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On the association between circulating biomarkers and atherosclerotic calcification in a cohort of arterial disease participants

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Cardiovascular disease

Abstract *Background and aims:* Atherosclerotic calcification is a powerful predictor of cardiovascular disease. This study aims to determine whether circulating levels of a local/systemic calcification inhibitor or a marker of bone formation correlate with measures of coronary or extracoronary calcification.
Methods and results: Clinical computed tomography (CT) was performed on 64 arterial disease participants undergoing carotid and lower extremity endarterectomy. Coronary artery calcium (CAC) scores and volumes were acquired from the CT scans (n = 42). CAC scores and volumes were used to derive CAC density scores. Micro-CT was performed on excised carotid (n = 36) and lower extremity (n = 31) plaques to quantify the volume and volume fraction of extracoronary calcification. Circulating levels of dephospho-uncarboxylated Matrix Gla Protein (dp-ucMGP), fetuin-A, carboxylated and uncarboxylated osteocalcin (ucOC) were quantified using commercial immunoassays. Carotid participant CAC density scores were moderately negatively correlated with plasma dp-ucMGP ($r_s = -0.592$, $P = 0.008$). A weak negative association was found between CAC scores and %ucOC for all participants ($r_s = -0.335$, $P = 0.040$). Another weak negative correlation was observed between fetuin-A and the volume of calcification within

Abbreviations: CAC, Coronary Artery Calcium; cOC, Carboxylated Osteocalcin; CT, Computed Tomography; CVD, Cardiovascular Disease; CVF, Calcified Volume Fraction; dp-ucMGP, dephospho-uncarboxylated Matrix Gla Protein; HU, Hounsfield Unit; ICH-GCP, The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use - Good Clinical Practice; MGP, Matrix Gla Protein; Micro-CT, Micro-Computed Tomography; ucOC, uncarboxylated Osteocalcin; VKDP, Vitamin K-Dependent Proteins.

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excised carotid specimens ($r_s = -0.366$, $P = 0.031$). Despite substantial differences in coronary and extracoronary calcium measurements, the levels of circulating biomarkers did not vary significantly between carotid and lower extremity subgroups.
Conclusion: Correlations identified between circulating biomarkers and measures of coronary and extracoronary calcium were not consistent among participant subgroups. Further research is required to determine the association between circulating biomarkers, coronary and extracoronary calcium.
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Introduction

Individuals with subclinical atherosclerosis are a priority for risk stratification and primary prevention of major adverse cardiovascular events including myocardial and cerebral infarctions [1]. The Coronary Artery Calcium (CAC) Score [2] is a widely used marker of atherosclerotic burden [3] and is a powerful predictor of cardiovascular morbidity and mortality [4]. It is well-documented that atherosclerosis is a systemic disease [5] and large volumes of calcification in distinct vascular territories are associated with increased cardiovascular disease (CVD) mortality [6]. Therefore, screening for calcification in the coronary arteries alone may have limited value in estimating the atherosclerotic burden [7]. However, utilising whole-body CT to quantify the calcific burden in all major atherosclerotic vessels is not without its challenges, including increased cost and radiation dose [8].

Blood-based biomarkers may provide an alternative diagnostic approach. The active formation of atherosclerotic calcification [9] provides a unique opportunity to examine the utility of circulating biomarkers of vascular calcification to characterise atherosclerotic plaques based on the calcified content. Arterial tissues are in immediate contact with circulating blood, making this a feasible approach for the subclassification of CVD [10].

Matrix γ-carboxyglutamate (Gla) protein (MGP) is a potent inhibitor of vascular calcification expressed locally by vascular smooth muscle cells [11]. The dephosphorylated and uncarboxylated isoform of this protein (dp-ucMGP) has a low affinity for intravascular calcium deposits and is readily released into circulation [12]. This inactive MGP species has been proposed as a biomarker of cardiovascular risk [13] and vascular calcification [12,14,15]. Fetuin-A is a circulating plasma glycoprotein produced by the liver, which contributes to the systemic calcification inhibitory capacity of the circulating blood [16]. Osteocalcin is a marker of bone turnover and osteoblast activity [17].

The main objective of this study was to determine the association between circulating levels of a local or systemic calcification inhibitor (dp-ucMGP and fetuin-A), a marker of bone formation (osteocalcin) and either coronary and extracoronary calcium measurements. A sub-objective of this study was to compare the calcium measurements and the levels of circulating biomarkers between participants undergoing carotid versus lower extremity endarterectomy procedures.

