Clinical Biochemistry 50 (2017) 1048–1055

Contents lists available at ScienceDirect

Clinical Biochemistry

journal homepage: www.elsevier.com/locate/clinbiochem

Relation between serum levels of chemotaxis-related factors and the presence of coronary artery calcification as expression of subclinical atherosclerosis

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ARTICLE INFO

Keywords:
Coronary artery calcification
Atherosclerosis
Biomarkers
Netrin-1
Gremlin-1
Macrophage inflammatory protein-1β

ABSTRACT

Background: Atherosclerotic plaque formation is characterized by recruitment of monocytes/macrophages, which contributes to its calcification by releasing pro-osteogenic cytokines. Chemotaxis-related proteins, including netrin-1, gremlin-1 and macrophage inflammatory protein-1β (MIP-1β), regulate immune cell migration. However, their relation with the presence of subclinical atherosclerosis, assessed by measures of coronary artery calcifications (CAC) in patients without known coronary artery disease (CAD), remains unclear.

Aims: To examine whether these chemoattractant-related proteins are associated with the presence of CAC in patients without known CAD.

Methods: A retrospective case-control observational study was conducted in 120 outpatients without CAD, undergoing a CAC evaluation by computed tomography with the Agatston Calcium score, categorized as CAC^- (none) and CAC^+ (≥ 1). Serum biomarkers were quantified by ELISA.

Results: Lpa, dyslipidaemia and smoking were significantly higher ($p = 0.006$, $p \leq 0.0001$ and $p = 0.001$, respectively) in CAC^+ patients. Serum netrin-1 levels were lower in CAC^+ than in CAC^- patients (196.8 ± 127.8 pg/ml versus 748.3 ± 103.2 pg/ml, $p \leq 0.0001$), and a similar pattern was found for gremlin-1 (1.14 ± 0.39 ng/ml versus 4.33 ± 1.20 ng/ml, $p \leq 0.0001$). However, TNFα and MIP-1β were strongly upregulated in CAC^+ patients (447.56 ± 74 pg/ml versus 1104 ± 144 pg/ml and 402.00 ± 94 pg/ml versus 905.0 ± 101.6 pg/ml, respectively, $p \leq 0.001$). Multivariate analyses revealed that low netrin-1 and gremlin-1 levels and high TNFα and MIP-1β amounts were associated with CAC presence, after adjustment for clinical and biochemical variables.

Conclusions: We found a netrin-1 and gremlin-1 deficiency and a TNFα and MIP-1β overproduction in CAC^+ patients' serum. These proteins may be used to identify individuals with subclinical atherosclerosis. Further research is warranted in a larger cohort of patients to establish these chemotactic-related proteins as biomarkers that improve CAD risk stratification.

1. Introduction

Cardiovascular atherosclerosis is the primary cause of morbidity and mortality in industrialized societies. Atherosclerosis is a chronic immuno-inflammatory disease characterized by the accumulation of lipids, inflammatory cells and fibrous elements in adolescence that frequently remains clinically dormant due to preservation of the arterial lumen [1,2]. However, some lesions undergo necrotic breakdown that leads to abrupt thrombosis and triggers myocardial infarction and stroke, as the first clinical presentation [3,4]. This grim reality is, at least in part, due to the lack of markers that accurately identify active atherosclerotic disease before complications occur. The identification of

Abbreviations: CAC, coronary artery calcifications; CAD, coronary artery disease; VSMC, vascular smooth muscle cells; MDCT, multiple detector computed tomography; TNFα, tumor necrosis factor α; MIP-1β, macrophage inflammatory protein-1β

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http://dx.doi.org/10.1016/j.clinbiochem.2017.08.012
Received 10 April 2017; Received in revised form 2 August 2017; Accepted 19 August 2017
Available online 19 August 2017

subjects at risk of such events is obviously important for implementing effective preventive measures. CAC is a non-invasive marker of subclinical vascular disease [5,6]. CAC quantity, measured via multiple detector computed tomography (MDCT) using the Agatston Score, correlates with increased burden of atherosclerotic plaque in the coronary arteries and is a predictor of future cardiovascular events [7].

Vascular calcification is a cell-regulated process that reflects an osteochondrogenic transformation of vascular smooth muscle cells (VSMC) and is associated with macrophage infiltration. The recruited macrophages may promote VSMC osteochondrogenic differentiation [8] through the secretion of pro-calcific cytokines. Diverse molecules acting as chemoattractans, chemorepellents or chemoattractant blocking agents may direct cell movement towards or away from the vessel wall, affecting this process [9,10]. In this regard, proteins such as macrophage inflammatory protein-1β (MIP-1β), gremlin-1 and netrin-1 have received particular attention in CAD patients because of their chemotaxis-regulatory effect on inflammatory cells; however, the relationship between these markers and the existence of detectable CAC (CAC score $>$ 0) in asymptomatic individuals has never been studied [11–14]. Therefore, our study aims to examine associations between the presence of CAC, quantified by MDCT, and a panel of chemotaxis-related factors that have recently been suggested to influence the development and progression of atherosclerosis [15–17].

