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Effects of dietary intervention and n-3 PUFA supplementation on markers of gut-related inflammation and their association with cardiovascular events in a high-risk population

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HIGHLIGHTS

- Markers of gut-related inflammation are not affected by diet intervention.
- LBP and sCD14 are associated with increased risk of cardiovascular disease.
- LBP predicts increased risk of cardiovascular disease independent of CRP.

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ABSTRACT

Background & aims: Dysbiosis of the gut microbiota is associated with increased levels of circulating lipopolysaccharide (LPS) and subsequent activation of systemic inflammation. Diet is an important modulator of the gut microbiome.

We aimed to investigate whether circulating markers of gut-related inflammation, LPS binding protein (LBP) and soluble CD14 (sCD14) can be modulated by n-3 PUFA supplementation and/or diet counselling, and whether these markers are related to cardiovascular (CV) outcome.

Methods: 484 men aged 65–75 years, at high CV-risk, were included and randomized in a 2 × 2 factorial design to 36-month intervention with dietary counselling, n-3 PUFA supplementation, or both. N-3 PUFA supplementation was placebo-controlled. ELISAs were used for determination of the biomarkers measured at baseline and study-end. A composite endpoint was defined as new CV-events and CV-mortality after 36 months.

Results: There were no significant differences in changes of either LBP or sCD14 in the intervention groups compared to their respective controls (n-3 PUFA *vs.* placebo: $p = 0.58$, $p = 0.15$, diet *vs.* no-diet: $p = 0.53$, $p = 0.59$, respectively). The group with LBP levels above median had about 2-fold unadjusted risk of suffering an endpoint compared to the group below (HR 2.22, 95% CI 1.25–3.96; $p = 0.01$). A similar tendency was seen for sCD14 (HR 1.72, 95% CI 0.97–3.03; $p = 0.06$). After adjusting for covariates, LBP remained significantly associated with a two-fold CV-risk, whereas sCD14 gained statistical significance, however, lost when hsCRP was added to the model.

Conclusions: In our population, markers of gut-related inflammation associated with 36-month CV outcome. However, neither n-3 PUFA nor diet intervention had an effect on these markers.

1. Introduction

Inflammation is believed to be a major contributor to the development of cardiovascular disease (CVD). It is implicated in all phases of atherosclerotic plaque development from initiation to the potentially devastating event of atherothrombosis [1–3]. Targeting

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inflammation is now looked upon as a feasible method for addressing the substantial residual risk observed after managing conventional risk factors. Studies like IMPROVE-IT have shown a convincing effect on secondary CV events when both LDL-cholesterol and high sensitive C-reactive protein (hsCRP) were lowered by statins and Ezetimibe [4]. The recent hallmark study CANTOS, where hsCRP was lowered independently of LDL-cholesterol, further provides evidence of the causal role of inflammation in CVD [5].

The innate immune system stands at the front line of vascular inflammation. Monocyte-derived macrophages present in the vascular wall initiates inflammation through identification of danger signals [6]. These danger signals may serve as ligands for Toll-like-receptors (TLRs) or primers/activators of the inflammasome. Downstream, they induce transcription of pro-inflammatory cytokines such as interleukin 6 (IL-6) and interleukin 1β (IL-1β), which are highly associated with cardiovascular disease [7,8]. Pathogens associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) are also known potent activators of innate immunity through essentially the same signaling receptor family [9].

Antibodies against LPS have been found to co-localize with antibodies to TLR4 and CD68 in atherosclerotic plaques, suggesting an active role in the atherosclerotic process [10]. Furthermore, LPS and other gut derived PAMPs have been suggested to play a role in atherothrombosis [11,12].

The source of circulating LPS in healthy individuals is thought to be derived from the gut. Unfavorable changes in the bacterial composition of the gut microbiome, namely dysbiosis, is suggested to increase the amount of LPS in the gut lumen as well as in systemic blood through translocation of whole bacteria or bacterial components [13].

