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Lipocalin-2 and Calprotectin Potential Prognosis Biomarkers in Peripheral Arterial Disease

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WHAT THIS PAPER ADDS

Peripheral arterial disease (PAD) is regarded as a chronic inflammatory disease of atherosclerotic aetiology presenting residual inflammation. The inflammatory markers lipocalin-2 and calprotectin are associated with increased risk of death or amputation or lower limb events in symptomatic and early stage PAD. Combined analysis of the inflammatory biomarkers lipocalin-2 and calprotectin might be useful for risk stratification in PAD at different stages.

Objective: Peripheral arterial disease (PAD) is the most prevalent cardiovascular (CV) condition globally. Despite the high CV risk of PAD patients, no reliable predictors of adverse clinical evolution are yet available. In this regard, previous transcriptomic analyses revealed increased expression of calprotectin (S100A8/A9) and lipocalin-2 (LCN2) in circulating extracellular vesicles (EVs) of patients with PAD. The aim of this study was to determine the prognostic value of LCN2 and calprotectin for CV risk assessment in PAD.

Methods: LCN2 and the S100A9 subunit of calprotectin were examined in human femoral plaques by immunohistochemistry and qPCR. LCN2 and calprotectin were determined by ELISA in PAD (CHN cohort, $n = 331$, Fontaine II–IV, serum), and PAD diagnosed by population based screening (VIVA trial, $n = 413$, the majority Fontaine 0–I, plasma). Patients were followed up for a mean of four years, recording the primary outcomes; CV death or amputation in the CHN cohort and CV death or major lower limb events (MALE) in the VIVA population. Secondary outcomes were all cause death or amputation, and all cause death or MALE, respectively.

Results: LCN2 and S100A9 were detected in human plaques in regions rich in inflammatory cells. LCN2 and calprotectin levels were 70% and 64% lower in plasma than in serum. In the CHN cohort, high serum levels of LCN2 and calprotectin increased the risk of primary and secondary outcomes 5.6 fold ($p < .001$) and 1.8 fold ($p = .034$), respectively, after covariable adjustment. Similarly, elevated plasma levels of LCN2 and calprotectin increased by three fold the risk of primary and secondary outcomes ($p < .001$) in the VIVA cohort. Moreover, addition of the combined variable to basal models, considering clinically relevant risk factors, improved reclassification for the primary outcome in both cohorts ($p \leq .024$).

Conclusion: Combined assessment of the inflammatory biomarkers LCN2 and calprotectin might be useful for risk stratification in advanced and early PAD.

Keywords: Amputation, Atherosclerosis, Inflammation, Mortality, Vascular disease
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INTRODUCTION

Peripheral arterial disease (PAD) is the most prevalent cardiovascular (CV) condition globally, with > 200 million cases worldwide and it is estimated to increase greatly with the ageing of the population.[1] Individuals with the most severe form of PAD, chronic limb threatening ischaemia (CLTI), are at heightened risk of trophic lesions, surgical and percutaneous revascularisation, amputation, and impaired quality of life.[2] Despite its poor prognosis, no reliable risk predictors are yet available.

PAD is regarded as a chronic inflammatory disease of atherosclerotic aetiology. Accordingly, several inflammatory molecules stand out as candidates for risk stratification and outcome assessment.[3] C reactive protein (CRP) and neutrophil to lymphocyte ratio (NLR) are predictors of poor outcome in PAD.[4,5] Nevertheless, both being non-specific markers of inflammation, their elevated levels could be attributed also to existing comorbidities. Extracellular vesicles (EVs) carry lipids, proteins and nucleic acids from the cell of origin, and their content varies according to the stimulus triggering their release, reflecting the physiopathological condition of the parental cell. Likewise, the analysis of their content has emerged as an alternative for new biomarker discovery (liquid biopsy). By transcriptomic analysis of circulating EVs, the present authors recently described expression of genes related to the immune response in PAD. In particular, calprotectin (S100A8/A9 heterodimer) and lipocalin-2 (LCN2) or NGAL (neutrophil gelatinase-associated lipocalin), a 25 kDa glycoprotein, were increased in EVs of patients with PAD.[6] Moreover, an association was reported between high circulating levels of calprotectin and CV mortality or amputation in patients with symptomatic PAD, with incremental prognostic value mainly when combined with hs-CRP. Interestingly, S100A9 expression was also detected in femoral plaque-derived EVs and femoral tissue, suggesting its possible involvement in lesion progression.[6] On the other hand, LCN2 is released into the circulation mostly by mature neutrophils; it is considered a sound kidney injury marker and has been suggested as a possible candidate for outcome prognostication in patients with CV diseases, including coronary atherosclerosis, heart failure, acute coronary syndromes, or diabetes.[7] In contrast, its role in PAD has been scarcely investigated.[8]

Based on transcriptional studies in EVs indicating that LCN2 might be upregulated in PAD along with calprotectin, the present study aimed to measure local expression of both proteins in femoral plaques, exploring their association in the circulation, and analysing their prognostic value in advanced and early PAD.