Methods

Recruitment

Arterial disease participants undergoing standard carotid and lower extremity endarterectomy at the University Hospital Limerick, Ireland were recruited for this study (n = 64). Written informed consent was acquired from all participants. This study was approved by the Research Ethics Committee at the University Hospital Limerick and conforms to the principles of ICH-GCP and the Declaration of Helsinki. Preoperative participant assessment included demographics, comorbid conditions, preoperative medication and cardiovascular history, as presented in Table 1.

Sample acquisition

Blood samples

Fasting venous blood was preoperatively collected by venepuncture (n = 64). Serum samples were prepared in serum gel tubes (S-Monovette 4.9 mL Z-Gel, Sarstedt, UK) and plasma samples were prepared in K3EDTA plasma tubes (S-Monovette 7.5 mL K3E, Sarstedt, UK) both by standard centrifugation (15 min, 1580×*g*). Serum and plasma samples were split into dedicated aliquots and stored at −80 °C until time of protein quantification.

Atherosclerotic plaques

Plaque specimens were acquired from 64 different participants undergoing carotid and lower extremity endarterectomy: 67 plaque specimens in total, 36 carotid and 31 lower extremity (2 aorto-iliac and 29 ilio-femoral). There were 3 participants that underwent two separate endarterectomy procedures (multiple [n = 3]). Within 24 h of surgical removal, the plaque samples were frozen in phosphate-buffered saline solution and stored at −20 °C until time of analysis.

Coronary artery calcium imaging

Coronary CT scanning was performed for 55 of the participants; 2 preoperatively and 53 postoperatively (postoperative duration 51 ± 54 days, maximum 32 weeks),

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Table 1 Participant characteristics.

Participant Variables	Total (n = 64)	Carotid (n = 35)	Lower Extremity (n = 26)	*P*-value[a]	Multiple (n = 3)
Demographics					
Age, years (mean ± SD[b])	68 ± 9	70 ± 9	66 ± 10	0.165	68 ± 11
Sex, Male, n (%)	45 (70)	22 (63)	20 (77)	0.570	3 (100)
Comorbidities					
Systolic Blood Pressure (mmHg) (mean ± SD)	147 ± 24	152 ± 24	139 ± 23	0.043	150 ± 31
Diastolic Blood Pressure (mmHg) (mean ± SD)	76 ± 15	80 ± 16	71 ± 11	0.011	67 ± 17
Body Mass Index (kg/m^2) (mean ± SD)	26 ± 5	27 ± 5	26 ± 5	0.370	25 ± 4
Obesity, n (%)	11 (17)	7 (20)	4 (15)	0.883	0 (0)
Smoker (current or ex), n (%)	56 (88)	29 (83)	24 (92)	0.280	3 (100)
Diabetes Mellitus, n (%)	19 (30)	6 (17)	12 (46)	0.014	1 (33)
Dyslipidemia, n (%)	52 (81)	27 (77)	23 (88)	0.285	2 (67)
Hypertension, n (%)	51 (80)	28 (80)	20 (77)	0.772	3 (100)
Atrial Fibrillation, n (%)	11 (17)	5 (14)	5 (19)	0.606	1 (33)
Chronic Obstructive Pulmonary Disease, n (%)	10 (16)	3 (9)	5 (19)	0.223	2 (67)
Osteoporosis, n (%)	4 (6)	2 (6)	2 (8)	0.758	0 (0)
Increased C_2H_5OH Levels, n (%)	5 (8)	3 (9)	2 (8)	0.901	0 (0)
Medication					
Ace Inhibitor, n (%)	19 (30)	8 (23)	9 (35)	0.311	2 (67)
Angiotensin 2 Receptor Blocker, n (%)	15 (23)	9 (26)	6 (23)	0.813	0 (0)
B Blocker, n (%)	29 (45)	15 (43)	12 (46)	0.798	2 (67)
Calcium Antagonist, n (%)	23 (36)	13 (37)	9 (35)	0.839	1 (33)
Nitrates, n (%)	2 (3)	1 (3)	1 (4)	0.968	0 (0)
Anticoagulant Therapy, n (%)	11 (17)	5 (14)	6 (23)	0.115	0 (0)
Antiplatelet, n (%)	20 (31)	8 (23)	11 (42)	0.105	1 (33)
Aspirin, n (%)	51 (80)	28 (80)	20 (77)	0.772	3 (100)
Insulin, n (%)	6 (9)	1 (3)	4 (15)	0.078	1 (33)
Oral Hypoglycaemic, n (%)	15 (23)	5 (14)	9 (35)	0.062	1 (33)
Statin, n (%)	54 (84)	29 (83)	22 (85)	0.854	3 (100)
Cardiovascular History					
Ischemic Heart Disease, n (%)	30 (47)	16 (46)	13 (50)	0.740	1 (33)
Previous Cardiac Event, n (%)	10 (16)	5 (14)	5 (19)	0.606	0 (0)
Lower Limb Peripheral Artery Disease, n (%)	36 (56)	7 (20)	26 (100)	<0.001	3 (100)
Previous Cerebrovascular Event, n (%)	36 (56)	32 (91)	2 (8)	<0.001	2 (67)