The effect of circulating LPS is mainly facilitated by LPS binding protein (LBP). LBP is essentially an acute phase protein, mainly produced by the liver and stimulated by LPS, but also IL-1, IL-6 and tumor necrosis factor α (TNFα) have been shown to increase expression of LBP [14,15]. In the circulation, LPS binds to LBP with high affinity. This step is suggested to be a rate-limiting step facilitating further binding to membrane bound cluster of differentiation 14 (CD14), which promotes atherogenic and proinflammatory properties through TLR4, which is a transmembrane receptor on a large variety of cells, including mononuclear immune cells and vascular cells [16].

Dysbiosis as well as several commensal gut bacteria has been associated with increased risk of CVD [17–19]. Diet is the foremost long-term modulator of the gut microbiota. High fat and high sugar diets have been associated with driving the microbiome into a pro-inflammatory phenotype associated with increased circulating levels of LPS [20,21]. Conversely, interaction between the gut microbiota and a low fat, high fiber diet, has been suggested to have protective roles in the development of atherosclerosis, when compared to western diet [22].

We therefore aimed to explore whether diet counselling and n-3 PUFA intervention would reduce markers of gut leakage, namely LBP and soluble CD14 (sCD14), thus reducing the activation of the innate immune system.

Furthermore, we investigated whether baseline levels of these markers were predictive of cardiovascular events in a high CVD risk population.

2. Materials and methods

2.1. Study population

In total 563 males, all aged between 65 and 75 years, deemed at high cardiovascular risk, were enrolled in the Diet and Omega-3 Intervention Trial on Atherosclerosis (DOIT), a prospective randomized trial initiated in 1997. The participants were randomized in a 2 × 2 factorial design with 36-month intervention with either dietary counselling, n-3 PUFA supplementation, or both. N-3 PUFA supplementation was placebo controlled with encapsulated corn oil [23].

Blood samples from 484 subjects were available for the present investigation. Of these, 241 subjects received 2.4 g n-3 PUFA daily, whereas 243 subjects received placebo. The n-3 PUFA capsules contained 35% eicosapentaenoic acid (EPA), 20% docosahexaenoic acid (DHA), and 3.5 mg tocopherols/g for prevention of fatty acid peroxidation. The n-3 PUFA capsules consisted of an ethyl esterified triglyceride formula.

According to randomization, 237 subjects underwent strict personal dietary counselling, guided by a clinical nutritionist. All of these subjects were advised to reduce intake of saturated fat and meat from animal sources and increase intake of vegetable oils, fruit, fish and vegetables. Calorie-restriction was advised for subjects deemed as overweight. At randomization, 30–45 min of counselling was given, and repeated after three months. They were followed either by telephone consultations or at the outpatient clinic every six months. Compliance of n-3 PUFA and diet intervention was obtained by measurement of serum fatty acids and comparison of food questionnaires obtained at baseline and at the end of the study [23]. Methods for randomization and intervention have previously been published [23].

All participants were followed for 36 months. Endpoints were registered as a composite of new CV events and CV mortality. New CV events were defined as acute myocardial infarction, need for revascularization, aortic aneurysm, peripheral arterial occlusive disease, cerebrovascular events and death due to cardiovascular causes.

The DOIT study was approved by the Regional Ethics Committee, and all subjects gave their informed written consent to participate.

The trial has been registered at clinicaltrials.gov (NCT00764010, www.clinicaltrials.gov).

2.2. Laboratory methods

Blood samples were obtained at inclusion in fasting condition (> 10 h) by standard venipuncture before daily intake of medication between 08:00 and 10:00 a.m. EDTA blood was separated by centrifugation at 4 °C within 1 h at 2500 × g for 10 min and plasma was kept stored at −80 °C until analyses. Serum lipids were determined by conventional enzymatic methods. LPB and sCD14 were analyzed by commercial ELISAs (Hycult Biotech, Uden, the Netherlands and R & D Systems Europe, Abingdon, Oxon, UK, respectively). The inter-assay coefficients of variation were for LBP 8.2% and for sCD14 8.9%. Method for high sensitivity (hs)CRP analysis has previously been described [24].