MATERIALS AND METHODS

Independent analyses were performed in patients with symptomatic PAD (CHN cohort[6]) and in patients with early stage PAD (VIVA cohort[9,10]). The Supplementary material and methods include a complete description of the cohorts, material and methods, and statistical analysis.

Human atherosclerotic lesions

Femoral endarterectomy samples (> 75% stenosis) were frozen for RNA analysis ($n = 8$), or fixed with 4% paraformaldehyde and embedded in paraffin for morphological analysis ($n = 5$). Immunostaining was performed with the following antibodies: rabbit anti-human NGAL (PA5-88079 ThermoFisher), rabbit anti-human S100A9 (PA5-82145, ThermoFisher), rabbit anti-human neutrophil elastase (ELANE, HPA066836, Sigma), and mouse anti-human CD68 (M0184, Dako).

Complejo Hospitalario de Navarra (CHN) cohort

Patients with symptomatic PAD ($n = 331$, CHN cohort)[6] were enrolled prospectively (Supplementary Fig. S1), and blood samples were collected at the time of clinical evaluation at the Complejo Hospitalario de Navarra (CHN, 2010—2019). Patients were classified into intermittent claudication (IC, Fontaine class II) diagnosed by haemodynamic study (Doppler ultrasound) or into chronic limb threatening ischaemia (CLTI) with lower limb rest pain and/or trophic lesions (Fontaine class III—IV) confirmed by imaging studies (arteriography, magnetic resonance angiography, or Doppler ultrasound). The mean average follow up was four years, recording amputation and death, either all cause or CV, to evaluate the primary composite endpoint, CV death or amputation, and the secondary outcome, all cause mortality or amputation.

The study was approved by the Institutional Review Board of the CHN (30/10), according to the standards of the Declaration of Helsinki on medical research, and written informed consent was obtained from all patients who were enrolled.

Viborg Vascular (VIVA) study patients

The Viborg Vascular (VIVA) Screening Trial was a population based, randomised, clinically controlled screening trial including 50 170 Danish men aged 65—74 years living in the Central Denmark Region, which ran from 2008 to 2011.[9,10] Briefly, study participants were randomly assigned 1:1 to triple screening or no systematic screening to evaluate abdominal aortic aneurysm (AAA), hypertension, and PAD. Diagnostic criteria were aortic diameter ≥ 30 mm for AAA, ABI < 0.9 or > 1.4 for PAD, and BP > 160/100 mmHg for hypertension. LCN2 and calprotectin were measured in 413 patients with PAD (most at Fontaine stages 0—I) age matched with the CHN cohort with a mean follow up of 4.5 years. Mortality, either all cause or CV, was registered. Major adverse lower limb events (MALE) were defined as major amputation ($n = 22$), acute lower limb ischaemia ($n = 3$), and revascularisation due to IC ($n = 13$) and CLTI ($n = 12$).[11—13] The primary outcome was a composite of CV death or MALE, and the secondary outcome was a composite of all cause mortality or MALE.

The trial was approved by the scientific, ethical committee of the Central Denmark Region (M20080018) and registered in the Clinical Trials Register (NCT00662480).[9]

Lipocalin-2 and calprotectin determination in blood

LCN2 and calprotectin determinations were performed in serum in the CHN cohort and older controls, and in plasma EDTA in the VIVA cohort, using the Human Lipocalin-2/NGAL ELISA (Biovendor) with inter- and intra-assay coefficients of variation of $< 10\%$ and $< 8\%$, respectively, and a limit of detection of 0.02 ng/mL, and the LegendMax Human MRP8/14 kit (BioLegend) with an inter- and intra-assay coefficients of variation of < 8 and $< 3\%$, and a detection limit of 0.62 ± 0.34 ng/mL, respectively. All serum samples (CHN cohort, $n = 331$), and later, all plasma samples (VIVA cohort, $n = 413$) were tested at the same time over an interval of a week.

Statistical analysis

Values are expressed as mean $\pm$ SD or median (interquartile range), and categorical variables as numbers and percentages. Associations were assessed by Pearson correlation test and linear regression analysis. Receiver Operating Characteristic (ROC) curves were plotted to assess the diagnostic performance of LCN2 and calprotectin for amputation (CHN cohort) and MALE (VIVA trial), and the cutoff values calculated by the Youden Index. Sub-hazard (SHR) and hazard ratios (HR) and their 95% CI were estimated using Fine-Gray competing risk models and Cox regression models, respectively, after adjusting for relevant covariables. The additional value of LCN2 and calprotectin for risk prediction was