We a priori hypothesized that elevated serum levels of the pro-inflammatory chemokine-like MIP-1β are significantly associated with the presence of CAC while proteins like netrin-1 and gremlin-1 with presumably anti-inflammatory functions such as inhibition of chemokine-mediated leukocyte migration and modulation of leukocyte accumulation into arterial walls were expected to be reduced in serum of patients with CAC. Tumor necrosis factor α (TNFα) levels were also evaluated as a reference inflammatory cytokine [18].

2. Material and methods

2.1. Study population

A retrospective case-control observational study was performed in 120 outpatients attending the Cardiology Department of the Hospital Universitario Rio Hortega de Valladolid. The study was approved by the Hospital Ethics Committee. All participants gave written informed consent. Exclusion criteria included impaired renal or liver function, cancer, inflammatory diseases or lack of patient consent.

Included subjects had no previous history of cardiovascular disease (heart failure, CAD or stroke). A clinical indication for performing coronary MDCT was established according to clinical criteria (ruling out CAD in patients with atypical chest pain, no definitive functional tests with a low to moderate risk of developing ischemic heart disease).

All participants underwent a complete medical examination and anthropometric measurements were taken.

2.2. Computed tomography image acquisition protocol and coronary artery calcium scoring

Subjects underwent prospectively ECG-triggered cardiac MDCT (Sensation 64, Siemens Medical Solutions, Forchheim, Germany). Data were reconstructed with 3 mm slice thickness. An experienced reader quantified the amount of coronary artery calcium using dedicated software (CaScore, Siemens). Calcified lesions were identified as areas of at least 130 Hounsfield Units (HU) attenuation. The Agatston score was computed as previously described [7]. Calcium score in Agatston units was calculated for each calcified lesion and the scores were summed across all lesions within a given artery and across all arteries to obtain the total calcium score.

Fig. S1 corresponds to representative images of non-calcified and calcified left anterior descending coronary artery.

2.3. Case/control groups

According to cardiac MDCT analysis, cases were defined as patients with coronary artery calcification (CAC^+, Agatston Score above 0) and control consisted of patients without calcifications (CAC^-, Agatston Score = 0).

To assess the effect of the presence of calcium on coronary arteries in each of the selected chemotaxis-related proteins, CAC was categorized in tertiles (none, 0; medium, above 0–399; and high, ≥ 400).

2.4. Biochemical analyses

Blood samples were collected in Vacutainer® tubes and allowed to clot before centrifugation at 1000 × *g*, 20 min and 4 °C. The supernatants (serum) were transferred into a polypropylene tube and stored in aliquots at − 80 °C.

Serum lipid profile (total cholesterol, LDL and HDL cholesterol, triglycerides, Apo B, ApoA1, Lpa), and general biochemical parameters (creatinine, bilirubin, c-troponin I, CRP) were measured in the Department of Clinical Analysis of the Hospital Universitario Rio Hortega, according to its routine protocols using validated assays, periodically tested and calibrated. Cholesterol, TG, HDL, CRP, Creatinine and Bilirubin were assayed on a Beckman Coulter AU5400 Chemistry Analyzer (Beckman Coulter, California, USA). LDL cholesterol was calculated using a Friedewald formula [19]. Apo A1, Apo B and Lpa concentrations were determined by nephelometry on a Beckman Coulter IMMAGE 800 Immunochemistry System (Beckman Coulter, California, USA). Cardiac Troponin I was determined using a paramagnetic particle chemiluminescent immunoassay on a Unicel DxI800 Immunoassay Analyzer (Beckman Coulter, California, USA), the upper limit of normal value (ULN, 99th percentile of healthy controls) was 0.04 ng/ml.

Serum biomarkers related to CAC were measured by commercial ELISA according to the manufacturer's instructions. All samples were assayed in duplicate. For some assays, serum was diluted as indicated so that the values would not exceed those of the standard curve. TNFα ELISA kit from Abnova (Heidelberg, Germany) was employed – inter and intra-assay variations were < 8.1% and 7.7%, respectively. The detection limit was 5.1 pg/ml. The detection range was 23–1500 pg/ml. Serum was diluted 1:1. MIP-1β ELISA kit from ENDOGEN (Massachusetts, USA) was employed – inter and intra-assay variations were < 10% and 10%, respectively. The detection limit was 4 pg/ml. The detection linear range was 9–600 pg/ml. Serum was diluted 1:4. Netrin-1 ELISA kit from CUSABIO (Wuhan, China) was employed – inter and intra-assay variations were < 10% and 8%, respectively. The detection limit was 7.81 pg/ml. The detection range was: 31.25–2000 pg/ml. Gremlin-1 ELISA kit from YH Biosearch Laboratory (Shanghai, China) was employed – inter and intra-assay variations were < 12% and 10%, respectively. The detection limit was 50 pg/ml. The detection range was: 0.1–16 ng/ml. Serum was used undiluted.

2.5. Statistical analysis

Continuous variables were expressed as mean ± standard deviation (SD) and categorical variables such as percentage. For the analysis of differences between CAC^- and CAC^+ patients, we used the Student *t*-test for continuous variables and the Chi-square test for categorical variables. Differences in TNFα, MIP-1β, gremlin-1 and netrin-1 levels were assessed according to CAC categories (none, above 0–399, > 400) by ANOVA, followed by Bonferroni test for post hoc comparisons between groups. To investigate the relationships among biomarkers we used Pearson correlation test. Multiple linear regression analyses were used to examine associations between TNFα, MIP-1β and netrin-1 levels and coronary calcification expressed by calcium score adjusted by clinical and biochemical variables included in the study. The B coefficient (unstandardized) and its 95% confidence interval were calculated.

