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# Circulating Gas6 is associated with reduced human carotid atherosclerotic plaque burden in high risk cardiac patients

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ABSTRACT

*Objective:* Pre-clinical studies suggest that growth arrest-specific protein 6 (Gas6), a member of the vitamin K dependent family of proteins, is implicated in atherosclerosis. A role for Gas6 in stabilizing atherosclerotic plaque has been suggested. Our aim was to determine the association between Gas6 and measures of carotid artery atherosclerosis in humans undergoing elective coronary angiography. Secondary aims were to determine the association between Gas6 and sex, diabetes, and obesity.

*Methods:* In 204 outpatients referred for coronary angiography, EDTA plasma was collected and a focused carotid ultrasound performed. Degree of angiographic coronary artery disease was scored. Carotid intima media thickness as well as maximum plaque height, plaque area, and grayscale median were measured by vascular sonography. Gas6 was assessed by enzyme-linked immunosorbent assay.

*Results:* We found that Gas6 concentrations were lower in males and were associated with diabetes, obesity, and lower kidney function. After adjustment for age, sex, kidney function, BMI and traditional cardiac risk factors; diabetes was associated with higher levels of Gas6, whilst there was a significant inverse relationship between Gas6 and total plaque area. Gas6 was inversely associated with maximum plaque height and total plaque area in adjusted multi-variable models.

*Conclusions:* We observed higher levels of Gas6 in participantswith adverse cardiovascular risk profiles (e.g. diabetes, obesity) yet Gas6 was independently associated with reduced plaque height and total plaque area. These findings suggest that Gas6 may play a role in human atherosclerotic plaque remodeling.

## 1. Introduction

Growth arrest-specific protein (Gas6) is a ligand for the TAM (Tyro-3/Axl/Mer) group of receptors which have been shown to play a role in a variety of biological processes involving inflammation, hemostasis and fat metabolism [1]. Gas6 is expressed in endothelial cells, vascular smooth muscle cells, and bone marrow. Gas6 is a gamma-carboxyglutamic (Gla) protein and thus, a member of the vitamin K dependent family of proteins. Vitamin K-mediated gamma-carboxylation of the Gla domain is critical for the binding of Gas6 to anionic phospholipids which re-locate to the cell surface under conditions of cell injury, activation or apoptosis [2]. The carboxylated Gla domain may thus, be of importance in targeting Gas6 to activate endothelial cells in a variety of disease states. Although the concentration of Gas6 in the circulation is much lower than that of other vitamin K-dependent proteins, it has been shown to be elevated in patients with chronic kidney disease [3], sepsis [4], peripheral arterial disease [5], systemic lupus erythematosus [6] and pre-eclampsia [7].

Higher levels of circulating Gas6 have been linked to obesity, insulin resistance, inflammation and endothelial dysfunction in adolescents [8] and in females [9]. In addition to the Gla domain, Gas6 contains four epidermal growth factor (EGF)-like repeats and a C-terminal steroid

*Abbreviations:* BMI, body mass index; CAD, coronary artery disease; CIMT, carotid intima media thickness; DICOM, Digital Imaging and Communications in Medicine; eGFR, estimated glomerular filtration rate; ELISA, enzyme-linked immunosorbent assay; Gas6, growth arrest-specific protein 6; GE, General Electric; Gla, gamma-carboxyglutamic; GSM, grayscale median; ICA, internal carotid artery; MPH, maximum plaque height; PIVKA-II, prothrombin induced by vitamin K absence-II; SHBG, C-terminal steroid hormone binding globulin; TAM, Tyro-3/Axl/Mer; TIFF, Tagged Image File Format; TPA, total plaque area; WC, waist circumference

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hormone binding globulin (SHBG)-like domain. The SHBG-like C terminal domain is critical for TAM receptor-binding. Signaling functions of TAM receptors include stimulation of hemostasis, modulation of inflammation and inhibition of apoptosis.