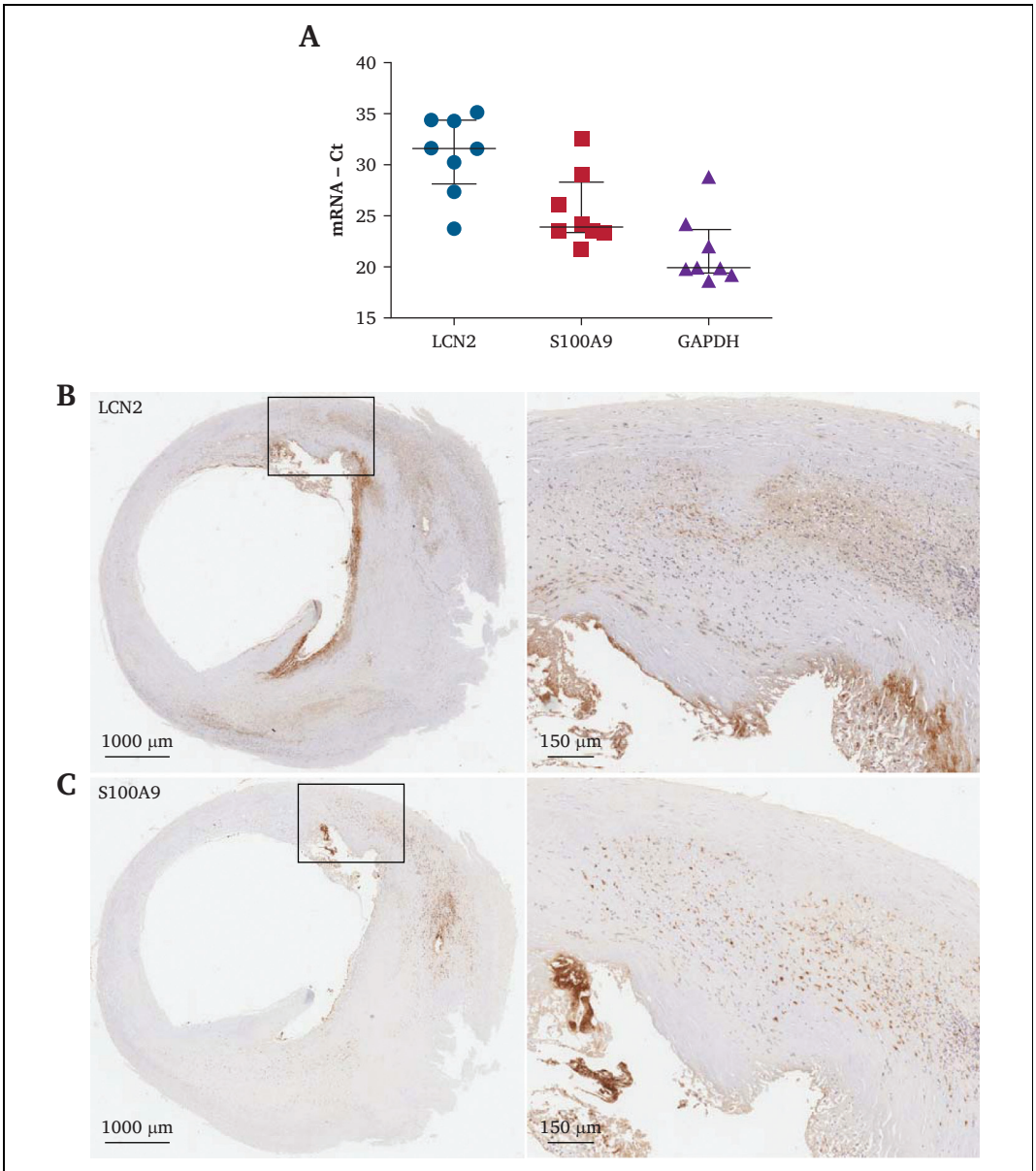

Figure 1. (A) Lipocalin-2 (LCN2) and S100A9 mRNA expression in human femoral plaques ($n = 8$). Glyceraldehyde 3-phosphate dehydrogenase (GAPHD) was used as housekeeping gene. (B, C) Representative immunohistochemistry for LCN2 (B, brown) and S100A9 (C, brown) in human femoral plaques.

assessed with Harrell's C statistics, the integrated discrimination index (IDI), and the continuous net reclassification index (NRI). Analyses were performed with STATA version 13 and SPSS version 15. All p values are two tailed, and statistical significance was set at $p < .05$.

RESULTS

Lipocalin-2 and calprotectin are expressed in human femoral plaques

Local expression of LCN2 and the calprotectin subunit, S100A9, was observed in femoral atherosclerotic plaques by mRNA determination (RT-qPCR, Fig. 1A) and by immunohistochemistry (Fig. 1B, C). LCN2 colocalised with neutrophils and weakly with macrophages, while S100A9 staining was observed in both inflammatory cell types (Supplementary Fig. S2).

