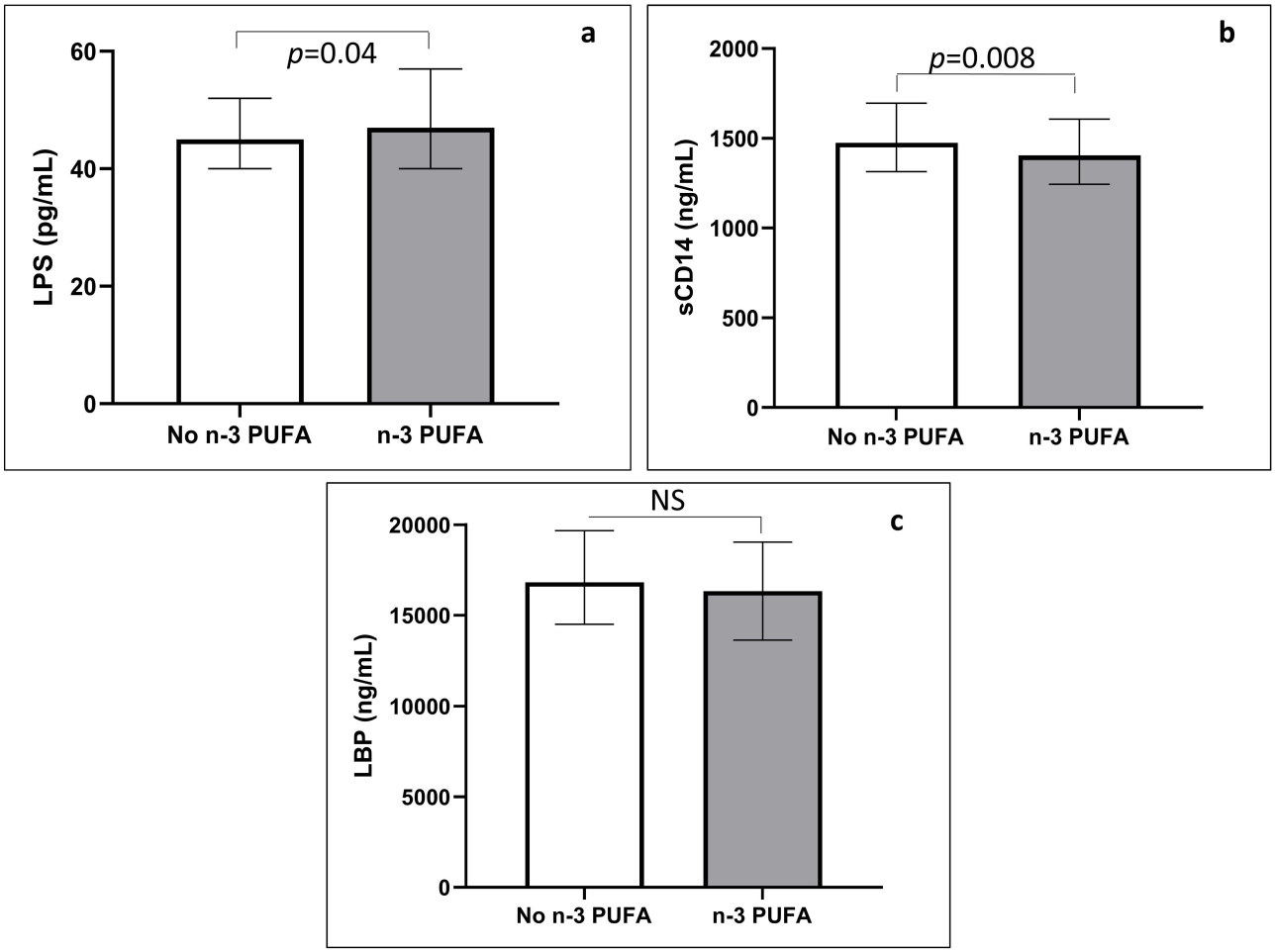

Figure 5 (**a–c**) Circulating gut leakage markers as related to intake of n-3 PUFA.
Abbreviations: sCD14, soluble cluster of differentiation 14; LBP, LPS-binding protein; LPS, lipopolysaccharide; PUFA, polyunsaturated fatty acid; NS, not significant.