Current research suggest that Gas6 may play a role in atherosclerosis, although an exact mechanism remains controversial as rodent, translational, and human studies support both pro- and anti-atherogenic functions. Atherosclerotic plaques that are prone to rupture and to cause clinical events have thin fibrous caps with large lipid cores and contain high levels of inflammatory cells (complex plaque) [10]. Interestingly, higher Gas6 levels have been found to be associated with less complexity (stability) of human carotid atherosclerotic plaque [11]. Gas6 has been found to be higher in subjects with other risk factors for atherosclerosis [3,8], thus its potential role as a protective agent has been postulated. The association of Gas6 with overall carotid plaque burden has never been studied in a systematic manner previously. With this background, we sought to determine the association of Gas6 with carotid atherosclerotic plaque.

## 2. Materials and methods

### 2.1. Study population

Males and females above the age of 18, who were referred for a non-emergency coronary angiogram between May and October 2013 were approached to participate in this study and blood samples collected. Coronary angiograms were clinically indicated for one of: non-specific chest pain evaluation, stable or unstable angina pectoris, positive stress test, pre-operative assessment, old or recent myocardial infarction (> 2 days). Two-hundred and four individuals agreed to have a carotid ultrasound performed. Demographic and medical data were obtained by direct participant interview and from the medical chart and/or hospital information database. The study was approved by Queen's University Health Sciences and Affiliated Teaching Hospitals Research Ethics Board. All participants gave informed consent to participate.

### 2.2. Angiographic score

Angiograms were scored as previously described [12,13]. In brief, an angiographic score of 0 = no or minimal disease (coronary artery disease [CAD] 0%–19% narrowing in any segment), 1 = mild disease (20%–49% narrowing in any segment), 2 = moderate disease (luminal narrowing of at least one segment of 50%–69%), and 3 = severe disease (≥70% narrowing within any segment of the main branches of the coronary artery or ≥ 50% in the left main coronary artery).

### 2.3. Carotid plaque quantification

A focused carotid ultrasound, to assess plaque, was performed using a GE Vivid E9 (General Electric [GE] Healthcare, Milwaukee, WI) vascular ultrasonography device equipped with a 9 L-D transducer [13]. Images were stored in Digital Imaging and Communications in Medicine (DICOM) format and analyzed offline using EchoPAC software (GE). Carotid intima media thickness (CIMT) was determined for both right and left vessels using the auto border detection function. The mean of the right and left side was used in the analysis. Plaque height was measured manually using callipers in the bulb/internal carotid artery (ICA) region. The maximum plaque height (MPH) of either side was used in the analysis. Plaque area was traced manually. The total area of both sides was used as the total plaque area (TPA).

### 2.4. Grayscale median (GSM)

Grayscale median (GSM) analysis is an accepted marker of plaque vulnerability and used to assess echolucency of the arterial plaque [14]. GSM was assessed in longitudinal sections of the bulb/ICA where carotid plaque was present. DICOM images were exported to uncompressed Tagged Image File Format (TIFF) files, and were opened in Adobe Photoshop (2015.0.1 Release, Adobe Systems Incorporated). Images were then converted to grayscale to discard colour information so that all pixel values fell between a range of 0 (black) and 255 (white). Images were normalized by linear scaling using the "curves" option so that a selected area within the lumen had a GSM of 0, and the brightest area of the adventitia had a GSM of 190. Plaque was outlined manually using the pen tool to create a region of interest (ROI) and the associated histogram of gray values was obtained to determine the GSM [15]. If more than one plaque lesion was present in an image, all lesions were combined to calculate a single GSM value. The average GSM of both sides was used in the analysis.

### 2.5. Measurement of laboratory variables

Blood was collected in EDTA (lavender top tubes) at the time of the angiogram, at the beginning of the procedure. The blood was spun to separate the plasma, then stored at $-80^{\circ}$C. Plasma was used to measure Gas6. Creatinine was measured by Abbott Diagnostics Enzymatic assay which is IDMS traceable at the hospital core lab.

