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Interaction between serum endotoxemia and proprotein convertase subtilisin/kexin 9 (PCSK9) in patients with atrial fibrillation: A *post-hoc* analysis from the ATHERO-AF cohort

Daniele Pastori[a,1], Evaristo Ettorre[b,1], Roberto Carnevale[c,e], Cristina Nocella[d], Simona Bartimoccia[a], Elisabetta Del Sordo[a], Vittoria Cammisotto[a], Francesco Violi[a,e,2], Pasquale Pignatelli[a,e,*,2], the Atherosclerosis in Atrial Fibrillation (ATHERO-AF) Study Group (Mirella Saliola, Marco Antonio Casciaro, Alessio Farcomeni, Luca Rubino, Cinzia Marchese, Maria Santulli, Fortunata Vasaturo, Valentina Castellani, Danilo Menichelli)

[a] *I Clinica Medica, Department of Internal Medicine and Medical Specialties, Sapienza University of Rome, Italy*
[b] *Department of Cardiovascular, Respiratory, Nephrologic, Anesthesiologic and Geriatric Sciences, Division of Gerontology, Sapienza University, Rome, Italy*
[c] *Department of Medical-Surgical Sciences and Biotechnologies, Sapienza University of Rome, Latina, Italy*
[d] *IRCCS Neuromed, Pozzilli, 86077, IS, Italy*
[e] *Mediterranea Cardiocentro, Napoli, 80122, Italy*

HIGHLIGHTS

- In patients with atrial fibrillation, LPS is directly associated with high PCSK9 levels.
- Nox2-related oxidative stress significantly correlated with PCSK9.
- Patients with elevated LPS and PCSK9 had an increased risk of cardiovascular events.
- Consumption of olive oil and wine was inversely associated with PCSK9.

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ABSTRACT

Background and aims: Lipopolysaccharides (LPS) is emerging as a novel risk factor for cardiovascular events (CVEs). Furthermore, *in vitro* evidence suggested that LPS may elicit proprotein convertase subtilisin/kexin 9 (PCSK9) expression, but their relationship *in vivo* has not been investigated.
Methods: We conducted a *post-hoc* analysis of a prospective, single centre cohort study of 907 patients with non-valvular atrial fibrillation (AF). At baseline, PCSK9, LPS and NADPH oxidase (sNox2-dp) were measured. PCSK9 and LPS were correlated with the incidence of CVEs.
Results: Median PCSK9 and LPS were 1200 [900–1970] and 49.9 [15.0–108.2] pg/ml, respectively. LPS and PCSK9 were significantly correlated (rS 0.378, $p < 0.001$). Logistic regression analysis showed that LPS was associated with PCSK9 above the median (odds ratio [OR] 1.727 95% confidence interval [CI] 1.147–2.600 $p = 0.009$). Other factors associated with PCSK9 above the median were sNox2-dp (OR 1.759 C.I. 95% 1.167–2.650, $p = 0.007$), use of antiplatelet drugs (OR 0.437 95%CI 0.219–0.871 $p = 0.017$) and high adherence to Mediterranean diet (OR 0.737 95%CI 0.643–0.845 $p = 0.001$). Olive oil (OR 0.376 95%CI 0.185–0.763, $p = 0.001$) and wine (OR 0.460 95%CI 0.289–0.733 $p = 0.007$) were negatively associated with PCSK9.
Patients with concomitant high PCSK9 and LPS (LPS ≥88 pg/ml and PCSK9 ≥1570 pg/ml) had an increased risk of CVEs compared to those with low levels (LPS < 24.3 pg/ml and PCSK9 < 1000 pg/ml, Log-Rank test,

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* Corresponding author. I Clinica Medica, Viale del Policlinico 155, Roma, 00161, Italy.
E-mail address: pasquale.pignatelli@uniroma1.it (P. Pignatelli).
[1] These authors contributed equally to this work.
[2] Joint senior authors.

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$p = 0.022$).
Conclusions: This study demonstrated, for the first time *in vivo,* that circulating levels of PCSK9 and LPS are associated with a mechanism possibly involving NADPH oxidase activation. Patients with concomitant increase of PCSK9 and LPS showed a higher risk of CVEs.