Table 1
Baseline characteristics of patient population.

Demographic data	
Number (M/F)	120 (61/59)
Age (yrs)	60 ± 11
Age range (yrs)	33–82
BMI (kg/m^2)	29.46 ± 10.14
Waist circumference	99.70 ± 12.77
Coronary risks factors	
Current smoking	42 (35%)
Type 2 diabetes	21 (17.5%)
Hypertension	61 (50.8%)
Dyslipidaemia	57 (47.5%)
Use of statins	41 (34.2%)
Serum markers	
Cholesterol (mg/dl)	215.17 ± 39.38
Triglycerides (mg/dl)	131.59 ± 76.38
HDL (mg/dl)	53.18 ± 12.99
LDL (mg/dl)	135.21 ± 35.61
ApoA1 (mg/dl)	148.08 ± 36.44
ApoB (mg/dl)	85.84 ± 29.91
ApoB/ApoA1	0.589 ± 0.16
Lpa (mg/dl)	43.09 ± 48.75
CRP (mg/dl)	0.373 ± 0.39
Creatinine (mg/dl)	0.92 ± 0.19
Bilirubin (mg/dl)	0.74 ± 0.36
c-Troponin I (ng/ml)	0.012 ± 0.013
TNFα (pg/ml)	830.58 ± 346.53
MIPβ (pg/ml)	677.44 ± 277.60
Gremlin-1 (ng/ml)	2.47 ± 1.78
Netrin-1 (pg/ml)	426.59 ± 297.35
Coronary calcification (Agatston)	
CAC score	415.80 ± 977.91
Number CAC score = 0	50 (41.7%)
Number CAC score > 1 ≤ 399	42 (35.0%)
Number CAC score ≥ 400	28 (23.3%)

Hypertension defined per JNC-VII criteria (SBP ≥ 140, DBP ≥ 90 or being treated). Type 2 diabetes defined according to American Diabetes Association guidelines (fasting glucose ≥ 126 mg/dl or being treated).

Table 2
Clinical characteristics and biomarker levels by CAC score. Univariate analysis.

	CAC$^-$	CAC$^+$	p Value
	(none)	(≥ 1)	
	n = 50	n = 70	
Variable			
Sex (% male)	46.0	54.3	0.373
Age (yrs)	56 ± 11	63 ± 10	0.002
BMI (kg/m^2)	28.83 ± 4.28	29.91 ± 12.79	0.567
Waist circumference	98.32 ± 12.16	100.69 ± 13.19	0.319
Coronary risks factors			
Current smoking	18.0%	47.1%	0.001
Type 2 diabetes	20.0%	15.7%	0.628
Hypertension	44.0%	55.7%	0.267
Dyslipidaemia	24.0%	64.3%	< 0.001
Use of statins	58.3%	68.9%	0.385
Serum markers			
Cholesterol (mg/dl)	209.43 ± 30.95	219.10 ± 44.03	0.192
Triglycerides (mg/dl)	121.91 ± 74.07	138.22 ± 77.75	0.256
HDL (mg/dl)	55.00 ± 10.61	51.94 ± 12.80	0.175
LDL (mg/dl)	130.64 ± 27.78	138.34 ± 40.00	0.250
ApoA1	148.79 ± 38.47	147.57 ± 35.19	0.857
ApoB	86.89 ± 31.64	85.10 ± 28.82	0.747
ApoB/ApoA1	0.596 ± 0.17	0.584 ± 0.17	0.823
Lpa (mg/dl)	28.82 ± 30.50	53.28 ± 56.44	0.006
CRP	0.39 ± 0.48	0.35 ± 0.30	0.621
Creatinine (mg/dl)	0.91 ± 0.18	0.93 ± 0.20	0.578
Bilirubin (mg/dl)	0.80 ± 0.45	0.70 ± 0.28	0.140
c-Troponin I (ng/ml)	0.012 ± 0.009	0.013 ± 0.015	0.486
TNFα (pg/ml)	447.56 ± 74.24	1104.17 ± 144.69	< 0.001
MIPβ (pg/ml)	402.00 ± 94.33	905.00 ± 101.60	< 0.001
Gremlin-1 (ng/ml)	4.33 ± 1.20	1.14 ± 0.39	< 0.001
Netrin-1 (pg/ml)	748.32 ± 103.23	196.78 ± 127.81	< 0.001

Hypertension defined per JNC-VII criteria (SBP ≥ 140, DBP ≥ 90 or being treated). Type 2 diabetes defined according to American Diabetes Association guidelines (fasting glucose ≥ 126 mg/dl or being treated). Statistically significance $p < 0.05$.

$P < 0.05$ was considered statistically significant. The SPSS version 12.0 (SPSS Inc., Illinois, USA) was used for statistical analyses.

3. Results

3.1. Characteristics of study subjects

A total of 120 patients were included. The mean age was 60 years (range 33–82 years), with 50.8% being male. The demographic and clinical characteristics of the study population are summarized in Table 1. Forty-seven percent had dyslipidaemia, 50.8% had hypertension, 17.5% had type 2 diabetes and current smokers represented 35%.