2.3. Statistics

As most data were not normally distributed, non-parametric statistics were used. Demographic data are provided as numbers with proportions or medians with 25, 75 percentiles. Pearson Chi-squared test was used to explore baseline differences in categorical variables, whereas Mann-Whitney u-test was used to explore differences between groups in continuous variables. Mantel-Haenszel chi-square statistics was used for trends across quartiles.

Within the treatment groups, Wilcoxon signed-rank test was used to compare baseline levels with levels after the 36-month intervention. Interventional effect of n-3 PUFA or dietary counselling between the groups was explored according to the factorial design with one-way ANCOVA adjusting for baseline levels.

A Cox regression model was used to analyze the risk of having an endpoint during the 3-year follow-up period when dichotomizing LBP and sCD14 levels at median. Levels below and above median was chosen as cut-off values after first dividing both markers into quartiles as related to the incidence of an endpoint. We then visually found a natural cut-off at median level (Supplementary Fig. 2). Both markers are presented with three proposed models. In the first model, covariates which are associated with both CV outcome and the marker in question with a p-value less than 0.2, were included. In the second model, conventional risk factors were included. In the third model, hsCRP was

Table 1
Baseline characteristics of the total study population (n = 484) and according to having and endpoint or not. Median values (25, 75 percentiles) or numbers (proportions) are given.

	Total	Endpoint (−)	Endpoint (+)	*p*
Age (years)	70.1 (67.5–72.7)	69.88 (67.4–72.7)	71.22 (67.9–72.7)	0.32
BMI (kg/m^2)	26.5 (24.2–28.6)	26.4 (24.1–28.4)	27.4 (24.8–29.4)	0.14
Triglycerides (mmol/L)	1.50 (1.12–1.98)	1.50 (1.12–1.97)	1.63 (1.12–2.13)	0.62
LDL (mmol/L)	4.02 (3.46–4.60)	3.99 (3.38–4.59)	4.05 (3.66–4.60)	0.24
HDL (mmol/L)	1.37 (1.16–1.62)	1.38 (1.17–1.63)	1.34 (1.14–1.55)	0.66
Fasting glucose (mmol/L)	5.6 (5.3–6.2)	5.6 (5.3–6.2)	5.8 (5.3–7.5)	0.07
HbA1C (%)	5.6 (5.3–5.9)	5.6 (5.3–5.9)	5.6 (5.3–6.3)	0.20
Systolic BP (mmHg)	147 (136–160)	147 (135–160)	153 (143–168)	**0.02**
Current smokers	160 (33)	135 (32)	23 (43)	0.09
History of diabetes mellitus	79 (16)	64 (15)	15 (28)	**0.01**
History of MetS	182 (38)	159 (37)	23 (43)	0.37
History of hypertension	150 (31)	133 (31)	17 (32)	0.87
Previous CVD	139 (29)	118 (28)	21 (40)	0.07
hsCRP (mg/L)	3.28 (1.78–5.89)	3.08 (1.67–5.80)	4.88 (3.10–6.69)	< **0.001**
Statin use	134 (28)	122 (29)	13 (25)	0.54
Antidiabetic use	21 (4)	16 (4)	5 (9)	0.06
Diuretic use	23 (5)	20 (5)	3 (6)	0.75
ACE-inhibitor use	78 (16)	66 (16)	13 (25)	0.09
Aspirin use	131 (27)	107 (25)	24 (45)	**0.002**
Betablocker use	79 (17)	64 (16)	15 (21)	0.22
Calcium blocker use	82 (17)	74 (17)	8 (15)	0.68
Nitrate use	43 (9)	32 (8)	11 (21)	**0.001**

Statistical significant differences ($p < 0.05$) are outlined in bold.