1. Introduction

Gut-derived metabolites such as trimethylamine-N-oxide and lipopolysaccharides (LPS) are emerging novel risk factors for cardiovascular diseases [1]. Increased levels of LPS have been described in patients with cardio-metabolic diseases characterized by systemic atherosclerosis, such as those with diabetes [2], obesity [3] and metabolic fatty liver disease [4].

Mechanisms suggesting serum LPS as a player in the onset of cardiovascular complications have been reported. For instance, LPS may favour vascular thrombosis by increasing platelet activation [5], and its presence has been recently described in human atherosclerotic plaques [6].

In addition to these mechanisms, recent experimental evidence suggested an interplay between LPS and proprotein convertase subtilisin/kexin 9 (PCSK9), which is a co-receptor of low-density lipoprotein cholesterol (LDL-C) regulating the uptake of these lipoproteins into the hepatocytes [7]. Thus, PCSK9 inhibition actually represents a key strategy for the management of patients with familial hypercholesterolemia or in those patients not reaching an appropriate LDL-cholesterol target in secondary prevention, despite an optimal lipid-lowering strategy [8].

In addition to its role in the regulation of lipid metabolism, recent evidence showed that PCSK9 may be involved in the onset and progression of atherosclerosis [9], disclosing pro-atherogenic properties [8]. Thus, PCSK9 is highly expressed in vascular smooth muscle cells and in human atherosclerotic plaques [10], in which it promotes the uptake of oxidized LDL in macrophages [11].

The expression of PCSK9 is regulated by several mediators, including inflammation, reactive oxygen species (ROS) produced upon NADPH oxidase activation [12], and damaged mitochondrial DNA [13,14]. The role of oxidative stress seems to be particularly important in this context, as experimental evidence showed that Nox2-derived ROS may mediate the synthesis of PCSK9 induced by LPS [14]. Although this hypothesis seems to be noteworthy, so far, the interplay between LPS and PCSK9 has never been investigated *in vivo*.

The potential interaction between LPS and PCSK9 may be particularly intriguing in patients at high cardiovascular risk, such as those suffering from with atrial fibrillation (AF). Thus, patients with AF have an increased oxidative stress, as shown by increased Nox2 activity and urinary isoprostanes excretion, which are significantly associated with an increased risk of cardiovascular events [15,16]. More recently, both PCSK9 and LPS levels have been found to be elevated in patients with AF and significantly associated with cardiovascular risk [17,18]. However, the interaction between PCSK9 and LPS in this patients' population has never been studied.

To this purpose, we performed a *post-hoc* analysis of a prospective study to investigate the relationship between PCSK9 and LPS, and their possible association with Nox2-related oxidative stress and CVEs in patients with AF.

2. Materials and methods

Post-hoc analysis of a prospective single centre cohort study including 907 patients with non-valvular AF treated with vitamin K antagonists oral anticoagulants referred to the Atherothrombosis Centre of the Department of Internal Medicine and Medical Specialties, Sapienza University of Rome.

All patients with non-valvular AF who were > 18 years of age were eligible for the study. All patients were treated with vitamin K antagonists after appropriate thrombotic risk stratification [19]. Exclusion criteria were the presence of metallic prosthetic heart valves or the presence of severe valvulopathy, severe cognitive impairment, chronic infectious disease, and systemic autoimmune disease. Patients were also excluded from the study if they had active cancer or liver insufficiency (e.g., cirrhosis) or acute infections. At study entry, medical history was recorded and cardiovascular risk factors were defined according to international guidelines [18].

The adherence to Med-Diet has been evaluated with a 9 items semi-quantitative questionnaire, as previously described [20].

All patients provided written informed consent at baseline. The study protocol was approved by the local ethical board of Sapienza-University of Rome (no. 1306/2007) and was conducted according to the principles of the Declaration of Helsinki.

Table 1
Characteristics of study population according to the median value of PCSK9.