Association between serum lipocalin-2 and calprotectin in symptomatic peripheral arterial disease (CHN cohort)

As shown in Table 1, 53% of the CHN patients (mean age 70 years, 90% men) had diabetes, 76% hypertension, 68% dyslipidaemia, and more than a third suffered chronic kidney disease (41%) (Table 1). The mean ABI was below 0.6. Patients with PAD were treated according to the guidelines, and total cholesterol and low density lipoprotein (LDL) levels were within target concentrations in subjects with high CV risk (Table 1).[14] Inflammatory markers hs-CRP, and the NLR were above the levels reported in healthy subjects.[6,15] Moreover, sex and age matched subjects with risk factors, but without CV disease ($n = 70$, Supplementary materials and methods) presented decreased serum levels of LCN2 (median [interquartile range] 54 [18] ng/mL controls *vs.* 84 [59] ng/mL PAD, $p < .001$) and calprotectin (3.2 [2.4] μg/mL controls *vs.* 3.8 [3] μg/mL PAD, $p = .074$) compared with patients with symptomatic PAD.

As shown in Table 2, both the NLR (B −0.110, 95% CI −0.215 — −0.005), $p = .040$) and calprotectin (B 0.438, 95% CI 0.348 — 0.528), $p < .001$) were independent predictors of serum LCN2 in the CHN cohort, although the associated partial R^2 indicated a stronger association with calprotectin.

Table 1. Demographic and clinical parameters in the Complejo Hospitalario de Navarra (CHN) cohort of 331 patients with symptomatic peripheral artery disease (PAD)

	Patients ($n = 331$)
Demographic and clinical data	
Men	289 (87)
Age — y	70 ± 10
Smokers	
Never	63 (19)
Current	111 (34)
Former	157 (47)
Diabetes mellitus	177 (53)
Hypertension	251 (76)
Dyslipidaemia	225 (68)
Ankle brachial index	0.56 ± 0.18
Treatment	
Anticoagulants	41 (12)
Antiplatelets	256 (77)
ACE inhibitors	115 (35)
Angiotensin II receptor antagonist	92 (28)
Calcium antagonists	73 (22)
Vasodilators	22 (7)
β blockers	85 (26)
Statins	231 (70)
Previous history	
Chronic obstructive pulmonary disease	45 (14)
Chronic kidney disease	137 (41)
Acute myocardial infarction	90 (27)
Cardiomyopathy	83 (25)
Cerebral ischaemia	34 (10)
Laboratory data	
Total cholesterol — mmol/L	4.4 ± 1.2
LDL-C — mmol/L	2.6 ± 0.9
HDL-C — mmol/L	1.1 ± 0.4
Triglycerides — mmol/L	1.7 ± 0.9
eGFR — mL/min/1.73m^2	73 ± 21
Median calprotectin (IQR) — μg/mL	3.8 (3)
Median NLR (IQR)	2.5 (1.8)
Median hs-CRP (IQR) — mg/L	4.6 (10)
Median LCN2 (IQR) — ng/mL	84 (59)

Data are presented as n (%) or as mean ± standard deviation, unless stated otherwise. ACE = angiotensin converting enzyme; IQR = interquartile range; LDL = low density lipoprotein; HDL = high density lipoprotein; eGFR = estimated glomerular filtration rate; hs-CRP = high-sensitivity C reactive protein; NLR = neutrophil to lymphocyte ratio; LCN2 = lipocalin-2.

Combination of lipocalin-2 and calprotectin improves outcome prediction in the CHN cohort

Ten per cent of patients with symptomatic PAD presented with worsening during the follow-up. Amputation ($n = 26$, 8%) and death, either all cause ($n = 129$, 39%) or CV ($n = 46$, 14%; $n = 33$ related to cardiac failure, $n = 7$ to stroke, and $n = 6$ to mesenteric ischaemia) were recorded during the follow-up. The primary composite outcome (CV death or amputation) occurred in 65 patients (20%), while the secondary outcome (all cause death or amputation) occurred in 137 (41%).

First, risk prediction was determined with LCN2 alone and a statistically significant association was found between high levels of LCN2 and the primary outcome before and after correcting by other covariables (Fine-gray model: SHR > 3.5, $p \leq .040$ for all tested models, Supplementary Table S1), while for all cause death or amputation the association was lost when adjusted by age, sex, diabetes, hypertension, dyslipidaemia, eGFR, and hs-CRP.

To address whether the combination of LCN2 and calprotectin could improve risk assessment, a ROC curve was performed for LCN2 assessing amputation (AUC 0.674 ± 0.06, 95% CI 0.56 — 0.79, $p = .003$) to mimic the analyses performed previously for calprotectin in this cohort (cutoff for calprotectin $\geq$ 7.4 μg/mL).[6] Youden index analysis rendered a cutoff of $>$ 86 ng/mL for LCN2 (77% sensitivity, 54% specificity).