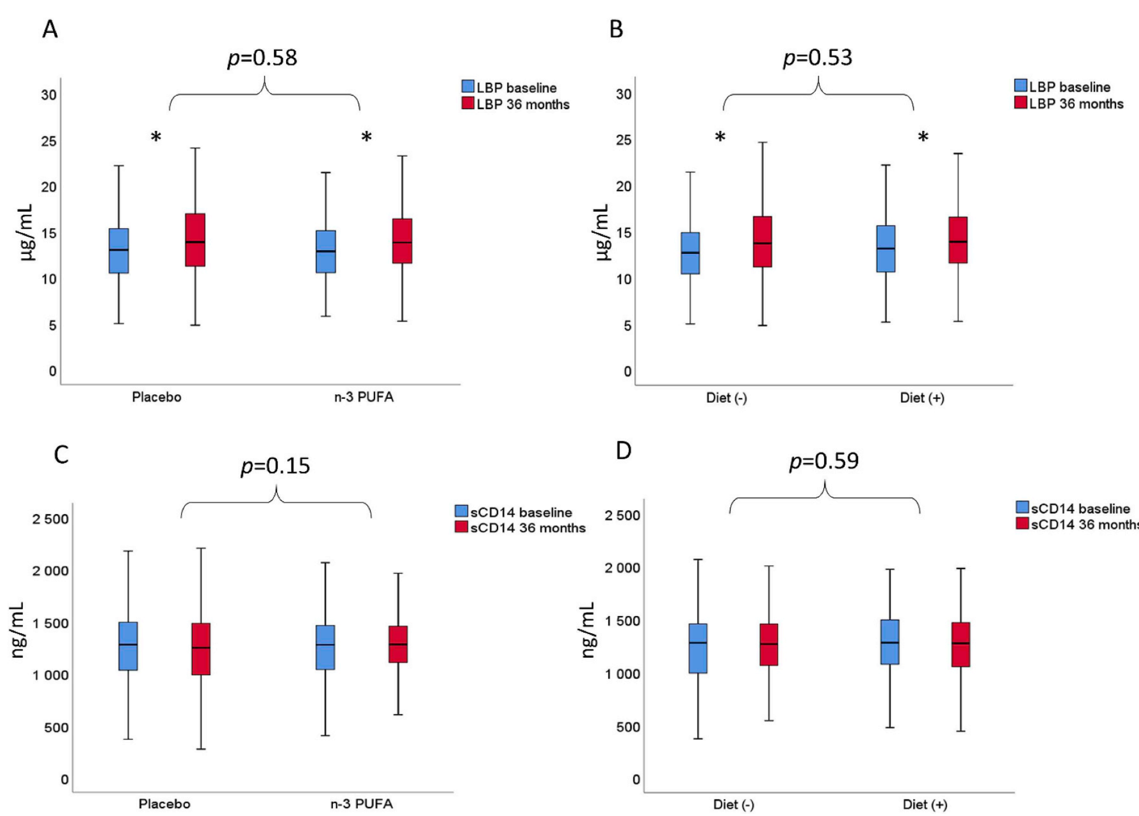

Fig. 1. Plasma levels of both markers at baseline and after 36-month intervention according to the factorial design.
(A and B) LBP and (C and D) sCD14. * Indicates statistically significant changes within groups with *p*-values of < 0.001. The *p*-values given refer to difference in changes between n-3 PUFA and placebo (A and C) and diet intervention vs no diet intervention (B and D) (One-way Ancova).

added. Risk is given as Hazard ratio (HR). Kaplan-Meier method was used for time-to-event analysis.

To analyze the discriminative ability of our markers, receiver operating characteristics (ROC) curves were used. We also compared incremental contributions of each marker and the combination of both markers when added to hsCRP. Area under the curves were calculated.

IBM SPSS statistics version 25.0 was used for all statistical analyses.

3. Results

3.1. Baseline characteristics

Of the 484 subjects, a total of 53 patients suffered the composite endpoint of a new CV event and CV mortality.

Baseline characteristics of the patients that experienced an endpoint compared to those who did not are given in Table 1. Levels of hsCRP and systolic blood pressure were higher in the endpoint group and proportionallythere were more diabetics in the endpoint group, as was also the use of aspirin and nitrates (Table 1).