	Total (n = 907)	PCSK9 below the median (n = 484)	PCSK9 above the median (n = 423)	*p*
Age (years)	73.5 ± 8.2	73.3 ± 8.1	73.7 ± 8.4	0.527
Female sex (%)	43.1	41.1	45.4	0.202
Persistent/permanent AF (%)	53.4	50.8	56.3	0.109
Arterial hypertension (%)	89.7	90.7	88.7	0.325
Systolic blood pressure (mmHg)	135.3 ± 21.3	135.0 ± 21.3	135.8 ± 21.4	0.644
Diastolic blood pressure (mmHg)	80.1 ± 11.8	80.5 ± 11.0	79.6 ± 12.6	0.488
Diabetes mellitus (%)	20.0	19.2	20.8	0.561
Heart failure (%)	17.2	15.7	19.0	0.217
Prior cerebrovascular events (%)	14.6	14.7	14.5	0.927
Prior cardiac events (%)	24.8	24.0	25.8	0.539
Antiplatelet therapy (%)	19.5	22.1	16.5	0.036
Statins (%)	41.8	41.3	42.3	0.786
Digoxin (%)	20.6	20.1	21.1	0.727
Proton pomp inhibitors (%)	46.6	49.4	43.4	0.079
CHA_2DS_2-VASc score	3.6 ± 1.5	3.6 ± 1.5	3.7 ± 1.5	0.240
Lipopolysaccharides (pg/ml)	49.9 [15.0–108.2]	34.9 [10.2–78.3]	77.5 [25.0–150.0]	< 0.001
sNox2-dp (pg/ml, n = 702)	10.0 [7.0–20.2]	8.0 [6.0–16.0]	14.0 [8.0–24.0]	< 0.001

AF: atrial fibrillation; sNox2-dp: soluble Nox2-derived peptide.

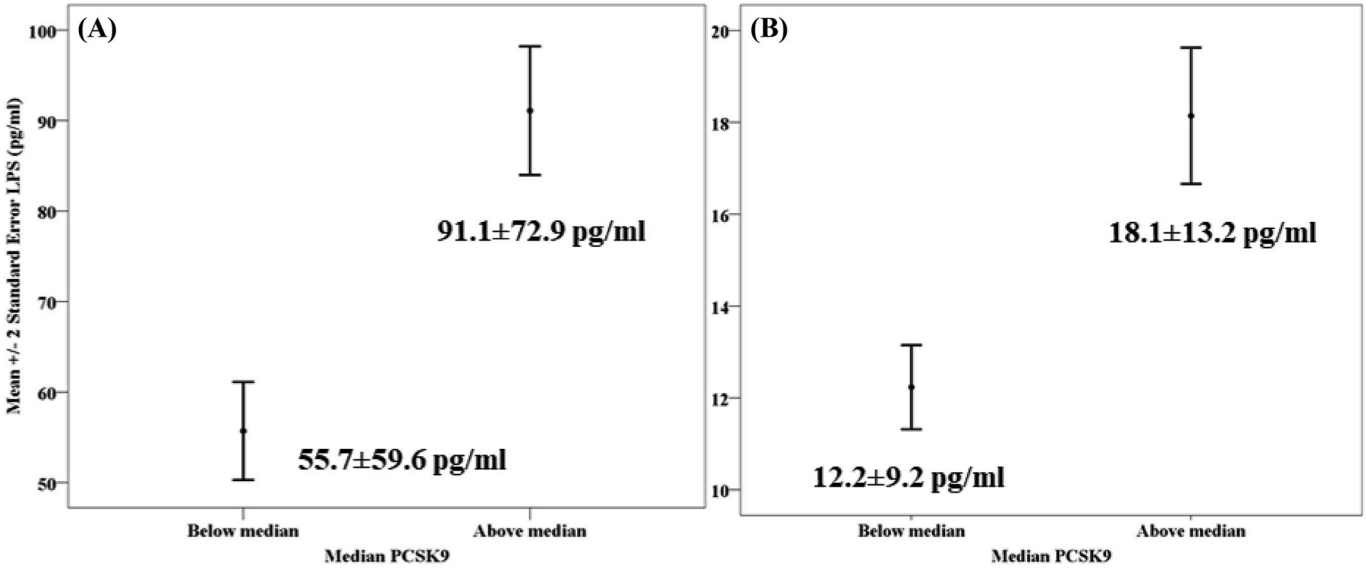

Fig. 1. LPS (A) and serum levels of sNox2-dp (B) according to PCSK9 above or below the median.

Table 2
Multivariable logistic regression of factors associated to PCSK9 above the median.