[a] *P*-value compares carotid and lower extremity groups.
[b] SD = Standard Deviation.

using a Siemens Somatom Sensation 64 (Erlangen, Germany). The scanning parameters were 200 mm field of view, 3 mm slice thickness, acquisition 24 × 1.2 mm and reconstruction increment of 1.5 mm. The x-ray source was operated at 120 kV and all tomographic slices were obtained with a rotation time of 0.33 s and pitch of 0.2. CAC scores and volumes were acquired using Syngo calcium scoring software (Siemens AG, Erlangen, Germany). CAC scan results for participants with coronary artery bypass grafts (CABG) (n = 8), pacemakers (n = 2), coronary stents (n = 2) or a CABG and a pacemaker (n = 1) were excluded. Based on the methodology of Criqui et al., the average CAC density scores were calculated by dividing the CAC scores by the average area scores for each participant, where the average score is estimated as the CAC volume divided by the CT slice thickness [18]. Participants with a CAC score of zero could not be included in CAC density score analysis (n = 3).

Plaque-specific image acquisition and post processing

As the standard preoperative image assessment of peripheral arterial disease varied on a case-by-case basis, the calcified content within the excised plaque samples was determined using *ex vivo* high-resolution micro-CT imaging postoperatively.

Chemical fixation

To prevent any destructive effects to the tissue structure under the intense micro-CT voltage source, the specimens underwent a previously described preservation process involving chemical fixation with methanol, dehydration with ethanol and drying with hexamethyldisilazane [19].

Scanning parameters

The scanning was performed with a 0.4x optical magnification, low energy pass filter and 2.5 s x-ray exposure time. The x-ray source was operated at 50 kV and 81 μA and all tomographic slices were obtained with a pixel resolution of 15.68 μm (Xradia versa 500, Zeiss, Germany). Images were calibrated to Hounsfield Unit values (HU).

Calcification quantification

The calcified portions of the excised plaques were quantified based on the standard radiographic density

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thresholds (≥130HU) using ImageJ (National Institutes of Health, Maryland, USA). The volume of calcification is calculated by multiplying the number of calcified pixels by the known voxel volume. The degree of excised plaque calcification is represented by the calcified volume fraction (CVF), the ratio of calcification volume to total plaque volume [20].

Protein quantification

Circulating plasma levels of dp-ucMGP levels were quantified using the inaKtif MGP iSYS kit analysed on the IDS-iSYS Multi-Discipline Automated System (Immuno-DiagnosticSystems, Boldon, UK). Sample aliquots underwent a minimum of 1 and a maximum of 3 freeze−thaw cycles before dp-ucMGP quantification. Circulating plasma levels of fetuin-A and serum levels of cOC and ucOC were quantified using commercially available ELISA test kits (Bio-techne, R&D Systems (MN, USA) Cat. No. DFTA00, and TaKaRa Bio Inc (Japan) Cat. No. MK111 and MK118, respectively). Samples were analysed in duplicate and ELISAs were carried out in accordance with the manufacturer's instructions. Results were read on a multi-mode plate reader (Synergy H1, BioTek, VT, USA). Samples underwent 1 freeze−thaw cycle prior to protein quantification. Circulating osteocalcin is described using percentage uncarboxylated osteocalcin to total osteocalcin (%ucOC). Both MGP and osteocalcin are vitamin K-dependent proteins (VKDP) that require carboxylation to become fully functional [21,22]. Any participants who were receiving warfarin (vitamin K antagonist) at the time of or prior to recruitment were excluded from the VKDP analysis (dp-ucMGP and %ucOC) (n = 5). Fig. 1 presents an overview of the sample acquisition,