Baseline characteristics according to the randomized groups were well matched (Supplementary Table 1). The diet intervention group was slightly older than controls (median 70.8 *vs*. 69.7 years, $p = 0.05$). There were borderline significantly more diuretic users in the n-3 PUFA intervention group (7% *vs*. 3%, $p = 0.05$). Antidiabetics and nitrates were more frequent in the diet control group compared to the diet counselling group ((7% *vs*. 2%), $p = 0.02$ and (12% *vs*. 6%), $p = 0.02$, respectively).

3.2. Interventional effects

The effects of 36 months with diet counselling or n-3 PUFA supplementation with regard to serum lipids and lipoproteins have previously been described in detail. In short, both diet and n-3 PUFA intervention significantly decreased serum triglyceride levels [23].

We observed a significant increase of LBP levels in all groups regardless of the intervention modality ($p < 0.001$). There was, however, no significant difference in changes in levels of LBP between groups receiving n-3 PUFA or diet counselling *vs*. their respective controls ($p = 0.58$ and $p = 0.53$, respectively), (Fig. 1, upper panel).

For the levels of sCD14, the group receiving n-3 PUFA had a non-significant increase over the 36-month intervention period ($p = 0.06$), while the group receiving placebo capsules had a non-significant reduction over the 36-month intervention period ($p = 0.34$). The difference in changes was not statistically significant ($p = 0.15$) (Fig. 1, lower panel). As for diet counselling, we observed no change in sCD14 within the intervention group ($p = 0.81$) and a non-significant increase in controls ($p = 0.28$). However, the difference in changes between the groups was not significant ($p = 0.59$) (Fig. 1, lower panel).

3.3. Clinical endpoint analyses

The concentration of LBP was found to be significantly higher in patients who suffered an endpoint compared to individuals that did not (median 14.09 *vs*. 12.68 µg/mL; $p = 0.01$). A trend towards increased concentration of sCD14 in the endpoint group was observed, however, not statistically significant (median 1354 *vs*. 1285 ng/mL, respectively; $p = 0.1$). Levels of CRP were significantly higher in subjects experiencing and endpoint, as previously reported [24].

When dividing LBP into quartiles, we observed a positive trend of increased prevalence of an endpoint across ascending quartiles ($p = 0.01$). This was not observed with sCD14 ($p = 0.10$).

When dichotomizing levels at median, there were 36 *vs*. 17 endpoints in the group above *vs*. below median LBP (12.88 µg/mL) and 32 *vs*. 19 for sCD14 (1294 ng/ml). In an univariate Cox regression model, the group above the median LBP had significantly higher risk of suffering an endpoint during the 36-month follow up (HR 2.22, 95% CI 1.25–3.96; $p = 0.01$), whereas we observed only a tendency towards higher risk with levels above median sCD14 (HR 1.72, 95% CI 0.97–3.03; $p = 0.06$) (Fig. 2). After adjusting for confounders in a multivariate model, LBP remained significantly associated with CV outcome (HR 2.00, 95% CI 1.11–3.58; $p = 0.02$) and sCD14 achieved statistical significance after adjustment (HR 1.82, 95% CI 1.02–3.23; $p = 0.04$). After adding traditional CV risk factors, including hsCRP in the model, sCD14 no longer remained significantly associated, whereas LBP prevailed (Tables 2 and 3).

We further investigated subjects with combined LBP and sCD14 above the median (n = 124). These subjects had no real added risk compared to LBP only when analyzed by univariate Cox regression (HR 2.25, 95% CI 1.29–3.91; $p = 0.004$).

ROC curve analyses revealed a similar AUC for hsCRP alone, LBP alone and LBP + CRP combined (0.63, 0.61 and 0.62). Thus, no incremental improvement of AUC was obtained when hsCRP was added (Supplementary Fig. 1).

4. Discussion

In our study of high CV risk individuals, neither n-3 PUFA nor diet counselling had any modifiable effects on levels of LBP or sCD14 after a 36-month intervention.