	Odds ratio	95% Confidence interval		*p*
Paroxysmal AF (*vs.* persistent/permanent AF)	1.110	0.740	1.664	0.615
Proton pomp inhibitors	1.107	0.732	1.674	0.629
Digoxin	0.953	0.584	1.555	0.847
Female sex	1.426	0.947	2.145	0.089
Age	0.977	0.952	1.002	0.072
Hypertension	0.966	0.505	1.850	0.918
Diabetes	0.806	0.472	1.377	0.430
Heart failure	0.812	0.451	1.463	0.488
Prior cerebrovascular events	1.209	0.686	2.129	0.512
Previous cardiac events	1.101	0.633	1.916	0.733
Antiplatelet therapy	0.437	0.219	0.871	0.019
Statins	1.109	0.728	1.692	0.630
sNox2-dp above median	1.759	1.167	2.650	0.007
Lipopolysaccharides above median	1.727	1.147	2.600	0.009
Mediterranean Diet score (continuous)	0.737	0.643	0.845	0.001

AF: atrial fibrillation; sNox2-dp: soluble Nox2-derived peptide.

2.1. Laboratory analysis

Blood samples obtained from patients after supine rest for at least 10 min were taken into tubes with 3.8% sodium citrate and centrifuged at 300*g* for 10 min to obtain supernatant, then immediately stored at −80 °C until use. Plasma levels of PCSK9 were measured by a commercial enzyme-linked immunoadsorbent assay (Cusabio, Wuhan, China). Plasma samples were diluted 1:10 in diluent buffer. Data are expressed as pg/ml, and the minimal detectable dose of PCSK9 was < 10 pg/ml in human plasma. The intra-assay and inter-assay coefficients of variance were 5.8% and 6.9%, respectively. Lipopolysaccharide serum levels were measured using a commercial ELISA kit (Cusabio, Wuhan, China). Standards of LPS, purified from *Escherichia coli,* and blood samples were plated for 2 h at room temperature onto a microplate precoated with the antibody specific for LPS. After incubation, samples were read at 450 nm. Values were expressed as picograms per milliliter; intra-assay and inter-assay coefficients of variation were 8% and 10%, respectively. Serum levels of sNox2-dp (soluble Nox2-derived peptide) were measured using an ELISA method as previously described [20] and the concentrations were expressed in pg/ml. The intra-assay and inter-assay coefficients of variation were 5.2% and 6%, respectively.

2.2. Statistical analysis

Categorical variables were reported as counts (percentage) and Pearson chi squared test was used to compare proportions. Kolmogorov-Smirnov test was used to test the distribution of continuous variables. Normally distributed continuous variables were expressed as mean and standard deviation and compared by the Student *t*-test and ANOVA. Non-normally distributed variables were reported as median and interquartile range (IQR) and categorized for multivariable analysis. Bivariate analysis was performed by Spearman rank correlation test.

The study cohort was divided according to the median value of PCSK9. A descriptive analysis of clinical characteristics was performed, and multivariable logistic regression analysis was used to determine factors independently associated with PCSK9 serum levels above the median through the calculation of odds ratio (OR) and 95% confidence interval (CI) for each variable.

To investigate the impact of concomitant high levels of both PCSK9 and LPS on CVEs, including fatal/nonfatal myocardial infarction and ischemic stroke, cardiac revascularization (stent or coronary artery bypass surgery), cardiovascular death, and transient ischemic attack (see Ref. [18] for definitions), we compared patients in the highest

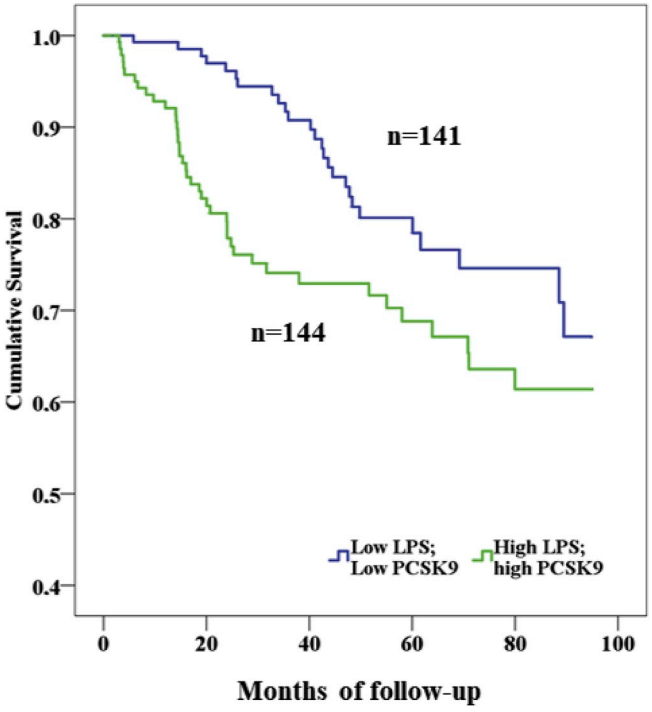

Fig. 2. Kaplan-Meier survival curves in patients with concomitant high PCSK9 and LPS compared to those with low levels of PCSK9 and LPS.

tertile of both LPS (≥88 pg/ml) and PCSK9 (≥1570 pg/ml) with those in the lowest tertile (LPS < 24.3 pg/ml and PCSK9 < 1000 pg/ml).