Gas6 was determined using the Human Gas6 DuoSet enzyme-linked immunosorbent assay [ELISA] (DY885, R&D Systems®, Minneapolis, MN, USA) [16,17] with analytical range of 0.13–8.00 ng/mL. IMMULON 4 HBX ELISA plates were coated overnight with a goat polyclonal antibody (R&D, AB885, Minneapolis, MN) at 0.4μg/mL and washed three times with phosphate buffered saline (PBS) containing 0.05% Tween 20 (PBST). They were then blocked for 1 h with 300 μL/well of 1% bovine serum albumin in PBS (reagent diluent). Plasma samples were originally run at 1:10 dilution, but were outside the analytical range (were in the top end and some were reading over). To ensure samples were within analytical detection range, samples were either diluted 1:30 (10 uL sample into 290 uL 1% BSA in PBS) or 1:50 (5 uL sample into 245 uL 1% BSA in PBS). All samples were well within the analytical range of the assay at both dilutions, and some samples were run across multiple plates to check for inter-assay variability. Diluted standards and plasma were added at 100 μL/well in duplicates, and incubated for 2 h at room temperature and washed as previously with PBST. Affinity-purified biotinylated goat polyclonal (R&D BAF885) was added at 0.1 μg/mL, incubated for 2 h at room temperature and then washed with PBST. Streptavidin peroxidase (R&D DY998) diluted 1:200 with reagent diluent was added and incubated for 20 min at room temperature without exposure to light. Substrate prepared according to the manufacturer's instructions (equal volumes of colour reagent A and B, R&D DY999) was added and colour development was monitored then terminated with 2 N H2SO4 once there was sufficient colour saturation. The absorbance at 450 nm was read with a Synergy microplate reader (SynergyHT Microplate Reader, Bio-Tek Instruments, Winooski, VT). The optical density for each sample was determined using Softmax Pro which calculated the concentration by reference to a four-parameter logistical regression from calibration curve of the standards. Wavelength correction was used at 570 nm to correct for optical imperfections of the plate. This DuoSet is calibrated against a highly purified NSO-expressed recombinant human Gas6 (aa 118–678) and also recognizes the longer form of recombinant Gas-6 (aa 49–678, R &D).

All samples were run in duplicate and had a coefficient of variation of < 10%. To assess inter-assay variability, a control with a known concentration was run on each of the plates. The coefficient of variation was also < 10% for the control across plates.

### 2.6. Statistical analysis

All data was analyzed using JMP®12.0.1 software (SAS Institute Inc., 2015). Fisher's exact test was used to compare nominal variables and the independent *t*-test was used for continuous variables.