For all groups, levels of LBP increased from baseline during the study period, however, with no differences in changes between the groups. For sCD14, there was no significant change in the diet intervention group and an increase in the n-3 PUFA group, however, not significantly different from their respective controls.

The effect of dietary intervention on prevention of CVD is thought to be through modification of known CV risk factors, however, interactions with the gut microbiota have been suggested to play an important role [25–27].

Diet-induced dysbiosis is characterized by increased LPS-containing luminal bacteria and increased leakage of bacteria, bacterial components or products through a dysfunctional intestinal barrier [21,28,29]. Overall, these changes facilitate leakage of LPS from the gut lumen and subsequent production of both sCD14 and LBP [30]. Conversely, we hypothesized that dietary intervention could counteract these changes. However, no signs of such counteractions were shown in our study.

The effect of n-3 PUFA on the human microbiome is largely unknown. There are experimental and clinical studies showing favorable changes and decreased intestinal wall leakage by supplementation, thus indicating reduced gut-related inflammation [31,32]. Recently, the landmark study REDUCE-IT showed remarkable effects of icosapent ethyl (Vascepa) on incident cardiovascular events [33]. These finding are in contrast to the contemporary meta-analysis by Aung et al., showing no effect of n-3 PUFA on cardiovascular endpoints [34]. In REDUCE-IT, a daily dose of 4 g of icosapent ethyl was used, whereas in the meta-analysis, the dosing displayed great heterogeneity, and in our study, a dose of 840 mg EPA daily was used. Thus, the lack of effect might be a dosing issue.

In line with our results, an intervention study using the Mediterranean diet showed a non-significant increase in LBP over a given time period [35].

We found that levels of LBP were significantly higher in individuals that experienced a CV event. Subjects with levels above median had a close to 2-fold increased risk of experiencing an endpoint during the 36-month follow up, also after correcting for known confounders, traditional risk factors and hsCRP. For sCD14, there was a tendency towards higher levels in the endpoint group and the group above median had a non-significant increased risk of experiencing an endpoint; however, gained significance after correcting for confounders. Statistical significance was, nonetheless, lost after adding other risk factors, including CRP. When analyzing the Kaplan-Meier survival curves for both markers, it is quite evident that the two curves diverge early for LBP, indicating a consistent reduction of event-free survival for the group with high levels. For sCD14, the two curves co-localize approximately until day 650. At this point, the rate of decline in event-free survival for the group with the lowest sCD14 levels is somewhat reduced. Prior studies have shown that high levels of sCD14 correlate well with both short and long-term mortality, more so than non-fatal cardiovascular events [36–38]. Thus, we may hypothesize that the decline in event-free survival is mainly driven by increased mortality; however, the current study was not powered to study mortality *per se*.

As for diagnostic accuracy, LBP did not outperform CRP or added incremental accuracy in predicting CV outcome, as judged by similar AUC levels in ROC curves.

Prior studies have identified differences in the microbial communities of individuals with CVD *vs*. individuals without, by metagenomic sequencing [18,39,40]. Recently, Bacteroides vulgatus and Bacteroides dorei were found to be depleted in patients with CAD. Supplementation