According to the result of CAC measure (Agatston score), patients were divided in two groups: CAC$^-$ (no calcium, Score = 0) and CAC$^+$ (Score ≥ 1). Table 2 displays the clinical characteristics of age, sex, risk factors, by absence of CAC versus presence of CAC.

These CAC$^+$ and CAC$^-$ groups were not significantly different with respect to gender, although patients with higher CAC levels were more likely to be men. Patients with coronary calcium were older (63 ± 13 versus 56 ± 11 years), with a higher proportion of current smoking and dyslipidaemia and similar presence of treated type 2 diabetes and hypertension. Due to the treatment with statins in 68,9% of patients with CAC$^+$ and hypercholesterolemia, we found a minimally higher concentration of cholesterol, triglycerides and LDL as well as lower HDL, although these differences failed to reach statistical significance.

The ApoB/ApoA1 ratio, a marker of cardiovascular risk that reflects the balance between atherogenic and antiatherogenic particles, did not differ between both groups and was lower than the cut-off value of 0.9 [20]. In each group, only a total of 0.8% of the analysed subject had the ApoB/ApoA1 ratio that exceeded 0.9. Further, CAC + hipercholesterolemic patients treated with statins did not have a significantly different ApoB/ApoA1 ratio than untreated ones ($p = 0.759$).

Due to the patients profile (asymptomatic individuals with a low risk of developing ischemic coronary event), serum concentrations of biomarkers of acute disease including CRP and c-Troponin I were within the normal range in all subjects and no significant differences were observed between the CAC$^-$ and CAC$^+$ groups (Table 2).

3.2. Association of selected plasma biomarkers with coronary artery calcification. Univariate analysis

We observed that netrin-1 and gremlin-1 concentrations were significantly lower in the CAC$^+$ group that in the CAC$^-$ ones. In contrast, the concentrations of the proinflammatory cytokine TNFα and of the chemoattractant MIP-1β were significantly higher in those patients with CAC$^+$ compared to those with CAC$^-$ (Table 2). The circulating concentrations of netrin-1 and gremlin-1 in serum from CAC$^-$ subjects were 748.32 pg/ml [range: 530.7–931.7] and 4.32 ng/ml [range: 1.93–6.61], respectively, whereas in serum from CAC$^+$ patients netrin-1 and gremlin-1 decreased to 196.78 pg/ml [range: 3.08–505.8] and 1.14 ng/ml [range: 0.41–1.79], respectively. In contrast, circulating TNFα and MIP-1β were lower in serum from CAC$^-$ patients (447.55 pg/ml [range: 269.45–579.15] and 402 pg/ml [range: 266.1–611.3], respectively); compared to serum from CAC$^+$ patients, (1104 pg/ml [range: 969.00–1489.00] and 905.00 pg/ml [range: 690.50–1082.00], respectively). No significant gender-specific difference was observed in any marker (Fig. 1 A-D).

We also evaluated the inflammatory marker CRP, and the cardiovascular risk marker ApoB/ApoA1 ratio in this population and no correlation with the presence of arterial calcification or with serum netrin-1, gremlin-1, TNFα or MIP-1β concentrations were found. There was no relation between serum netrin-1 levels and hypertension,