The cumulative incidence for CVEs was estimated using Kaplan-Meier curves, which were formally compared using the Log-Rank test. Univariate and multivariate Cox's proportional hazards regression analysis was used to calculate the adjusted relative hazard ratios (HR) of CVEs by each clinical variable. We entered as covariate CHA_2DS_2 VASc score, groups of PCSK9-LPS (high *vs.* low), persistent/permanent AF (*vs.* paroxysmal), amiodarone, antiplatelets, digoxin, ACE inhibitors/sartans, beta blockers, Calcium channel antagonists, HDL cholesterol, triglycerides, statins.

Only p values < 0.05 were considered statistically significant. All tests were two tailed and analysis was performed using software package computers (SPSS-18.0, SPSS Inc.).

3. Results

Of the initial 1,138 patients, 150 patients did not meet entry criteria and were excluded, 26 patients refused to participate, and 55 patients were lost to follow-up (28 were diagnosed with cancer and 27 underwent cardiac valve surgery or organ transplantation) and data were censored. Thus, the final study cohort was composed of 907 patients with AF. Median PCSK9 values in the entire cohort were 1200 [900–1970] pg/ml.

Characteristics of patients according to PCSK9 above and below the median serum concentration are reported in Table 1. Mean age was 73.5 ± 8.2 years, and 43.0% were women. The prevalence of cardiovascular risk factors was similar between patients with PCSK9 below and above the median and no difference in the thromboembolic risk was found between the two groups (Table 1).

Patients with PCSK9 above the median had a significant higher serum concentration of LPS (p < 0.001, Fig. 1A) and sNox2-dp (p < 0.001, Fig. 1B).

PCSK9 was directly correlated with LPS (rS 0.378, p < 0.001) and sNox2-dp (rS 0.353, p < 0.001). Moreover, LPS and sNox2-dp were significantly correlated (rS 0.321, p < 0.001).

At multivariate logistic regression analysis, LPS concentrations (OR 1.727 C.I. 95% 1.147–2.600 p = 0.009) and sNox2-dp (OR 1.759 C.I. 95% 1.167–2.650, p = 0.007) were directly associated with PCSK9 above the median (Table 2). Conversely, adherence to Med-Diet (OR 0.737 C.I. 95% 0.643–0.845 p = 0.001 for each point of the Med-Diet score) and antiplatelet treatment (OR 0.437 C.I. 95% 0.219–0.871 p = 0.017) were inversely associated with PCSK9 above the median.

When we used single items of Med-Diet questionnaire as covariates instead of the total score, we found that use of extra virgin olive oil (OR 0.376 C.I. 95% 0.185–0.763, p = 0.001) and moderate wine consumption (OR 0.460 C.I. 95% 0.289–0.733 p = 0.007) were inversely associated with high PCSK9 levels.

3.1. Survival analysis

Median follow-up was 40.5 (IQR 20.9–67.6) months, yielding 3,865 patient-years of observation. Patients with concomitant high PCSK9 and LPS (LPS ≥88 pg/ml and PCSK9 ≥1570 pg/ml) had an increased risk of CVEs as compared to those with low levels (LPS < 24.3 pg/ml and PCSK9 < 1000 pg/ml) with 48 and 29 CVEs in each group, respectively (Log-Rank test, p = 0.022) (Fig. 2). This association remained significant in multivariable Cox proportional regression analysis (HR 1.728, 95%CI 1.042–2.867, p = 0.034) after adjustment for potential confounders (Table 3).

3.2. Adherence to Mediterranean diet

To investigate the relationship between adherence to Med-Diet and PCSK9, we divided the cohort into three groups according to tertiles of the Med-Diet score: first tertile was the low-adherence group (0–4 points), second tertile was the intermediate adherence group (5 points), and third tertile was the high-adherence group (6–9 points). PCSK9

Table 3
Multivariable Cox proportional regression analysis of factors associated with cardiovascular events.