Next, Cox regression analyses were performed using categorised LCN2 and calprotectin (Table 3). Three groups

Table 2. Linear regression analysis to explore the association between cardiovascular risk factors and lipocalin-2 levels (dependent variable) in the Complejo Hospitalario de Navarra (CHN) cohort of 331 patients with symptomatic peripheral artery disease

	Univariable		Multivariable		
	B (95% CI)	*p*	B (95% CI)	Partial R2 − %	*p*
Age − y	0.001 (-0.001−0.003)	.46			
Men	0.082 (0.008−0.156)	.030	0.097 (0.032−0.162)	2.13	.003
ABI	-0.238 (-0.388− -0.088)	.002	-0.170 (-0.291− -0.048)	1.85	.006
Smoker	-0.026 (-0.089−0.038)	.43			
Diabetes mellitus	0.039 (-0.011−0.088)	.13	-0.002 (-0.046−0.043)	0.002	.94
Hypertension	0.020 (-0.038−0.078)	.50			
Dyslipidaemia	-0.085 (-0.137− -0.032)	.002	-0.019 (-0.063−0.025)	0.18	.40
eGFR − mL/min/1.73m^2	-0.004 (-0.005− -0.003)	<.001	-0.004 (-0.005− -0.003)	12.0	<.001
HDL − mg/dL	-0.004 (-0.005− -0.002)	<.001	0.001 (-0.002−0.001)	0.08	.56
Previous history					
COPD	-0.011 (-0.083−0.062)	.78			
CKD	0.138 (0.090−0.186)	<.001			
AMI	0.019 (-0.037−0.075)	.51			
Cardiomyopathy	0.075 (0.018−0.131)	.010	-0.023 (-0.075−0.030)	0.18	.39
Stroke	-0.020 (-0.102−0.062)	.63			
Treatment					
Anticoagulants	0.035 (-0.040−0.110)	.36			
Antiplatelets	0.001 (-0.060−0.059)	.99			
ACE inhibitors	0.048 (-0.004−0.100)	.071	0.013 (-0.030−0.056)	0.08	.56
ARA-2	-0.031 (-0.086−0.025)	.27			
Calcium antagonists	0.024 (-0.036−0.084)	.43			
Vasodilators	-0.028 (-0.127−0.072)	.59			
β-Blockers	0.004 (-0.052−0.061)	.88			
Statins	-0.033 (0.086−0.021)	.24			
Calprotectin − μg/mL*	0.464 (0.385−0.543)	<.001	0.438 (0.348−0.528)	22.6	<.001
NLR*	0.297 (0.197−0.397)	<.001	-0.110 (-0.215− -0.005)	1.04	.040
Hs-CRP − mg/L*	0.144 (0.105−0.183)	<.001	0.022 (-0.024−0.067)	0.22	.35

B = regression coefficient B; ABI = ankle brachial index; ACE = angiotensin converting enzyme; AMI = acute myocardial infarction; ARA-2 = angiotensin II receptor antagonist; CKD = chronic kidney disease; COPD = chronic obstructive pulmonary disease; HDL = high density lipoprotein; eGFR = estimated glomerular filtration rate; hs-CRP = high-sensitivity C reactive protein; NLR = neutrophil to lymphocyte ratio; CI = confidence interval.
* Logarithmically transformed variables.

of patients were defined presenting: (1) low LCN2 and low calprotectin, (2) either high LCN2 or high calprotectin, and (3) high LCN2 and high calprotectin. As shown in Table 3, patients in group 3 presented a five-fold increase in the risk of CV death or amputation in all tested models (SHR > 5, $p < .001$ in all models), outperforming hs-CRP in model 4 (hs-CRP SHR 0.99, 95% CI 0.98 − 41.01, $p = .79$). Risk prediction with the combined variable was lower, although still statistically significant, when assessing the secondary outcome, even when eGFR and hs-CRP were included as covariables (HR 1.83, $p = .034$, model 4, Table 3).

Furthermore, addition of the combined variable showed reclassification and discrimination improvements for the primary outcome beyond other covariables (age, sex, diabetes, hypertension, dyslipidaemia, PAD severity, eGFR, and hs-CRP) in fully adjusted models (Harrell's C, $p \leq .023$; NRI, $p \leq .024$; and IDI, $p \leq .07$; Supplementary Table S2). Finally, no prognostic added value was observed to predict the secondary outcome in the fully adjusted models.

When dividing the population by LCN2 and calprotectin quartiles, patients in the fourth quartiles of both proteins (Supplementary Table S3) presented a higher risk of the primary outcome separately (SHR 3.74, $p = .005$ for LCN2; and SHR 2.64, $p = .007$ for calprotectin), and in combination (SHR 3.01, $p = .001$ for LCN2 and calprotectin fourth quartiles *vs.* the rest).

Combination of lipocalin-2 and calprotectin improves risk prediction in patients with early stage peripheral arterial disease (VIVA cohort)

In general, the levels of LCN2 and calprotectin detected in the plasma of patients from the VIVA cohort were lower than those detected in serum of patients from the CHN cohort (LCN2, median (IQR), 84 (59) ng/mL CHN and 27 (10) ng/mL VIVA; calprotectin, 3.8 (3) μg/mL CHN and 1.3 (1.1) ng/mL VIVA). To account for the potential interference of plasma components, levels of LCN2 and calprotectin were examined in serum and plasma EDTA in a small group of CHN patients with plasma and serum availability. As shown in Supplementary Fig. S3, plasma detection rendered smaller concentrations of LCN2 and calprotectin compared with serum, but there was still a statistically significant correlation found between both measurements.