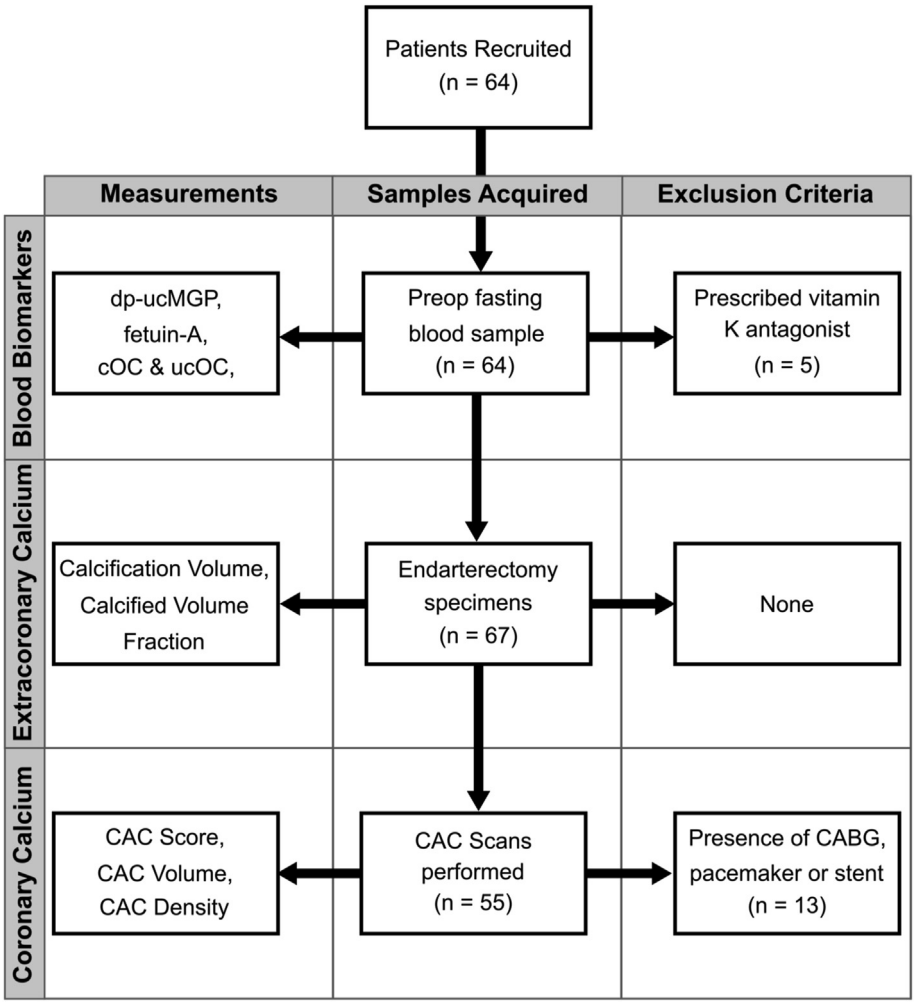


Figure 1 Flow chart of participant sample acquisition, measurements performed and any exclusion criteria. Sixty-four participants were recruited. Blood samples were acquired from the participants preoperatively (n = 64). Circulating levels of dephospho-uncarboxylated Matrix Gla Protein (dp-ucMGP), fetuin-A and carboxylated or uncarboxylated osteocalcin (cOC and ucOC) were measured. Any participants that were prescribed warfarin were excluded from the vitamin K-dependent protein analysis (dp-ucMGP and %ucOC) (n = 5). Plaques were acquired from endarterectomy procedures (n = 67). Calcification volume and calcified volume fraction within the excised plaques was determined. Coronary artery calcium (CAC) scans were performed (n = 55). Participants with the presence of coronary artery bypass grafts (CABG), pacemakers or stents were excluded from the CAC analysis (n = 13). CAC scores, CAC volumes and average CAC density scores were calculated from the scans.