	Hazard ratio	95% Confidence interval		p
CHA_2DS_2 VASc score	**1.396**	**1.197**	**1.629**	**< 0.001**
Groups of PCSK9-LPS (high *vs.* low)	**1.728**	**1.042**	**2.867**	**0.034**
Persistent/permanent AF (*vs.* paroxysmal)	0.738	0.446	1.221	0.237
Amiodarone	1.040	0.617	1.754	0.882
Antiplatelets	0.776	0.426	1.411	0.405
Digoxin	1.464	0.828	2.587	0.190
ACE inhibitors/sartans	0.892	0.520	1.530	0.677
Beta blockers	0.963	0.578	1.604	0.883
Calcium channel antagonists	1.362	0.796	2.332	0.259
HDL cholesterol	**0.982**	**0.964**	**1.000**	**0.048**
Triglycerides	0.998	0.993	1.004	0.574
Statins	1.294	0.780	2.145	0.318

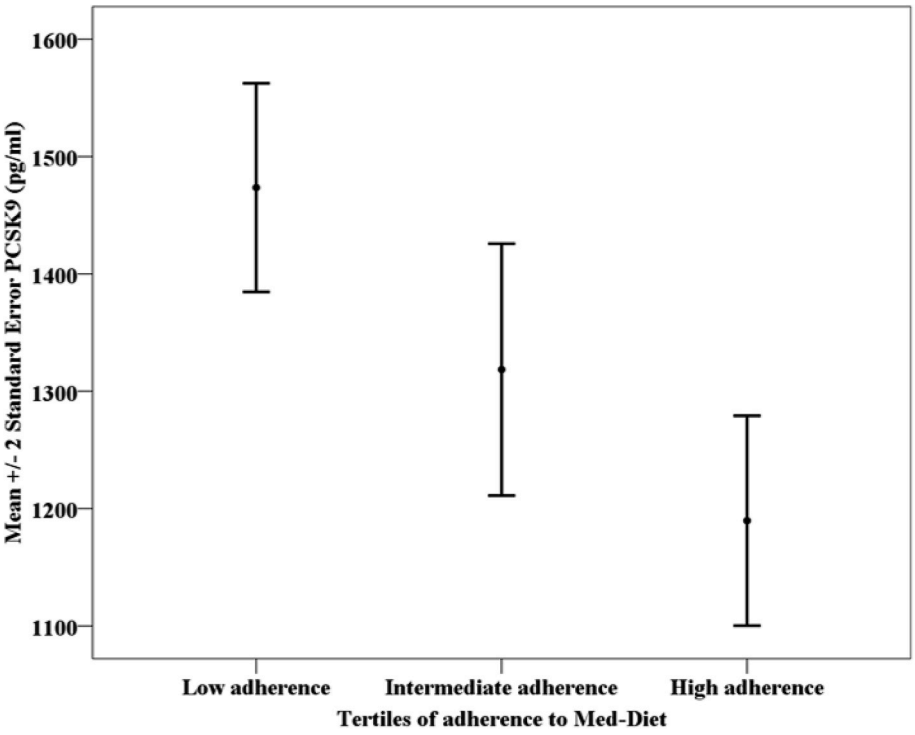

Fig. 3. Serum concentrations of PCSK9 according to groups of adherence to Mediterranean diet.

levels decreased significantly across tertiles of adherence to Med-Diet (low 1473.5 ± 676.6, intermediate 1318.4 ± 747.2, high adherence 1189.7 ± 738.9 pg/ml, ANOVA, $p < 0.001$, Fig. 3). Thus, PCSK9 and Med-Diet score were significantly and inversely correlated (rS −0.215, $p < 0.001$). Univariate logistic regression analysis showed an OR of 0.789 (95%CI 0.713–0.874, $p < 0.001$) for each point of Med-Diet score for PCSK9 above the median.

4. Discussion

This study demonstrated that, in a clinical setting of patients at high cardiovascular risk, such as those with AF, circulating levels of PCSK9 are significantly associated with LPS concentration, suggesting that LPS may have a pro-atherothrombotic property by enhancing PCSK9 expression.