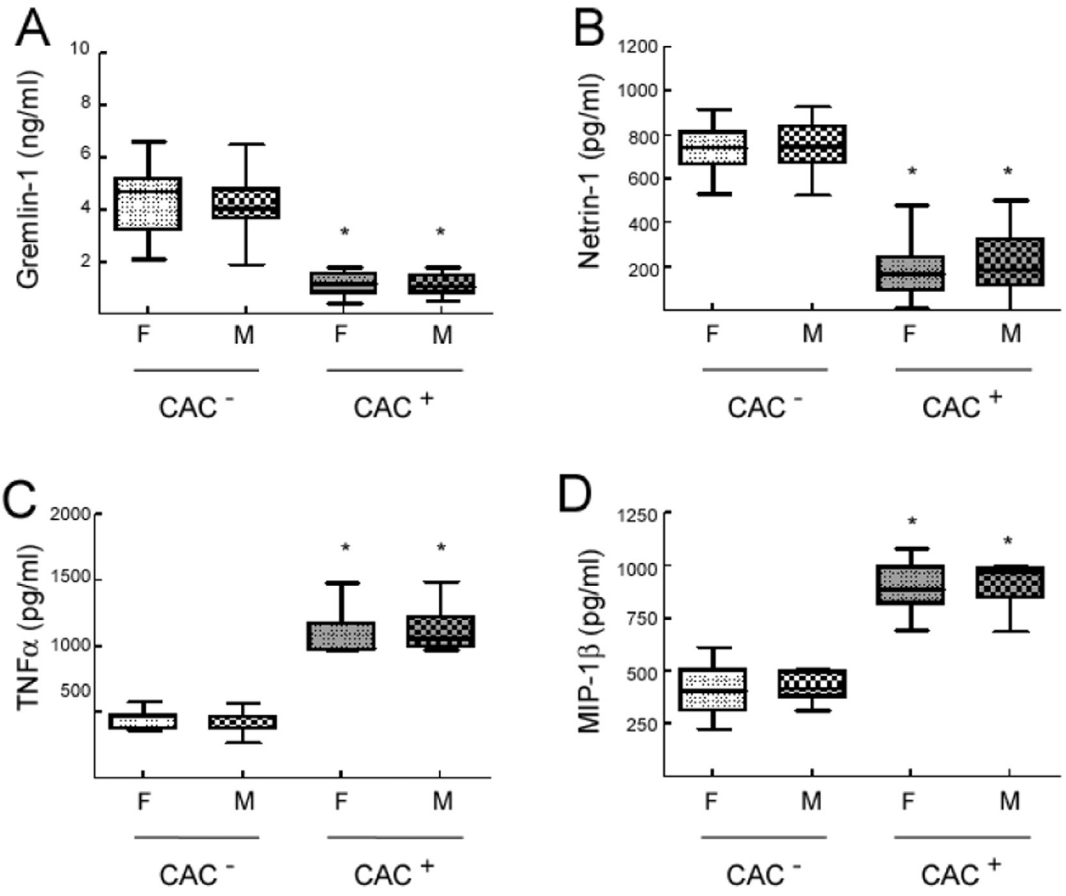

Fig. 1. Boxplots of gremlin-1, netrin-1, TNFα and MIP-1β serum levels for the CAC^- and CAC^+ groups. Levels of (A) gremlin-1, (B) netrin-1, (C) TNFα and (D) MIP-1β in serum of patients with (CAC^+) or without (CAC^-) coronary artery calcium. Data from female CAC^-: white dotted box, $n = 27$; male CAC^-: white gridded box, $n = 23$; female CAC^+: grey dotted box, $n = 32$; male CAC^+: dark gridded box, $n = 38$. Boxes show medians and interquartile ranges. *$p \leq 0.001$ CAC^+ versus CAC^-.

diabetes, lipids or lipoproteins.

3.3. Association of biomarkers with the coronary artery calcification extent

Next, we looked for a relationship between the distinct selected biomarkers and the extent of coronary artery calcifications. As shown in Fig. 2 A–D, variations in the biomarkers concentrations were determined by the presence of coronary artery calcifications but not by calcium content since no differences were observed in the concentrations of these markers between patients in medium or high CAC tertiles. Furthermore, in the subgroup of CAC^+ patients, the biomarkers netrin-1, gremlin-1, TNFα or MIP-1β did not show correlation with the amount of coronary calcification ($r = 0.069$, $p = 0.567$; $r = 0.211$, $p = 0.079$; $r = 0.088$, $p = 0.469$; $r = 0.008$, $p = 0.958$, respectively).

3.4. Intercorrelation between serum biomarkers

The relationships among individual chemotactic-related markers concentrations were examined using Pearson's rank correlational analyses. In the total study population, netrin-1 and gremlin-1 were both inversely and significantly correlated with the inflammatory cytokine TNFα ($r = -0.853$, $p \leq 0.0001$ and $r = -0.816$, $p \leq 0.0001$, respectively); similarly, a significant negative correlation between netrin-1 and gremlin-1 with the chemoattractant MIP-1β was observed ($r = -0.775$, $p \leq 0.0001$; and $r = -0.781$, $p \leq 0.0001$; respectively), whereas gremlin-1 levels correlated positively and significantly with those of netrin-1 ($r = 0.81$, $p \leq 0.0001$). To visually represent the relationship between the individual biomarkers, their serum concentrations were plotted pair-wise. Curiously, as shown in Fig. 3, two clusters rather than a correlation was observed, reflecting the presence (orange circle) and the absence (red circle) of CAC. When the subgroups (CAC^- and CAC^+) were separately analysed, no significant correlations were observed. The pairwise correlation analysis between the inflammatory biomarkers netrin-1, gremlin-1, MIP-1β, TNFα, and CRP in the CAC^+ group are summarized in Table 3. Only a trend towards a significantly weak correlation between gremlin-1 and the traditional marker of inflammation CRP was observed.

We also evaluated the ApoB/ApoA1 ratio in this population and no correlation with the presence of arterial calcification or with serum netrin-1, gremlin-1, TNFα or MIP-1β levels were found.

3.5. Association of selected serum biomarkers with coronary artery calcification. Multivariate analysis

According to multivariate linear regression analysis, only the presence of CAC was a significant and independent predictor of serum netrin-1, gremlin-1, MIP-1β and TNFα concentrations (Table 4) after adjustment for anthropometrical variables (age, gender, BMI), traditional cardiovascular risk factors (dyslipidemia, hypertension, diabetes and smoking) and lipid profile (LDL and HDL cholesterol, triglycerides, Apo B, ApoA1, Lpa). The CAC^+ score was significantly associated with low concentrations of netrin-1 (B: −564.35; CI95%: −620.25 to −508.46; $p \leq 0.0001$) and gremlin-1 (B:-3.16; CI95%:-3.53 to −2.79; $p \leq 0.0001$), as well as with high levels of TNFα (B:656.18; CI95%:598.67 to 713.69; $p \leq 0.001$); and MIP-1β (B:537.87; CI95%:456.63 to 619.12; $p \leq 0.0001$).