To assess the prognostic value of the combination of LCN2 and calprotectin in earlier PAD stages, plasma LCN2 and

Table 3. Cox regression analyses to evaluate the associations of the combination of lipocalin-2 (LCN2) and calprotectin (Calp) with the outcomes of interest in the Complejo Hospitalario de Navarra (CHN) cohort of 331 patients with symptomatic peripheral artery disease

	CV death or amputation		All cause death or amputation	
	HR (95% CI)	*p*	HR (95% CI)	*p*
Unadjusted				
Low LCN2 and Calp	1 (reference)		1 (reference)	
High LCN2 or Calp	1.70* (0.95–3.03)	.075	1.51 (1.04–2.20)	.030
High LCN2 and Calp	5.90* (3.05–11.4)	<.001	2.71 (1.68–4.39)	<.001
Model 1				
Low LCN2 and Calp	1 (reference)		1 (reference)	
High LCN2 or Calp	1.47* (0.82–2.63)	.195	1.27 (0.87–1.85)	.22
High LCN2 and Calp	6.25* (3.22–12.1)	<.001	3.09 (1.91–5.01)	<.001
Model 2				
Low LCN2 and Calp	1 (reference)		1 (reference)	
High LCN2 or Calp	1.37* (0.77–2.43)	.290	1.25 (0.86–1.83)	.24
High LCN2 and Calp	5.48* (2.63–11.40)	<.001	2.92 (1.79–4.76)	<.001
Model 3				
Low LCN2 and Calp	1 (reference)		1 (reference)	
High LCN2 or Calp	1.21* (0.64–2.29)	.561	1.01 (0.68–1.50)	.96
High LCN2 and Calp	5.02* (2.43–10.39)	<.001	2.82 (1.72–4.62)	<.001
Model 4				
Low LCN2 and Calp	1 (reference)		1 (reference)	
High LCN2 or Calp	1.29* (0.68–2.47)	.437	0.92 (0.61–1.39)	.70
High LCN2 and Calp	5.64* (2.70–11.77)	<.001	1.83 (1.05–3.20)	.034

Categorised variables: LCN2 > 86 ng/mL, calprotectin ≥ 7.4 μg/mL. Model 1: age and sex. Model 2: age, sex, diabetes, hypertension, dyslipidaemia. Model 3: age, sex, diabetes, hypertension, dyslipidaemia, estimated glomerular filtration rate (eGFR), PAD severity (intermittent claudication/chronic limb threatening ischaemia). Model 4: age, sex, diabetes, hypertension, dyslipidaemia, eGFR, high-sensitivity C reactive protein (log transformed). CV = cardiovascular; HR = hazard ratio; CI = confidence interval.
* Sub-hazard ratio (SHR, Fine-Gray model).

calprotectin were measured in 413 men included in the Danish VIVA screening trial. The VIVA patients were of a similar mean age (70 years, Table 4) as the CHN patients, but there was a lower proportion of patients with diabetes (23% VIVA *vs.* 53% CHN) or hypertension (56% VIVA *vs.* 76% CHN). ABI was within the range of PAD diagnosis (mean ABI 0.9) but more preserved than in the CHN cohort (mean ABI 0.56, Table 1). During follow up, MALE ($n = 50$, 12%) and death, either all cause ($n = 194$, 47%) or CV ($n = 29$, 7%) were recorded defining two composite endpoints; the primary outcome CV mortality or MALE ($n = 137$, 33%), and the secondary outcome all cause death or MALE ($n = 207$, 50%).

Like in the CHN cohort, high LCN2 levels were associated with CV death or MALE (SHR > 2 for all, $p < .001$, Supplementary Table S4), and all cause mortality or MALE (HR > 2, $p < .001$ for all) in the VIVA patients with PAD.

ROC curves were performed for MALE obtaining cutoff values of > 32.9 ng/mL (41% sensitivity, 81% specificity, Youden index) for LCN2, and ≥ 1.2 μg/mL (68% sensitivity, 54%) for calprotectin, and three subgroups were defined according to the obtained cutoffs. As shown in Table 5, high levels of LCN2 and calprotectin (group 3) increased risk prediction three-fold in patients with early stage PAD when assessing either the primary or the secondary outcome ($p < .001$ for all models). In this line, the addition of the combined variable showed discrimination (Harrel's C, $p \leq .001$) and reclassification (IDI, $p \leq .001$; NRI, $p \leq .007$) improvements both for the primary and secondary outcomes in fully adjusted models (Supplementary Table S5).

VIVA patients at the fourth quartiles of LCN2 and calprotectin presented with increased risk of the primary outcome, when assessed independently (SHR 1.76, $p = .012$ for LCN2; and SHR 2.12, $p = .004$ for calprotectin) or in combination (SHR 2.11, $p = .001$ for LCN2 and calprotectin fourth quartiles *vs.* the rest, Supplementary Table S3).