The interplay between PCSK9 and LPS was previously investigated only *in vitro* and in animal models. In mice injected intraperitoneally with LPS (5 mg/kg body weight), a progressive increase of PCSK9 levels was found [21], suggesting LPS as a potential trigger for PCSK9 expression. Our finding that circulating levels of PCSK9 and LPS are elevated and mutually associated in patients with AF seems in accordance with this previous experimental report. In this context, it is of note that Ding and colleagues evidenced that the increase of PCSK9 levels in endothelial cells treated with LPS was blunted by treatment with a specific inhibitor of the NADPH oxidase (Nox2) [14].

Furthermore, recent data showed that LPS localized into human atherosclerotic plaque and dose-dependently induced Nox2 up-regulation by human monocytes via interaction with its receptor TLR4[6]. Based on this, we hypothesized that LPS may induce PCSK9 *in vivo* through an up-regulation of Nox2 activity. Indirect support to this hypothesis is provided by the fact that LPS correlated with Nox2, which in turn was significantly associated with PCSK9.

To evaluate if concomitant increase of PCSK9 and LPS could increase the risk of CVEs, we grouped patients according to values of PCSK9 and LPS. Noteworthy, we found that patients with concomitant high levels of both PCSK9 and LPS disclosed an enhanced risk of cardiovascular events, as compared to patients with low levels of both biomarkers.

An intriguing result from our study is represented by the interplay between PCSK9 and Med-Diet, a well-recognized healthy dietary pattern [22,23]. We found that high adherence to Med-Diet is associated with lower levels of PCSK9. In particular, extra virgin olive oil and wine, two foods rich in antioxidant compounds, showed an independent, inverse association with PCSK9 levels, suggesting another mechanism potentially accounting for the inverse association between Med-diet and CVEs [20].

Our study translates previous experimental evidence on the relationship between PCSK9 and oxidative stress in a human model of patients at high residual cardiovascular risk, such as those with AF, who experience a high rate of cardiovascular complications despite current antithrombotic treatments [24].

Thus, our findings suggest that LPS may represent a risk factor for cardiovascular disease not only by increasing platelet activation, as previously demonstrated [25], but also by inducing an upregulation of PCSK9 expression/synthesis, with a mechanism potentially involving Nox2-derived oxidative stress. Thus, strategies aimed at modulating gut microbiota, and the study of gut permeability in patients with AF may provide novel insights into the role of LPS in this population.

In this context, the modification of lifestyle factors (i.e. diet, physical activity and smoking) [26,27], improving adherence to Med-Diet, should represent the first therapeutic approach to patients with atherosclerosis.

The study has limitations to be acknowledged. The study is a *post-hoc* analysis from a single center cohort study and should be confirmed in a multicenter cohort study with larger sample size. Furthermore, only a randomized interventional nutritional study will confirm if Med-Diet could affect directly LPS and PCSK9 concentrations. The inclusion of only Caucasian elderly patients limits the generalizability of the results to other populations. Even if it is arguable that enhanced LPS stems from gut microbiota (we excluded patients with acute infections), we have not definite evidence in support of this hypothesis and further studies are to be done to assess the presence of gut dysbiosis or

enhanced gut permeability. Finally, despite we performed a multivariable analysis including a number of covariates representing the principal clinical characteristics of patients, we cannot exclude that some potential confounders may be still present. Among others, previous studies showed that PCSK9 levels may be modified by renal function, increasing in patients with end stage renal disease or undergoing dialysis [28,29]. Furthermore, patients with different degrees of systemic and vascular inflammation may disclose different levels of PCSK9 [30].

In conclusion, this study demonstrated that circulating levels of LPS are associated with enhanced PCSK9 and that their concomitant high concentration increased cardiovascular risk.

Conflicts of interest

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

Author contributions

Daniele Pastori: study design; data analysis; interpretation of data; drafting of manuscript; critical revision.

Evaristo Ettorre, Elisabetta Del Sordo: data collection; interpretation of data; drafting of manuscript; critical revision.

Roberto Carnevale, Cristina Nocella, Simona Bartimoccia, Vittoria Cammisotto: laboratory analysis; interpretation of data; drafting of manuscript; critical revision.

Francesco Violi, Pasquale Pignatelli: study design; interpretation of data; drafting of manuscript; critical revision.

All authors read and approved the final version of the manuscript.

All authors in the ATHERO-AF Group participated in the enrolment of patients and data collection.

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