waist circumference as a measure of central obesity in another study, which is a feature of obesity more closely associated to inflammation and a dysregulated metabolism.[32] Also, we found the expression of LBP in SAT to be higher with higher BMI. The genetic expression is assessed by equal amount of tissue in the samples, and is thus independent of the amount of adipose tissue in each individual. This finding is in accordance with a previous study showing LBP in SAT to be higher in obese vs non-obese individuals,[19] reflecting that obese and overweight individuals have both higher circulating levels and increased expression of LBP in AT. Although circulating CD14 did not correlate to BMI, the elevated SAT expression in overweight and obese patients supports the role of LBP–CD14-mediated inflammatory activation in obese SAT. As blood samples were collected 2–8 weeks after the MI in this cohort, the inflammatory burst in the acute setting is avoided. Whether this reflects gut-leakage-mediated inflammation cannot be extrapolated from these results. It seems, however, that circulating LBP is a possible marker for identifying patients at risk of chronic inflammation linked to metabolic disorders.[33]

We found no significant associations between circulating LPS and SAT expression of the gut-related inflammatory markers. This may be explained by LPS being highly volatile in the circulation and subject to large circadian variation, and a single sample does not reflect the total burden of endotoxemia.[34] However, significant correlations were not seen between circulating levels of LBP or sCD14, which are more stable, and their corresponding genes in SAT. It may be argued that gut leakage does not contribute to inflammation in AT as the same pathway can be activated by an array of other agents. More probably, however, the finding reflects that SAT is not the primary source of circulating LBP and sCD14, as they are synthesized and released mainly by the liver.[35] It seems, nevertheless, that they play a role in obesity-related inflammation,