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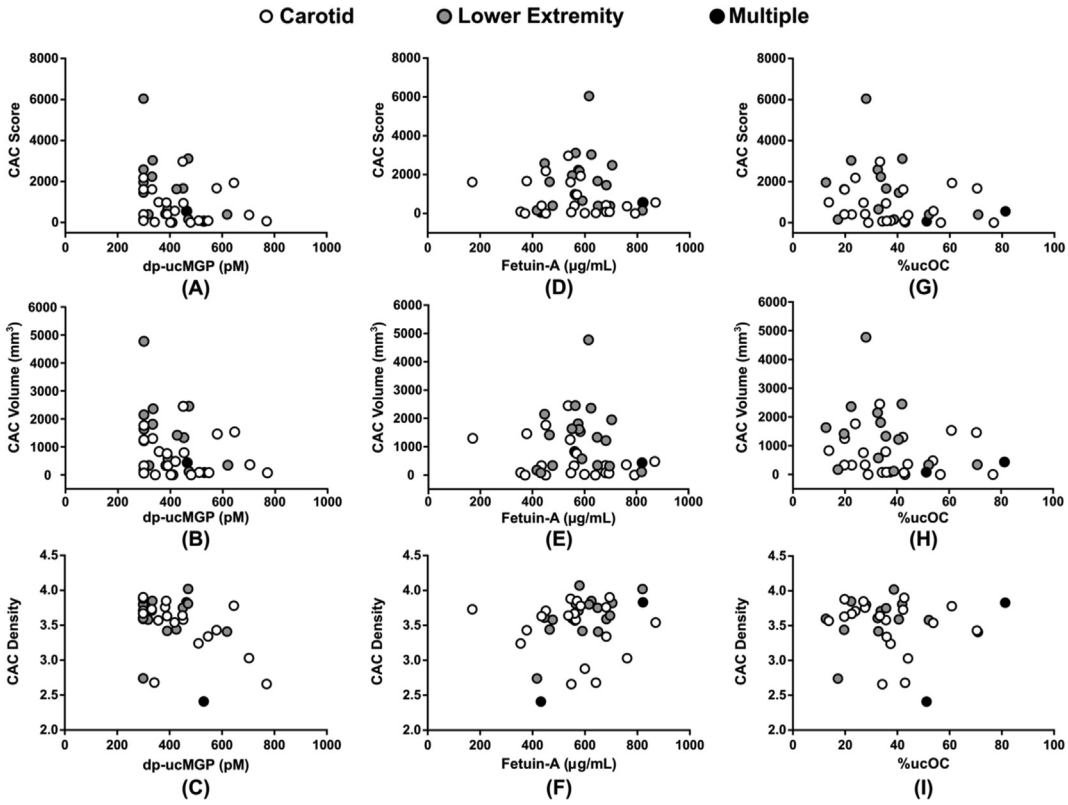


Figure 2 Scatterplots of circulating biomarkers versus measures of coronary calcification. Dephospho-uncarboxylated Matrix Gla Protein (dp-ucMGP) versus (A) coronary artery calcium (CAC) score (B) CAC volume and (C) CAC density. Fetuin-A versus (D) CAC score (E) CAC volume and (F) CAC density. Percentage uncarboxylated osteocalcin (%ucOC) versus (G) CAC score (H) CAC volume and (I) CAC density.

recorded measurements and any exclusion criteria that were utilised in this study.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics version 26. Descriptive statistics were used to summarise participant characteristics. Participants are grouped according to endarterectomy location (carotid, lower extremity and multiple). Categorical data were compared across groups using Chi-squared tests. Shapiro–Wilk analysis was performed to assess the distribution of continuous data. Associations in levels of circulating proteins (dp-ucMGP, fetuin-A and %ucOC) and measures of atherosclerotic calcification (CAC score, CAC volume, CAC density, calcification volume and CVF) were examined using Spearman's rank-order correlation coefficient (r_s). Comparisons in the calcium measurements and levels of circulating proteins between carotid and lower extremity participants are presented in the supplementary file. All data determined to be normally distributed were summarised as mean ± SD, presented in boxplots and compared between the carotid and lower extremity groups using independent t-tests. Similarly, all data determined to be not normally distributed were summarised as median values with 25th to 75th percentiles in square brackets, presented in boxplots and compared between groups using Mann–Whitney U tests. A two-tailed *P*-value less than 0.05 was deemed statistically significant in this study.

Results

Participant characteristics

The descriptive characteristics for participants, according to endarterectomy location, are presented in Table 1. Three participants underwent both carotid and lower extremity endarterectomies and were recorded as "multiple" location. Participants were an average of 70 ± 9 years (63% male), 66 ± 10 years (77% male) and 68 ± 11 years (100% male), respectively. Carotid participants had higher systolic (152 ± 24) and diastolic (80 ± 16) blood pressure than lower extremity participants (139 ± 23, 71 ± 11), $P = 0.043$ and $P = 0.011$. Additionally, 46% of lower extremity participants were diabetic, versus 17% of carotid ($P = 0.012$). Unsurprisingly, 91% of carotid participants had a preoperative cerebrovascular event while only 20% exhibited lower limb peripheral arterial disease, versus 8% and 100% of the lower extremity participants, respectively ($P < 0.001$ in both cases). Twelve samples had dp-ucMGP concentrations below the detection limit (<300 pM). These samples were therefore assigned a dp-ucMGP value of 299 pM, in line with previous work [23].