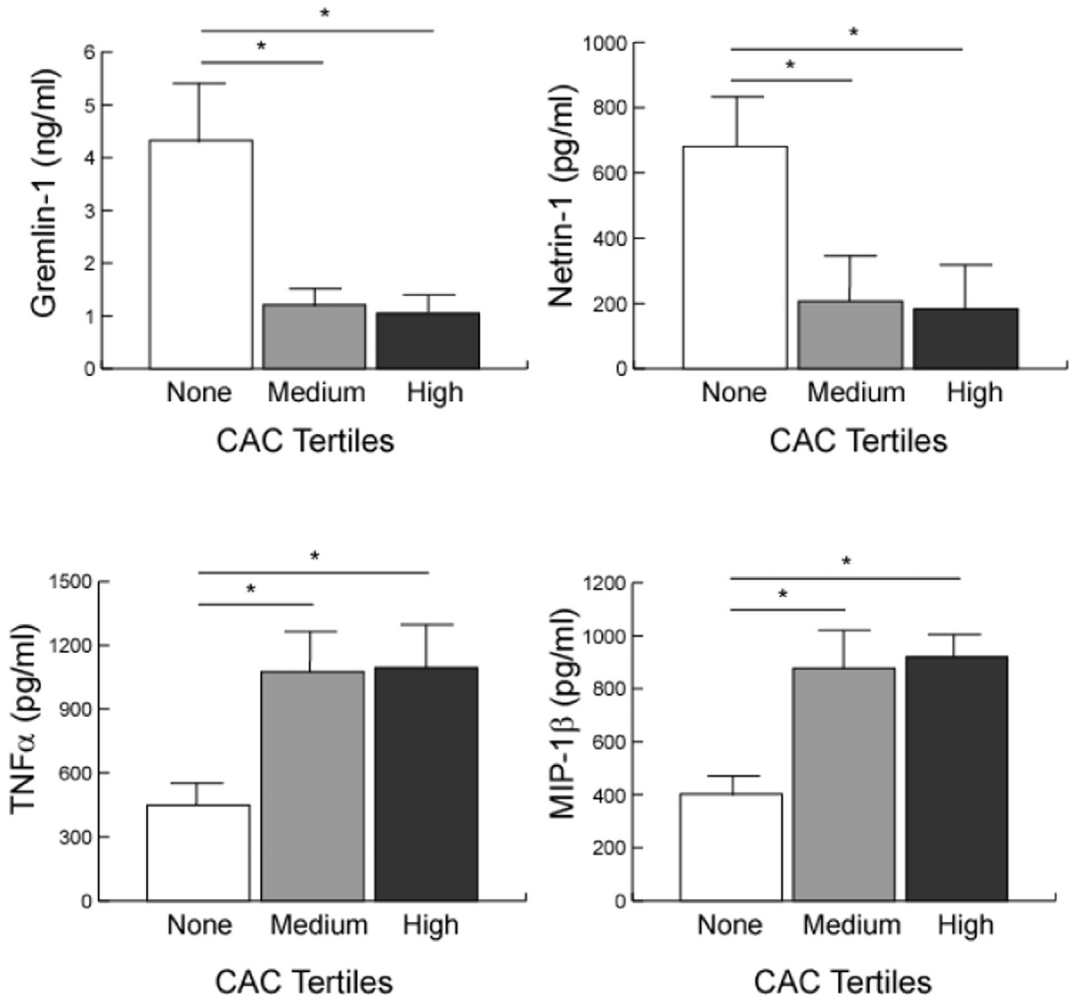

Fig. 2. Distribution of netrin-1, gremlin-1, TNFα and MIP-1β levels by CAC Tertiles. Mean netrin-1, gremlin-1, TNFα and MIP-1β levels are shown according to CAC category. CAC tertiles were defined as none calcium, 0; medium, ≥ 1 to 399; and high, ≥ 400. *$p \leq 0.0001$ medium and high CAC versus none CAC. There were no significant differences between the intermediate and highest CAC tertiles.

4. Discussion

The aim of this observational study was to identify novel risk factors/markers for coronary atherosclerosis in the clinically asymptomatic phase. For this proposal, we analysed a panel of serum proteins related to cell recruitment into tissues - netrin-1, gremlin-1, TNFα and MIP-1β - in patients without previous history of CAD, and we try to identify whether some of them could be associated with the presence of clinically silent atherosclerosis, as measured by the CAC score.

Our findings demonstrated that elevated concentrations of MIP-1β and TNFα and low concentrations of netrin-1 and gremlin-1 in serum were associated with the presence of CAC, after multivariate analyses. Nevertheless, netrin-1, gremlin-1, TNFα and MIP-1β were not associated with each other. The correlation among these inflammatory markers was absent, suggesting that they might reflect different aspects of inflammatory response or different inflammation-related functional (sub) pathways linked to the CAC process. Neither patient gender nor their age or clinical characteristics significantly influenced netrin-1, gremlin-1, MIP-1β and TNFα concentrations.

Netrin-1 is a neuronal guidance protein that outside of the central nervous system modulates leukocyte migration in a broad range of situations. Numerous studies have documented that netrin-1 is dysregulated in cardiac and kidney injuries, being detected in damaged tissues and biological fluids [21,22]. As far as we know there is not much information about netrin-1 in the systemic circulation in disorders related to CAD [14,17,23,24]. Some investigations in pre-clinical models have associated reduced serum netrin-1 concentrations with neutrophil infiltration and tissue damage [25,26]. In the context of cardiovascular diseases, several studies have found that circulating netrin-1 may act as a potent protective and therapeutic agent [16,27].