**Table 1**
Demographic, laboratory, and atherosclerosis variables in the sample population.

| Variables | Overall ($n$ = 204) | Male ($n$ = 134) | Female ($n$ = 70) | $p$-value |
|---|---|---|---|---|
| Demographic | | | | |
| Age (years, mean ± SD) | 65.5 ± 9.4 | 65.8 ± 9.8 | 65.0 ± 8.7 | 0.51 |
| Diabetes (n, %) | 63 (31%) | 38 (28%) | 25 (36%) | 0.34 |
| Hypertension (n, %) | 154 (75%) | 96 (72%) | 58 (83%) | 0.09 |
| Dyslipidemia (n, %) | 168 (82%) | 110 (82%) | 58 (83%) | 0.85 |
| Tobacco use (n, %) | 32 (16%) | 19 (14%) | 13 (19%) | 0.42 |
| BMI (mean ± SD) | 30.1 ± 6.7 | 29.7 ± 6.1 | 30.8 ± 7.6 | 0.29 |
| BMI ≥ 30 (n, %) | 79 (39%) | 51 (38%) | 28 (40%) | 0.88 |
| Waist circ. (cm, mean ± SD) | 106.6 ± 14.1 | 107.9 ± 13.1 | 103.9 ± 15.9 | 0.09 |
| Laboratory variables | | | | |
| Creatinine (umol/L, mean ± SD) | 85.1 ± 27.2 | 91.8 ± 28.6 | 72.4 ± 18.5 | < 0.0001 |
| eGFR (ml/min/$m^2$, mean ± SD) | 77.4 ± 21.8 | 78.5 ± 22.4 | 75.2 ± 20.5 | 0.29 |
| Gas6 (ng/mL) | 7.15 ± 2.71 | 6.84 ± 2.25 | 7.74 ± 3.36 | 0.046 |
| Angiographic score (mean ± SD) | 2.1 ± 1.2 | 2.3 ± 1.2 | 1.8 ± 1.2 | 0.001 |
| 0 (normal, no CAD) [n,%] | 32 (16%) | 20 (15%) | 12 (17%) | 0.69 |
| 1 (mild CAD) | 38 (19%) | 15 (11%) | 23 (33%) | 0.0003 |
| 2 (moderate CAD) | 9 (4%) | 5 (4%) | 4 (6%) | 0.50 |
| 3 (severe CAD) | 125 (61%) | 94 (70%) | 31 (44%) | 0.0005 |
| Cardiac medications | | | | |
| ASA (n, %) | 148 (73) | 104 (78) | 44 (63) | 0.045 |
| Beta Blocker (n, %) | 111 (54) | 80 (60) | 31 (44) | 0.045 |
| ACE Inhibitor (n, %) | 67 (33) | 50 (37) | 17 (24) | 0.08 |
| ARB (n, %) | 26 (13) | 13 (10) | 13 (19) | 0.08 |
| Calcium Channel Blocker (n, %) | 59 (29) | 36 (27) | 23 (33) | 0.41 |
| Statins (n, %) | 140 (69) | 97 (72) | 43 (61) | 0.11 |
| Carotid ultrasound measures (mean ± SD) | | | | |
| Mean CIMT (mm) | 0.81 ± 0.15 | 0.82 ± 0.16 | 0.79 ± 0.14 | 0.12 |
| Maximum plaque height (mm) | 2.97 ± 1.35 | 3.01 ± 1.33 | 2.88 ± 1.39 | 0.52 |
| Total plaque area ($mm^2$) | 55.7 ± 40.6 | 59.4 ± 43.0 | 48.5 ± 34.8 | 0.05 |
| Mean GSM | 58.3 ± 13.6 | 58.1 ± 13.3 | 58.9 ± 14.2 | 0.71 |

The Fisher's exact test (two tailed) was used to compare nominal variables and the independent t-test was used for continuous variables. ASA: Acetylsalicylic acid, ACE: angiotensin converting enzyme, ARB: angiotensin II receptor blockers, BMI: body mass index, CAD: coronary artery disease, eGFR: estimated glomerular filtration rate, CIMT: carotid intima media thickness, GSM: grayscale median, Waist circ.: waist circumference.

Spearman's correlation coefficient was used to evaluate the bivariate (unadjusted) associations of continuous variables. We used a backward selection criteria of $p < .25$ to select independent factors associated with Gas6. The candidates included all atherosclerosis variables: CIMT, maximum plaque height, total plaque area, GSM, and demographics: age, sex, estimated glomerular filtration rate (eGFR, Modification of Diet in Renal Disease [MDRD]), body mass index (BMI), traditional cardiac risk factors (tobacco use, diabetes, hypertension, and dyslipidemia). Multiple linear regression models were used to examine the independent associations between maximum plaque height and total plaque area and demographic (age, sex, BMI), Gas6 and traditional cardiac risk factors (diabetes, tobacco use, hypertension, and dyslipidemia). All tests were 2-sided without correction for multiplicity, and statistical significance was accepted at $p < .05$.

## 3. Results

Table 1 describes the demographic, clinical, and laboratory variables in the entire cohort and in males and females separately. Of the 204 stable out-patients undergoing elective coronary angiography, 31% had diabetes and 75–82% had either hypertension or dyslipidemia. Males had significantly lower levels of Gas6 and higher angiographic scores, compared to females.