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Circulating biomarkers and coronary calcium

Figure. 2 presents scatterplots of circulating dp-ucMGP, fetuin-A and %ucOC correlated with coronary artery calcium (CAC) scores, CAC volumes and average CAC density scores. A moderate negative association was observed between dp-ucMGP and CAC density score for the carotid participants ($r_s = -0.592$, $P = 0.008$). Similarly, a weak negative correlation was also found between dp-ucMGP and CAC density score for all participants ($r_s = -0.338$, $P = 0.047$). Another weak negative correlation was observed between %ucOC and CAC scores for the total cohort ($r_s = -0.335$, $P = 0.040$). No significant associations were found between circulating fetuin-A and coronary calcium measures. Spearman's correlation coefficients and *P*-values are presented in Table 2.

Circulating biomarkers and extracoronary calcium

Figure. 3 presents scatterplots of circulating dp-ucMGP, fetuin-A and %ucOC correlated with extracoronary calcium volume and the calcified volume fraction (CVF) of excised plaques. A weak negative correlation was found between circulating fetuin-A levels and the volume of calcification within excised carotid specimens ($r_s = -0.366$, $P = 0.031$). No other significant correlations were found between circulating dp-ucMGP or %ucOC and extracoronary calcium measures. Spearman's correlation coefficients and *P*-values are presented in Table 2.

Comparison between carotid and lower extremity participant calcium and biomarkers

Comparisons between carotid and lower extremity groups are presented in full in the supplementary file. Briefly, lower extremity endarterectomy participants had significantly higher CAC scores and CAC volumes than carotid participants (Figs. S1A and B). However, the CAC average density scores were not statistically different between the two groups (Fig. S1C). Additionally, excised lower extremity specimens had much higher volumes and volume fractions of calcium than carotid plaques (Fig. S2). Despite these differences in the amounts of coronary and extracoronary calcium for carotid and lower extremity participants, the levels of circulating dp-ucMGP, fetuin-A and % ucOC were not statistically different between the cohorts (Fig. S3).

Discussion

Atherosclerotic calcification is a robust marker of cardiovascular disease risk. The purpose of this study was to correlate circulating biomarkers of vascular calcification with measures of atherosclerotic calcification derived from *in vivo* coronary artery calcium scans and *ex vivo* micro-CT scans of endarterectomy specimens in participants undergoing carotid and lower extremity endarterectomy. A moderate negative correlation was observed between dp-ucMGP and CAC density scores for carotid participants.

Table 2 Spearman's correlation coefficients and associated *P*-values describing the significance of the associations between circulating protein levels and measurements of coronary and extracoronary calcification for carotid, lower extremity and total groups (r_s; *P*).

		Coronary									Extracoronary					
		CAC Score			CAC Volume			CAC Density			Calcification Volume			Calcified Volume Fraction		
		Carotid	Lower	Total	Carotid	Lower	Total	Carotid	Lower	Total	Carotid	Lower	Total	Carotid	Lower	Total
dp-ucMGP	r_s	−0.139;	−0.258;	−0.308;	−0.060;	−0.227;	−0.249;	**−0.592;**	0.178;	**−0.338;**	−0.034;	0.217;	−0.031;	0.069;	0.113;	−0.005;
	P	0.536	0.372	0.060	0.792	0.435	0.132	**0.008**	0.544	**0.047**	0.854	0.320	0.823	0.708	0.607	0.970
	n	*(n=22)*	*(n=14)*	*(n=38)*	*(n=22)*	*(n=14)*	*(n=38)*	*(n=19)*	*(n=14)*	*(n=35)*	*(n=32)*	*(n=23)*	*(n=55)*	*(n=32)*	*(n=23)*	*(n=55)*
Fetuin-A	r_s	−0.236;	−0.167;	−0.065;	−0.227;	−0.174;	−0.064;	−0.078;	0.431;	0.266;	**−0.366;**	0.044;	−0.034;	−0.248;	−0.063;	−0.033;
	P	0.277	0.523	0.681	0.299	0.504	0.681	0.743	0.084	0.102	**0.031**	0.831	0.796	0.151	0.759	0.799
	n	*(n=23)*	*(n=17)*	*(n=42)*	*(n=23)*	*(n=17)*	*(n=42)*	*(n=20)*	*(n=17)*	*(n=39)*	*(n=35)*	*(n=26)*	*(n=61)*	*(n=35)*	*(n=26)*	*(n=61)*
%ucOC	r_s	−0.263;	−0.248;	**−0.335;**	−0.207;	−0.270;	−0.293;	−0.282;	0.064;	−0.133;	−0.083;	0.042;	0.005;	0.023;	−0.006;	0.029;
	P	0.237	0.392	**0.040**	0.354	0.350	0.074	0.241	0.829	0.448	0.651	0.847	0.973	0.899	0.979	0.836
	n	*(n=22)*	*(n=14)*	*(n=38)*	*(n=22)*	*(n=14)*	*(n=38)*	*(n=19)*	*(n=14)*	*(n=35)*	*(n=32)*	*(n=23)*	*(n=55)*	*(n=32)*	*(n=23)*	*(n=55)*