Here we observed negative associations between netrin-1 and the presence of CAC. In line with such observations is the fact that studies in ApoE$^{-/-}$ mice demonstrated that upregulation of netrin-1 at systemic level leads to reduced arterial accumulation of monocytes, as well as small lesion size and minus cytokines and chemokines production [16,17,28]. In contrast, genetic and pharmacological inhibition of circulating netrin-1 enhances monocyte trafficking into atherosclerotic lesions. Therefore, we proposed that low netrin-1 concentrations could be favouring immune cell entry into the arterial wall and increasing local inflammation, which contribute to plaque formation and arterial calcification. Interestingly, low netrin-1 concentrations have been associated in recent studies with obesity and type 2 diabetes, which are recognized as risk factors for atherosclerosis [29].

Noteworthy is the fact that netrin-1 within the atherosclerotic plaque, may inhibit both macrophage egress and/or monocyte ingress into atherosclerotic lesions, depending on its cellular source and the disease stage [17,24]. Nevertheless, in the periphery, at systemic level, netrin-1 inhibits inflammation by reducing circulating leukocyte recruitment into tissues.

Contrary to netrin-1, we have found high TNFα concentrations in serum from CAC^{+} patients. Previous studies have associated the presence of CAC with elevated TNFα concentrations in various medical

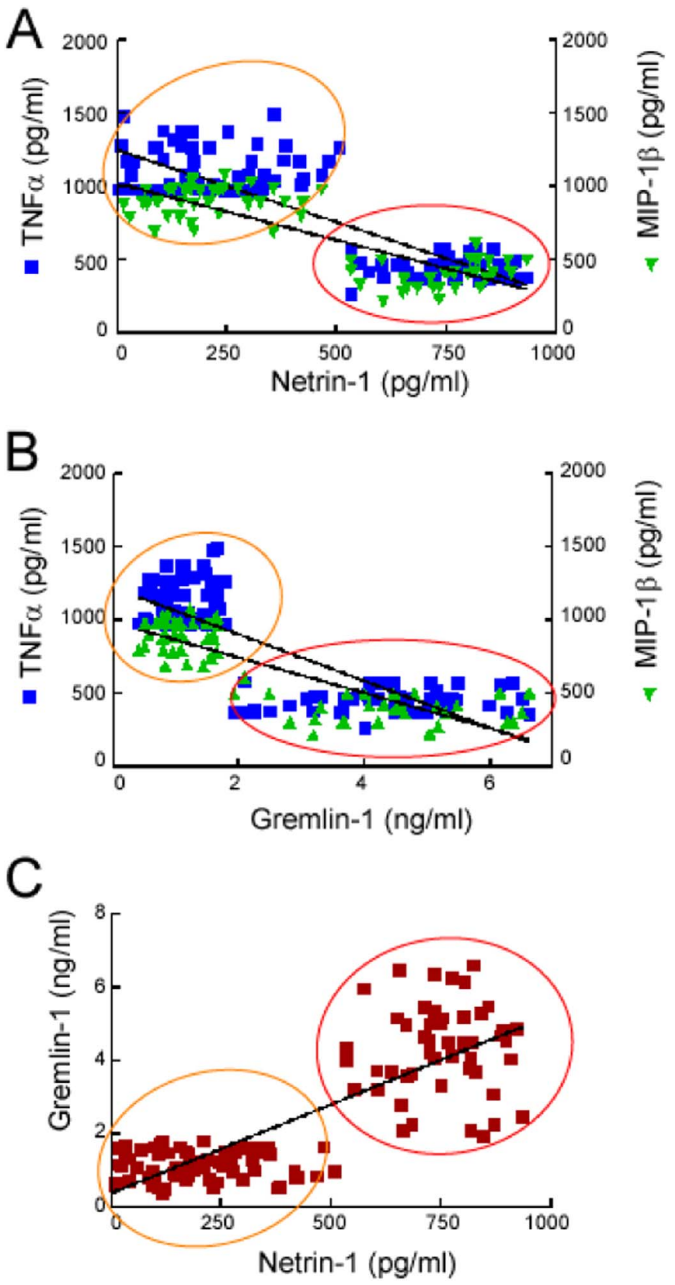

Fig. 3. Correlation of serum biomarkers in the study populations. Scatter plots comparing: (A) TNFα or MIP-1ß concentrations with netrin-1 concentrations; (B) TNFα or MIP-1ß concentrations with gremlin-1 concentrations; (C) gremlin-1 concentrations with netrin-1 concentrations.

conditions that can contribute to CAD [30–32]. However, there is not much information available about the association between TNFα and coronary calcium in asymptomatic subjects without known CAD.

A negative correlation between the circulating concentrations of TNFα and netrin-1 has previously been highlighted in experimental models of infection, where there is a rapid cell recruitment. Accordingly, in vitro studies have also shown that TNFα reduces netrin-1 expression in vascular endothelium [26]. These data taken together point to a putative role for TNFα in the down-regulation of netrin-1, a mechanism that maybe mediating, at least in part, leukocyte infiltration into tissues.

Another novel finding of our study was that CAC^+ patients had lower gremlin-1 serum levels than those who were CAC^-. Gremlin-1 may act as a chemoattractant blocking factor. Thus, its reduction, like

Table 3
Pair-wise correlations among biomarkers in patients with CAC^+.