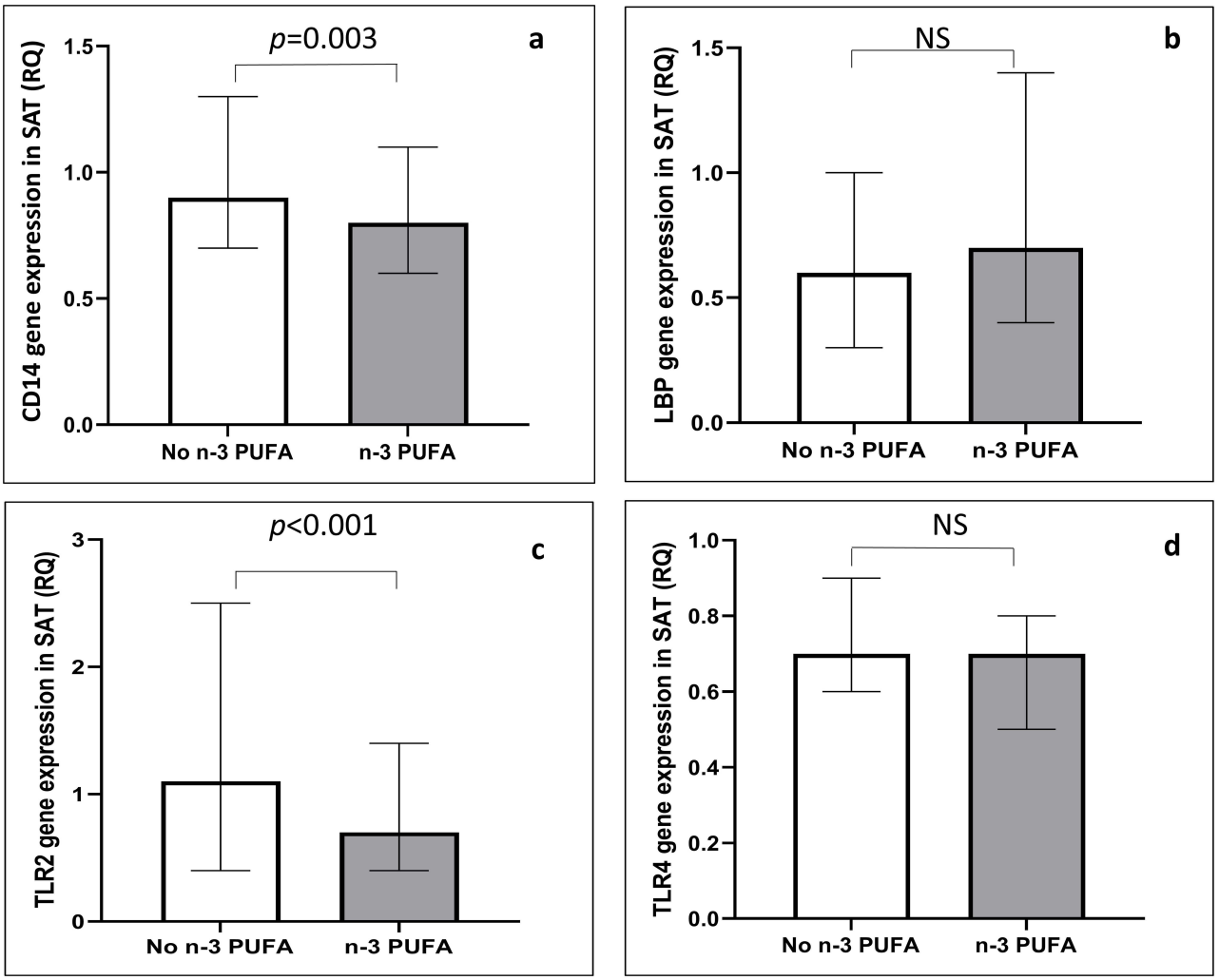

Figure 6 (**a**–**d**) Gene expression of gut leakage markers in SAT according to intake of n-3 PUFAs.
Abbreviations: CD14, cluster of differentiation 14; LBP, LPS-binding protein; PUFA, polyunsaturated fatty acid; TLR2/4, toll-like receptor 2/4; RQ, relative quantification; SAT, subcutaneous adipose tissue; NS, not significant.

both systemically and locally, and contributes to adipose tissue dysfunction.[19] In contrast to the study by Ahmad et al, showing both TRL2 and TLR4 to be increased in SAT of overweight and obese individuals,[21] neither of these receptors was differently expressed in groups of BMI in our population. Their study performed immunohistochemical analyses of the SAT, highlighting that the staining intensity of TLR2 and TLR4 was increased in areas containing inflammatory cells, thus suggesting that infiltrating immune cells may be the source of TLR2 and TLR4. It has, however, been shown that both TLR2 and TLR4 are present on human adipocytes,[36] and as both adipocytes and immune cells would be present in our SAT samples, this should not affect the result.

We further studied the relationship to other cardiometabolic risk factors. Circulating LBP was higher in patients with T2DM, HT, and MetS, confirming what has previously been shown.[37] SAT expression of both CD14 and TLR2 was also elevated in T2DM and MetS, while CD14 was also significantly higher in HT. This supports a possible LPS-mediated inflammatory pathway in SAT of patients with CAD and coexisting metabolic dysfunction. Somewhat surprisingly, TLR4, which is the main LPS receptor,[38] did not have elevated expression in SAT with cardiometabolic risk factors. However, it has been seen that TLR2 expression is tenfold higher compared to TLR4 on adipocytes from SAT.[36] TLR2 may be upregulated in SAT as it is able to bind LPS in the presence of helper molecules.[18] Additionally, it may reflect exposure to other gut-related insults, such as Gram-positive bacterial wall compounds, which we have not measured. LBP gene expression was also not elevated, contrary to what we hypothesized. A study found LBP in supernatant from SAT to be elevated in patients with MetS compared to healthy

controls.[39] However, it may be argued that as central obesity is a dominant feature of MetS and related metabolic abnormalities,[11] visceral adipose tissue samples may have shown different results. Visceral adipose tissue seems to exhibit a more pro-inflammatory profile than that of SAT, although, in both, some of the same traits are evident and similar in patients with obesity.[40]

We found LPS to inversely correlate to BMI, contrary to previous findings of fasting LPS levels to be higher in obese individuals vs healthy controls.[41] An explanation may be the higher LBP levels in obese and overweight individuals, contributing to higher LPS-binding capacity. In addition to the large circadian variation previously discussed, also, the method for LPS detection has limitations, as it is not able to detect sequestered LPS.[42]