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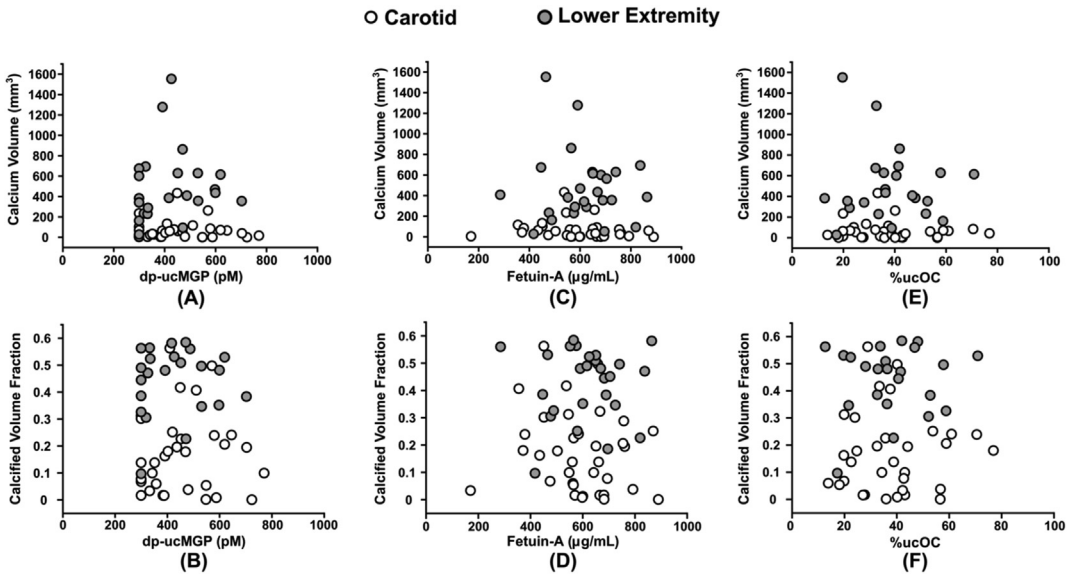


Figure 3 Scatterplots of circulating biomarkers versus measures of extracoronary calcification. Dephospho-uncarboxylated Matrix Gla Protein (dp-ucMGP) versus (A) calcium volume and (B) calcified volume fraction. Fetuin-A versus (C) calcium volume and (D) calcified volume fraction. Percentage uncarboxylated osteocalcin (%ucOC) versus (E) calcium volume and (F) calcified volume fraction.

Weak negative associations were found between %ucOC and CAC scores and between fetuin-A with and carotid plaque calcification volume.

There is conflicting evidence on the use of dp-ucMGP as a biomarker of cardiovascular disease morbidity and mortality for the general population [24] and those of high atherogenic status [25]. This study is the first to correlate circulating non-functional MGP with CAC density measurements. CAC density is inversely and significantly associated with coronary heart disease and CVD risk [18], while circulating dp-ucMGP has been positively associated with coronary artery calcification in healthy women [15] and cardiovascular mortality in participants with chronic stable vascular disease [13]. Therefore, a negative correlation would be expected between CAC density scores and circulating dp-ucMGP. However, a significant association was only observed for the carotid participants.

A weak negative association was found between %ucOC with CAC scores for the total cohort. Similarly, serum ucOC levels have been inversely related to abdominal aortic calcification scores in male diabetic participants [26]. In contrast, higher measures of uncarboxylated osteocalcin and osteocalcin ratio (ucOC:OC) have been associated with CAC scores > 0, for male subjects only [27]. However, the association between %ucOC and CAC scores is not significant for the carotid and lower extremity subgroups, only for the total cohort. Recent reviews also highlighted that there is no definitive association between osteocalcin and vascular calcification [28] and there is limited data available on undercarboxylated OC [29].