DISCUSSION

This study reports an association between blood levels of two inflammatory biomarkers, LCN2 and calprotectin, that in combination might improve risk prediction for death or amputation in patients with symptomatic PAD. Moreover, both proteins were expressed in advanced atherosclerotic femoral plaques. Most interesting, however, was to find similar results in subjects at earlier stages of PAD. As such, increased levels of both biomarkers were associated with death or MALE in men of the VIVA screening trial.

Patients with PAD are at high risk of CV complications, amputation and death, while predicting outcomes remains challenging.[3] Inflammation is a crucial player in the early phases of atherogenesis and a critical factor for plaque development, rupture and thrombus formation during disease progression.[16] CRP, IL-6 and the NLR have attracted special attention as possible biomarkers for PAD, showing associations with PAD incidence, diagnosis, and

Table 4. Demographic and clinical parameters in the Viborg Vascular (VIVA) cohort of 413 patients with peripheral artery disease

	Patients (n = 413)
Demographic and clinical data	
Men	413 (100)
Age − y	70.4 (67.6, 72.6)
Smokers	
Never	43 (10.4)
Current	189 (45.8)
Former	181 (43.8)
Diabetes mellitus	95 (23.0)
Hypertension	230 (55.7)
Dyslipidaemia	246 (59.6)
ABI right	0.8 (0.6, 0.9)
ABI left	0.8 (0.6, 0.9)
Treatment	
Anticoagulants	44 (10.7)
Antiplatelets	238 (57.6)
ACE inhibitors	132 (32.0)
Angiotensin II receptor antagonist	61 (14.8)
Calcium antagonists	114 (27.6)
Vasodilators	NA
β blockers	125 (30.3)
Statins	228 (55.2)
Previous history	
Chronic obstructive pulmonary disease	24 (5.8)
Chronic kidney disease	NA
Acute myocardial infarction	24 (5.8)
Cardiomyopathy	NA
Cerebral ischaemia	NA
Laboratory data	
Total cholesterol − mmol/L	5.1 (4.4, 5.8)
LDL-C	NA
HDL-C	NA
Triglycerides	NA
eGFR − mL/min/1.73m^2	NA
Calprotectin − μg/mL	1.3 (0.8, 1.9)
NLR	NA
hs-CRP	NA
LCN2 − ng/mL	27.1 (23.1, 33.5)

Data are presented as n (%) or as median (interquartile range). ABI = ankle brachial index; ACE = angiotensin converting enzyme; LDL = low density lipoprotein; HDL = high density lipoprotein; eGFR = estimated glomerular filtration rate; hs-CRP = high-sensitivity C reactive protein; NLR = neutrophil to lymphocyte ratio; LCN2 = lipocalin-2; NA = not available.

prognosis,[3,5,17] although a consensus has not been reached for their translation into clinical practice. Moreover, growing evidence suggests that a single biomarker approach might be too simplistic to predict complex multifactorial diseases such as PAD. In a recent study from the present authors, the transcriptomic analysis of circulating EVs showed an increased expression of genes related to the immune response in lower limb PAD patients compared with controls, including LCN2 and calprotectin, supporting a possible role for sustained low grade inflammation in development and progression of atherosclerotic vascular pathologies.[6] LCN2 and the calprotectin components, S100A8 and S100A9, are expressed by macrophages, neutrophilic granulocytes, endothelial cells, and smooth muscle cells of human carotid plaques,[18–20] and appeared to be associated with rupture prone regions, while in non-pathological tissues, calprotectin is undetectable in the intima of apparently normal mural areas of endarterectomy or aortic reconstruction tissues,[21] and LCN2 is expressed in areas free of macrophages in normal mammary arteries.[22] In this regard, LCN2 and S100A9 expression was found in areas rich in inflammatory cells in advanced human femoral plaques.

There is little evidence on the role of LCN2 and calprotectin in PAD. In one report elevated levels of LCN2 were associated with CLTI,[8] and in others, calprotectin increased according to PAD severity.[23,24] Additionally, the present study group observed an association between calprotectin and CV mortality or amputation in patients with PAD, improving risk prediction when combined with hs-CRP.[6] More abundant, however, are the data concerning other CV pathologies. In this line, blood LCN2 and calprotectin levels were associated with symptomatic carotid artery stenosis[25] and disease severity in acute coronary syndrome (ACS),[26,27] and with the incidence of CV disease and all cause death in middle aged, and older subjects.[28–31] In the present study, it was found that the combination of LCN2 and calprotectin was able to improve risk evaluation for CV mortality or amputation independently of other covariables even outperforming CRP, in patients with symptomatic PAD, presenting a high frequency of concomitant comorbidities, while the association was weaker for all cause death or amputation. Importantly, increased levels of LCN2 and calprotectin were also related to a higher mortality or MALE risk, either when evaluating the primary or the secondary outcome, in men from the VIVA trial at earlier stages of the disease. These data indicate that risk evaluation in PAD, a multifactorial pathology, might benefit from the combined analysis of inflammatory molecules, involved in different physiopathological processes. As such, by assessing multiple biomarkers, it might be possible to obtain a more precise picture of the processes underlying vascular remodelling. In this regard, not only multiple marker approaches, but also the application of computational methods, such as machine learning, in future studies might provide new opportunities for classification and treatment of patients with PAD.