Lastly, we investigated gut leakage markers and the corresponding genes in SAT as related to diet. We found no relationship between dietary habits and any markers of gut leakage, neither in the circulation nor as expressed in SAT. This was surprising, as especially a high-fat diet has been linked to both gut dysbiosis and to increased endotoxemia.[23] This could be due to the recall-based dietary registration, which is vulnerable as patients have to remember exactly what they have been eating. Thus, such a registration form may be subject to both under- and overestimation bias. However, patients who used n-3 PUFA supplements, which is common in the Norwegian population, had lower sCD14 levels and lower SAT expression of CD14 and TLR2, which may reflect the proposed anti-inflammatory effect of n-3 PUFAs.[43] As n-3 PUFAs are thought to improve the gut microbiota and the intestinal barrier,[44] we also expected lower LPS levels in those using supplements. The higher LPS levels surprisingly observed may be explained by the property of fatty acids to promote the translocation of LPS.[45]

Limitations

As this is a subset of patients included in a larger trial,[25] any power calculation was not performed for the current investigation. It has a cross-sectional design, thus the data are solely descriptive and explorative, and do not allow for any causative extrapolation. Any down-stream inflammatory signalling induced by LPS and the TLR4 receptor has not been studied. The patients had previously had a MI, and although stable they were heavily medicated. Almost all were on statins and aspirin, both having anti-inflammatory properties, which may have masked the results. NSAIDs and prednisolone are also suggested to associate to gut leakage; however, there were no significant differences between patients on such medication and patients without (data not shown). The patients were elderly, and there is limited knowledge on the gene regulatory capacity in ageing subjects. The adipose tissue samples were subcutaneous, which is the only mehod possible in this clinical setting. However, a strength of the study is the rather large number of adipose tissue samples, allowing realistic associations.

Conclusion

In this study, we show that, in elderly patients with established CAD, circulating levels of LBP and gene expression in SAT correlate significantly to anthropometric measures. Circulating levels of LBP furthermore associated significantly with MetS, T2DM, and HT. Additionally, CD14 and TLR2 expression in SAT was increased in patients with MetS and T2DM. Together, these findings suggest that gut-related inflammation assessed by the LPS–LBP–CD14 inflammatory pathway is activated systemically and in adipose tissue in the chronic low-grade inflammatory state associated with obesity and cardiometabolic diseases. The higher LPS levels in patients taking n-3 PUFA supplement seem to be counteracted by lower levels of sCD14 as well as lower CD14 gene expression, suggesting a net anti-inflammatory response. Diet composition in general did not significantly associate with the gut-related markers in this population.

Abbreviations

AT, adipose tissue; B2M, β2-microglobulin; CAD, coronary artery disease; CVD, cardiovascular disease; HDL, high-density lipoprotein; HT, hypertension; I-FABP, intestinal fatty-acid binding protein; LDL, low-density lipoprotein; LPS, lipopolysaccharide; LBP, LPS-binding protein; MetS, metabolic syndrome; MI, myocardial infarction; n-3 PUFA, omega-3 polyunsaturated fatty acids; RNA, ribonucleic acid; SAT, subcutaneous adipose tissue; sCD14, soluble cluster of differentiation 14; TAG, triacylglycerol; TLR4, toll-like receptor 4; TLR2, toll-like receptor 2; T2DM, type 2 diabetes mellitus.

Data Sharing Statement

The datasets used during the current study are available from the corresponding author on reasonable request.

Consent to Participate and Ethics Approval

The study was conducted in accordance with the Declaration of Helsinki. It was approved by the Regional Committee of Medical Research Ethics in South-Eastern Norway. All patients gave their written informed consent to participate.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work. SKA was involved in planning of the study, statistical and laboratory analyses, interpretation of results, and prepared the initial manuscript drafts. AAK and KL contributed majorly in the conduction of the OMEMI-trial, and in manuscript revision and intellectual content. SÅ was a major contributor in planning and conduction of the laboratory analyses, interpretation of laboratory results, and in the intellectual content and revision of the manuscript. HA and SS contributed in the planning of the study and to the intellectual content and discussion of the manuscript. IS and RH were both major contributors in planning and conduction of the study, involved in the statistical analyses and interpretation of the results, and major contributors to the intellectual content and manuscript revision. The manuscript have been read and approved by all authors.

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Disclosure

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