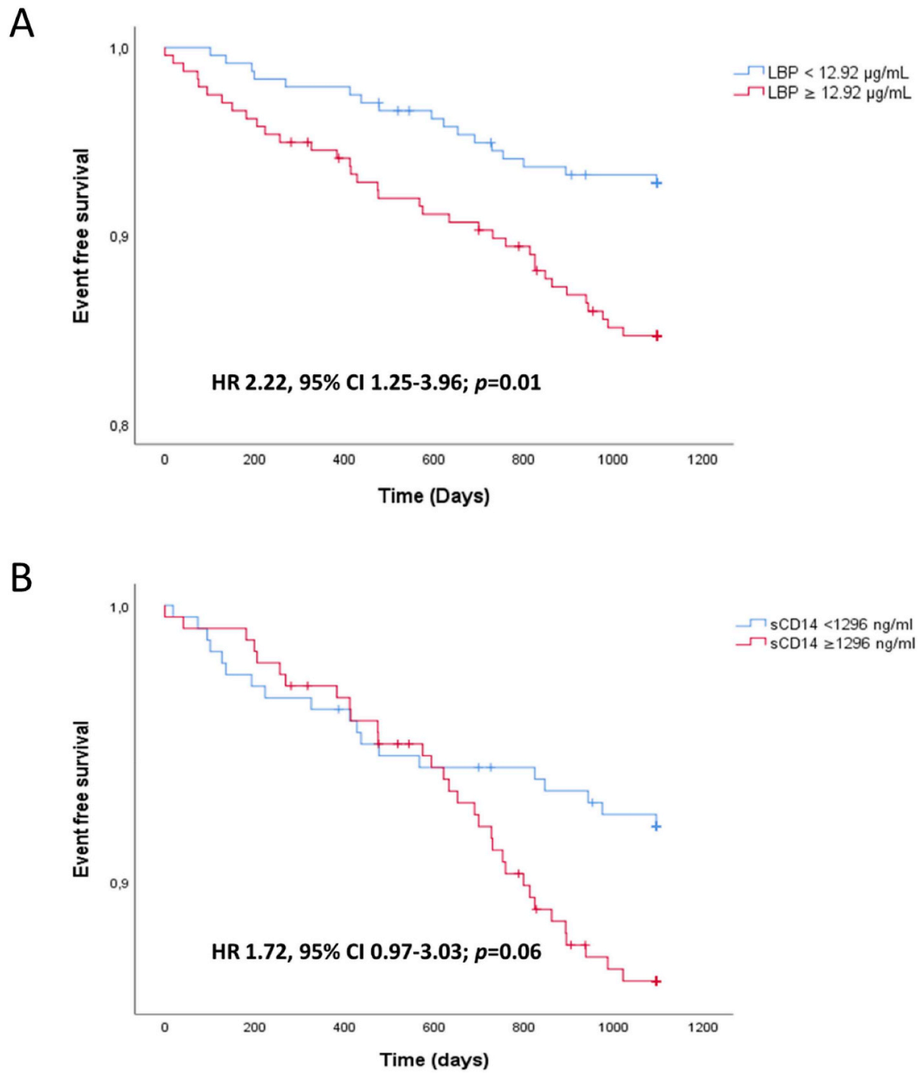

Fig. 2. Kaplan-Meier curves for event free CV survival, comparing levels of LBP (A) and sCD14 (B) above and below median values.

Table 2
Multivariate Cox regression model of LBP dichotomized at median for the prognosis of CV outcome.

	Model 1[a]		Model 2[b]		Model 3[c]	
	HR (95% CI)	*p*	HR (95% CI)	*p*	HR (95% CI)	*p*
LBP - below median	Ref.		Ref.		Ref.	
LBP - above median	2.00 (1.11–3.58)	**0.02**	1.98 (1.10–3.55)	**0.02**	1.90 (1.05–3.42)	**0.03**
Aspirin use	2.76 (1.56–4.88)	**< 0.001**	2.33 (1.25–4.32)	**0.01**	2.34 (1.26–4.35)	**0.01**
Current smokers	1.56 (0.90–2.70)	0.11	1.64 (0.94–2.87)	0.08	1.61 (0.92–2.83)	0.1
Total cholesterol	1.35 (1.01–1.79)	**0.04**	1.43 (1.06–1.91)	**0.02**	1.41 (1.05–1.90)	**0.02**
Age			1.06 (0.96–1.16)	0.24	1.05 (0.96–1.15)	0.32
History of diabetes			2.27 (1.24–4.16)	**0.01**	2.26 (1.24–4.14)	**0.01**
History of hypertension			0.91 (0.49–1.67)	0.75	0.86 (0.46–1.60)	0.63
Prior CVD			1.59 (0.84–3.00)	0.16	1.60 (0.84–3.04)	0.15
hsCRP					1.04 (0.98–1.11)	0.22

Statistical significance ($p < 0.05$) are outlined in bold.
[a] Model 1: Including confounders only.
[b] Model 2: Confounders and traditional risk factors added.
[c] Model: Confounders, traditional risk factors and hsCRP added.

of the aforementioned bacteria in rodents had anti-inflammatory effects by reducing fecal and blood levels of LPS, however, levels of LBP were not measured [17].