The association between cardiac risk factors and atherosclerosis measures and Gas6 is presented in Table 2. Significantly higher levels of Gas6 were measured in participants with diabetes, with an elevated BMI or with a waist circumference that exceeded the sex-specific high risk cut-off. There was no association between Gas6 and a history of hypertension, dyslipidemia, or smoking. The continuous bivariate measures are presented in Table 3. Gas6 was inversely correlated with eGFR and positively correlated with BMI and waist circumference.

Factors independently associated with Gas6 are presented for the entire cohort and in males (Table 4). No independent factors were observed in females after backward selection. Overall, after adjustment for age, sex, eGFR, BMI, and traditional cardiac risk factors, diabetes was associated with higher levels of Gas6 whilst there was a significant inverse relationship between Gas6 and total plaque area. When examined in males separately, the predictors remained the same. Overall, Gas6 was statistically different between males and females. This relationship remained significant when examined in the sub-group of non-diabetic subjects only. (Fig. 1).

The independent associations between traditional cardiac risk factors and Gas6 with maximum plaque height and total plaque area were also examined by multi-variable models. Higher maximum plaque height was associated with age, the presence of diabetes, dyslipidemia, and lower levels of Gas6 (Table 5A). Results were similar for total plaque area, where a lower level of Gas6 was independently associated with greater total plaque area after adjustment for important co-variates including age, sex, eGFR, as well as traditional cardiac risk factors (Table 5B).

## 4. Discussion

In this study of patients undergoing elective coronary angiogram, a higher level of Gas6 was associated with reduced carotid arterial plaque burden, as measured by maximum plaque height and area, after adjustment for cardiac risk factors for atherosclerosis, kidney function and age. In males Gas6 was significantly lower and these levels may be associated with higher increased atherosclerosis, especially in diabetics. Our results suggest that Gas6 may play a protective role in the