	Gremlin-1	Netrin-1	TNFα	MIP-1β	CRP
Gremlin-1					
Coefficient r	1	− 0.051	0.055	0.013	0.226
p Value		0.675	0.650	0.936	0.059
Netrin-1					
Coefficient r	− 0.051	1	0,046	0,063	− 0.047
p Value	0.675		0.706	0.700	0.699
TNFα					
Coefficient r	0.055	0.046	1	0.095	− 0.108
p Value	0.650	0.706		0.559	0.373
MIP-1β					
Coefficient r	0.013	0.063	0.095	1	− 0.081
p Value	0.936	0.700	0.559		0.621
CRP					
Coefficient r	0.226	− 0.047	− 0.108	− 0.081	1
p Value	0.059	0.699	0.373	0.621	

Statistical significance $p < 0.05$.

Table 4
Association of selected serum biomarkers with CAC score. Multivariate analysis.

	Variable	B	CI (95%)	*p* Value
Netrin-1	CAC^+	− 564.35	− 620.25 to − 508.46	≤ 0.0001
Gremlin-1	CAC^+	− 3.16	− 3.53 to − 2.79	≤ 0.0001
TNFα	CAC^+	656.18	598.67 to 713.69	≤ 0.0001
MIP-1β	CAC^+	537.87	456.63 to 619.12	≤ 0.0001
	Apo A1	1.70	0.44 to 2.95	0.009
	Lp a	− 0.61	− 1.29 to − 0.08	0.081

CI, confidence interval; CAC^+, coronary artery calcium ≥ 1. Statistical significance, $p < 0.05$.

that of the netrin, could be favouring immune cell entry into the arterial wall. Cellular assays have shown that gremlin-1 attenuates monocyte migration and adhesion to activated endothelium [12,33]. Gremlin-1 also counteracted several chemoattractant-dependent effects on monocytes and reduced release of TNFα from MIF-activated macrophages. In addition, Gremlin-1 administration to $ApoE^{-/-}$ mice reduced lesion formation, lesional macrophage content and the production of TNFα [12,15]. Consistent with this latter finding is our observation of an inverse correlation between gremlin-1 and TNFα in the serum of our study population. Thus, it is tempting to speculate that gremlin-1 reduction associated to TNFα increase contributes to a pro-inflammatory state leading to CAC.

However, a recent study has shown that symptomatic CAD patients have high gremlin-1 serum levels [13]. This may be indicating a different gremlin-1 regulation/role in asymptomatic versus symptomatic CAD. It can thus be hypothesized that reduced gremlin-1 levels in asymptomatic subjects points to an early stage of the atherosclerotic process, where its absence may be facilitating leukocytes trans-endothelial migration. Conversely, its enhanced levels in symptomatic CAD points to a protective effect in advance stages of the disease, where its presence may be counterbalancing the pro-inflammatory/pro-thrombotic action of chemotactic factors present in atherosclerotic lesions. Therefore, it can be speculated that it may be possible to differentiate the stage and acuity of CAD based on expression levels of gremlin-1.

Finally, the other mediator analysed, MIP-1β, was also associated with the presence of CAC. In various diseases, including atherosclerosis, MIP-1β has been correlated with the recruitment of certain leukocyte subsets into the inflamed tissue [34–36]. Heightened MIP expression has been found in serum of patients with atherosclerosis, as well as in VSMC and macrophages of the atherosclerotic plaque [34]. Conversely, MIP-1β receptor blockage reduces atherosclerotic lesion formation and

macrophage infiltration [37]. Plasma MIP-1β concentrations have recently been shown to possess predictive value for adverse major events in patients with either intermediate coronary artery lesions or hypertension [11,38,39]. Moreover, high plasma concentrations of MIP-1β and TNF-α, have been associated with the presence of inflamed plaques. Despite these reports, information about whether MIP-1β may predict the presence of clinically asymptomatic CAC is lacking. Our findings confirm the up-regulated presence of MIP-1β in a relatively early stage of atherosclerotic vascular disease – i.e. the clinically silent phase.

The fact that CAC^+ patients showed increased serum MIP-1β and TNFα concentrations but decreased netrin-1 and gremlin-1 expression suggests that the observed immune activation does not represent a nonspecific inflammatory response, rather being more specific in nature.

5. Conclusions

This population-based study shows that the selected factors were strongly associated with the presence of CAC but without any universal CAC^+-associated upregulation Rather, we observed upregulation of TNFα and MIP-1β but downregulation of netrin-1 and gremlin-1. However, this was an exploratory study for finding novel biomarker predictors of silent CAD. Further research should be performed in a larger cohort of subjects – with long-term follow-up for adverse cardiovascular outcomes – in order to establish these proteins as useful biomarkers for predicting the presence of coronary calcifications and improving CAD risk stratification.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.clinbiochem.2017.08.012.

Conflict of interest

The authors declare that they have nothing to disclose regarding conflict of interest with respect to this manuscript.

Financial support

This work was supported by MINECO: grant SAF2016-81063 to MLN; and by the Government of Castilla y León: grants GRS923/A/14, BIO/VA35/15 and BIO/VA45/14 to JCM and MLN, and the FPI Program co-funded by FSE to BG.

Acknowledgements

We thank Isabel Cabero, Beatriz Valle and Henar Sanz for their technical support. We thank Dr Victoria Cachofeiro for reading the manuscript and providing numerous helpful comments and Anthony DeMarco for his help in editing the article.

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