Danziger et al. previously did not find a relationship between circulating VKDPs (including MGP and osteocalcin) and vascular calcification [30]. Another review also highlighted the lack of associations evident between VKDPs and cardiovascular morbidity and mortality [31]. Here, we also found no associations between circulating fetuin-A and any coronary calcium measures. There is previous conflicting evidence regarding the relationship between serum fetuin-A and CAC scores in haemodialysis and chronic kidney disease populations [32,33].

It has been postulated that multisite imaging may be able to predict multiple CVD outcomes [8]. We did find a weak negative correlation between circulating fetuin-A and the absolute volume of carotid plaque calcification. No other associations were determined for dp-ucMGP or % ucOC with extracoronary calcium measures. The authors did not encounter any previous studies which examined a relationship between either dp-ucMGP, fetuin-A or ucOC and extracoronary calcification. The assessment of CVD risk using measures of extracoronary calcification requires further research and standardisation.

The dp-ucMGP and fetuin-A levels reported in this study are not different from values reported for healthy reference populations; dp-ucMGP: 525 $\pm$ 243 pM (age 66–80 years) [12] or $<$300–532 pM [23], and fetuin-A: 400–800 µg/mL [34]. These findings extend the observations of previous studies which did not demonstrate associations between dp-ucMGP and chronic heart disease, stroke risk [24] or calcification [30,35,36]. Conversely, other studies have demonstrated the potential utility of circulating dp-ucMGP levels to indicate arterial stiffness or calcification in specific cohorts including end-stage renal disease, aortic valve disease [12,37] and heart failure [38]. No participants included in this study were diagnosed with either chronic kidney disease, end-stage renal disease, or aortic valve disease.

Carotid and lower extremity participants have significantly different coronary and extracoronary calcium measures. The differences in excised plaque calcium content have been previously described [39,40]. Of interest,

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this study does not confirm that circulating calcification inhibitors can distinguish between carotid and lower extremity populations. The implications of these findings (supplementary file) have yet to be revealed. It is apparent that the association between circulating levels of calcification inhibitors and atherosclerotic calcification remains incompletely characterised [41].

Limitations

This study has some associated limitations that merit consideration. Firstly, quantification of calcification within endarterectomy specimens precludes that of calcium at other subclinical sites, such as medial calcification [42]. However, a strength of this study includes the measurement of CAC density, which has not been previously correlated with circulating MGP, fetuin-A or osteocalcin. Secondly, this is a single time point study therefore the prognostic value of the targeted biomarkers could not be determined. It is also necessary to consider that circulating levels of blood protein do not necessarily emulate tissue levels [36,43]. Given this, and the lack of consistent statistical associations determined in this study, future work would be beneficial which examines protein expression within the diseased tissue.

Conclusions

Improved quantification of atherosclerotic calcification will optimise cardiovascular disease risk assessment. A moderate negative correlation was observed between CAC density scores and plasma dp-ucMGP ($r_s = -0.592$, $P = 0.008$) for carotid participants. Additionally, a weak negative correlation was observed between circulating % ucOC and CAC scores for the total cohort ($r_s = -0.335$, $P = 0.040$). Carotid plaque calcification volume is negatively associated with circulating levels of fetuin-A ($r_s = -0.366$, $P = 0.031$). However, these associations were not consistent among all participant cohorts. The relationship between circulating blood-biomarkers and the calcified atherosclerotic burden remains incompletely characterised. Of note, while carotid and lower extremity endarterectomy groups had considerably different amounts of coronary and extracoronary calcium, the levels of circulating proteins were not significantly different between the groups. Future studies should conduct whole-body examination to quantify calcium in all arterial beds to truly explore if there is a link between atherosclerotic calcification and circulating biomarkers. Moreover, the correlation between protein expressions within calcified arterial tissue versus the levels of clinically targetable species in the circulating blood requires examination.

Author contributions

RC, HB, EK, MM, MT and EOC: conceptualization; RC, HB, AR, LS, JOB, EK, MM, SE, FL, TG and MI: data curation; RC, HB, AR, JM, HP, POS, MT and EOC: formal analysis and investigation; RC, HB and AR: writing - original draft; AR, JM, HP, LS, JOB, EK, MM, SE, FL, TG, MI, POS, MT and EOC: writing - review & editing; RC, HB, MW and EOC: funding acquisition; SE and FL: project administration; MW and EOC: supervision.

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Declarations of competing 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.numecd.2021.02.005.

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