Limitations of the study

LCN2 and calprotectin were determined in serum in the CHN cohort, and in plasma EDTA in the VIVA cohort, rendering higher values for both proteins in serum than in plasma as already reported by other authors.[32,33] The differences in LCN2 and calprotectin levels between serum and plasma could be related to the matrix on which blood was collected. The collection of blood in serum separating tubes enables aggregation and activation of platelets to facilitate clot formation, that in turn might result in the release of pro-inflammatory proteins or coagulation products contained in platelets, but also in other blood cells, such as neutrophils. This might render elevated levels of the tested proteins when compared with plasma samples, where platelet aggregation and coagulation is prevented by the added anticoagulants, at least EDTA.[33] Nevertheless,

Table 5. Cox regression analyses to evaluate the associations between the combination of lipocalin-2 (LCN2) and calprotectin (Calp) and the outcomes of interest in the Viborg Vascular (VIVA) cohort of 413 patients with peripheral artery disease

	CV death or MALE		All cause death or MALE	
	HR (95% CI)	*p*	HR (95% CI)	*P*
Unadjusted				
Low LCN2 and Calp	1 (reference)		1 (reference)	
High LCN2 or Calp	1.65* (1.09–2.51)	.018	1.79 (1.28–2.51)	.001
High LCN2 and Calp	3.40* (2.18–5.31)	<.001	3.13 (2.17–4.51)	<.001
Model 1				
Low LCN2 and Calp	1 (reference)		1 (reference)	
High LCN2 or Calp	1.65* (1.09–2.51)	.018	1.79 (1.28–2.51)	.001
High LCN2 and Calp	3.37* (2.16–5.25)	<.001	3.11 (2.16–4.50)	<.001
Model 2				
Low LCN2 and Calp	1 (reference)		1 (reference)	
High LCN2 or Calp	1.76* (1.14–2.70)	.010	1.82 (1.30–2.57)	.001
High LCN2 and Calp	3.30* (2.07–5.24)	<.001	3.03 (2.08–4.41)	<.001

Categorised variables: LCN2 > 32.9 ng/mL, calprotectin ≥ 1.2μg/mL. Model 1: age. Model 2: age, diabetes mellitus, hypertension, dyslipidaemia. MALE = major adverse limb events; HR = hazard ratio; CI = confidence interval; CV = cardiovascular.
* Sub-hazard ratio (Fine-Gray model).

the substudy performed in patients from the CHN cohort (Supplementary Fig. S3), where serum and plasma EDTA were obtained at the same time, showed a positive correlation for LCN2 and calprotectin in both biological fluids. Measuring LCN2 and calprotectin in a single blood sample does not allow for assessing the stability of these variables over time and an effect of long term storage on LCN2 and calprotectin stability cannot be ruled out. In this regard, no statistical differences were found when analysing LCN2 and calprotectin levels by recruitment year (Supplementary Fig. S4). Amputation incidence in the CHN cohort was low (8%); therefore, multivariable Cox regression analysis could not be performed for this outcome independently. Nevertheless, the percentage of events for the combined outcomes CV death or amputation (20%) and all cause death or amputation (41%) provides the statistical power required to support the conclusions. LCN2 has been postulated as a marker of chronic kidney disease (CKD), a pathology with high prevalence in the CHN population (41%); however, the association between LCN2 and calprotectin and the tested outcomes remained statistically significant even when eGFR was included in the model. The VIVA cohort is based on a screening trial, and consequently, patients were diagnosed at earlier stages of PAD and presented a low frequency of comorbidities. Some of the clinical and biochemical parameters were not recorded at the time of recruitment, including those assessing renal function. However, being a screening trial and considering the low percentage of people with diabetes, a minimal number of subjects with CKD could be expected. If any, they might be at stages I or II of the disease presenting preserved renal function. In this line, amputations in the VIVA patients were rare; thus, major lower limb events was considered as the outcome. The VIVA trial included exclusively men.[9,10] It cannot be determined whether the elevated levels of LCN2 and calprotectin are cause or consequence of a heavier atherosclerotic burden. As such, no causal relationship between high levels of the studied proteins and the analysed outcomes can be inferred from this study, and no consensual cutoffs for LCN2 and calprotectin can be defined. Finally, the administration of different therapy regimens during follow up may have influenced the study result.

Conclusions

An association is described between increased levels of LCN2 and calprotectin with worse outcomes in symptomatic PAD, and in patients at earlier pathological stages. The results suggest the possible role of the inflammatory biomarkers, LCN2 and calprotectin, for risk stratification in PAD at the early and severe stages. Moreover, risk assessment increased when LCN2 and calprotectin were combined, supporting the usefulness of multiple marker approaches to evaluate multifactorial pathologies. Nonetheless, future mechanistic and prospective studies must be performed to confirm the results of the present study.

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CONFLICT OF INTEREST

None.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejvs.2022.01.012.

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