Zhou and colleagues reported increased bacterial richness measured in blood in patients after ST-elevation infarction (STEMI) compared to controls. Circulating LPS was also increased and associated with adverse cardiovascular events. The same study showed that experimental treatment of STEMI mice with polymyxin B, an antibiotic with LPS-inhibitory properties, reduced infarct size [41]. Conceptually, inhibition of LBP could also be an interesting way to inhibit the effects of

Table 3
Multivariate Cox Regression Model of sCD14 dichotomized at median level for the prognosis of CV outcome.

	Model 1		Model 2		Model 3	
	HR (95% CI)	*p*	HR (95% CI)	*p*	HR (95% CI)	*p*
sCD14 - below median	Ref.		Ref.		Ref.	
sCD14 - above median	1.82 (1.02–3.23)	**0.04**	1.68 (0.95–2.99)	0.08	1.62 (0.91–2.89)	0.10
Aspirin use	2.10 (1.16–3.81)	**0.01**	1.98 (1.04–3.77)	**0.04**	2.02 (1.06–3.84)	**0.03**
Nitrate use	2.60 (1.22–5.55)	**0.01**	2.55 (1.18–5.54)	**0.02**	2.55 (1.18–5.52)	**0.02**
Current smokers	1.93 (1.09–3.41)	**0.02**	2.09 (1.17–3.74)	**0.01**	2.03 (1.13–3.65)	**0.02**
Age			1.06 (0.96–1.16)	0.24	1.05 (0.96–1.15)	0.29
History of diabetes			2.33 (1.26–4.31)	**0.01**	2.35 (1.27–4.35)	**0.01**
History of hypertension			0.72 (0.39–1.35)	0.31	0.68 (0.36–1.29)	0.24
Prior CVD			1.18 (0.62–2.26)	0.62	1.21 (0.63–2.31)	0.57
hsCRP					1.05 (0.98–1.12)	0.18

[a] Model 1: Including confounders only.
[b] Model 2: Confounders and traditional risk factors added.
[c] Model: Confounders, traditional risk factors and hsCRP added.
Statistical significance ($p < 0.05$) are outlined in bold.

circulating LPS.

Associations between LBP and CVD have previously been studied in the Ludwigshafen Risk and Cardiovascular Health Study (LURIC) [42], in which 2959 subjects who were scheduled for coronary angiography were followed for 8 years. They found that LBP was associated with severity of coronary artery disease (CAD), prevalent CAD and both total and CV mortality. However, the predictive value of LBP was lost after controlling for levels of CRP, in contrast to our study in which LBP remained significantly associated with new CV events independently of CRP.

There are many factors that may explain the differences between the studies. Most importantly, we studied an older group of individuals with higher degree of inflammatory burden measured by CRP, and we observed specifically new CV events and CV mortality.

Our study has shown that none of the suggested markers of gut related inflammation was influenced by diet or n-3 PUFA supplementation. However, both markers seem to play a predictive role in CVD. In the context of the utility as biomarkers for clinically evident CVD, both markers show relatively low predictive value. However, as both LBP and sCD14 play important roles in the LPS-LBP-CD14-TLR4 signaling pathway [43], they may represent novel biomarkers of atherosclerosis or even potential treatment targets in CVD. Further studies will be needed to clarify the role of LBP and sCD14 as either having a causal role in cardiovascular inflammation or as innocent bystanders associated with CVD.

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Author contributions

All authors made substantial contributions to conception, design, drafting and critically revising the manuscript. Dr. Marius Trøseid, Prof. Ingebjørg Seljeflot and Prof. Harald Arnesen have also been involved in acquisition of data. All authors have approved the final manuscript.

Conflicts of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atherosclerosis.2019.05.004.

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