**Table 2**
Comparison of atherosclerosis measures and Gas6 with presence of cardiac risk factors.

| | CIMT (mm) | MPH (mm) | TPA (mm2) | GSM | Gas6 (ng/mL) |
|---|---|---|---|---|---|
| Risk Factor | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD | Mean ± SD |
| Diabetes | | | | | |
| Yes | 0.81 ± 0.16 | 3.41 ± 0.97 | 71.0 ± 43.7 | 61.6 ± 13.3 | 7.88 ± 2.60 |
| No | 0.81 ± 0.15 | 2.77 ± 1.45 | 48.9 ± 37.3 | 56.7 ± 13.5 | 6.83 ± 2.70 |
| p-Value | 0.71 | 0.0003[a] | 0.0007[a] | 0.02[a] | 0.01[a] |
| Hypertension | | | | | |
| Yes | 0.82 ± 0.15 | 3.07 ± 1.29 | 58.0 ± 40.4 | 58.1 ± 13.8 | 7.32 ± 2.79 |
| No | 0.78 ± 0.16 | 2.65 ± 1.49 | 48.6 ± 40.7 | 59.3 ± 12.9 | 6.63 ± 2.37 |
| p-Value | 0.16 | 0.08 | 0.16 | 0.59 | 0.09 |
| Dyslipidemia | | | | | |
| Yes | 0.82 ± 0.15 | 3.17 ± 1.21 | 61.1 ± 38.9 | 58.8 ± 13.3 | 7.26 ± 2.75 |
| No | 0.75 ± 0.16 | 2.02 ± 1.54 | 30.7 ± 39.7 | 55.4 ± 15.3 | 6.68 ± 2.48 |
| p-Value | 0.02[a] | 0.0001[a] | 0.0001[a] | 0.27 | 0.22 |
| Tobacco use | | | | | |
| Yes | 0.82 ± 0.15 | 3.14 ± 1.38 | 63.8 ± 43.0 | 60.5 ± 57.9 | 7.37 ± 2.93 |
| No | 0.81 ± 0.15 | 2.94 ± 1.34 | 54.2 ± 40.1 | 57.9 ± 14.0 | 7.11 ± 2.67 |
| p-Value | 0.74 | 0.44 | 0.25 | 0.29 | 0.64 |
| BMI ≥ 30 | | | | | |
| Yes | 0.82 ± 0.15 | 2.96 ± 1.36 | 59.8 ± 43.1 | 59.0 ± 13.9 | 7.83 ± 3.12 |
| No | 0.81 ± 0.15 | 2.97 ± 1.34 | 53.1 ± 38.9 | 57.9 ± 13.5 | 6.73 ± 2.32 |
| p-value | 0.66 | 0.96 | 0.27 | 0.61 | 0.008[a] |
| WC > 88 cm (F) and > 102 cm (M) | | | | | |
| Yes | 0.82 ± 0.16 | 3.04 ± 1.33 | 54.7 ± 37.3 | 58.9 ± 13.6 | 7.60 ± 2.81 |
| No | 0.79 ± 0.15 | 2.74 ± 1.35 | 51.8 ± 42.0 | 57.3 ± 14.7 | 6.39 ± 2.35 |
| p-Value | 0.12 | 0.15 | 0.65 | 0.49 | 0.003[a] |
| CAD | | | | | |
| Score 2&3 | 0.82 ± 0.79 | 3.28 ± 1.21 | 64.8 ± 41.2 | 60.0 ± 13.5 | 7.20 ± 2.32 |
| Score 0&1 | 0.79 ± 0.14 | 2.37 ± 1.41 | 38.4 ± 33.4 | 57.7 ± 13.3 | 7.06 ± 0.40 |
| p-value | 0.10 | < 0.0001[a] | < 0.0001[a] | 0.01[a] | 0.76 |
| Statins | | | | | |
| Yes | 0.83 ± 0.15 | 3.21 ± 1.27 | 63.9 ± 40.1 | 59.6 ± 14.1 | 7.23 ± 2.83 |
| No | 0.77 ± 0.15 | 2.44 ± 1.38 | 37.9 ± 36.1 | 55.2 ± 14.1 | 6.99 ± 2.42 |
| p-value | 0.01[a] | 0.0003[a] | < 0.0001[a] | 0.049 | 0.53 |

Independent t-test was used.
[a] Significant. BMI: body mass index, CAD: coronary artery disease, CIMT: carotid intima media thickness, eGFR: estimated glomerular filtration rate, GSM: grayscale median, MPH: maximum plaque height, TPA: total plaque area, and WC: waist circumference.

**Table 3**
Bivariate associations between Gas6 and demographic and atherosclerotic variables.

| Gas-6 by Variable | Spearman rho | p-value |
|---|---|---|
| Age (years) | −0.01 | 0.90 |
| eGFR (mL/min/1.73 m$^2$) | −0.15 | 0.04[a] |
| BMI (kg/m$^2$) | 0.15 | 0.03[a] |
| Waist circumference (cm) | 0.16 | 0.03[a] |
| CIMT (mm) | −0.02 | 0.74 |
| MPH (mm) | −0.05 | 0.47 |
| TPA (mm$^2$) | −0.08 | 0.28 |
| GSM | −0.04 | 0.55 |

[a] Significant, CIMT: carotid intima media thickness, eGFR: estimated glomerular filtration rate, GSM: grayscale median, MPH: maximum plaque height, TPA: total plaque area.

**Table 4**
Predictors of Gas6 following stepwise backward selection regression analysis.

| Correlates of Gas6 (ng/mL) | Estimate | SE | Lower 95% | Upper 95% | Std. Beta | p-value |
|---|---|---|---|---|---|---|
| All participants | | | | | | |
| Diabetes | 0.63 | 0.21 | 0.22 | 1.05 | 0.22 | 0.003 |
| TPA (mm$^2$) | −0.01 | 0.005 | −0.02 | −0.004 | −0.21 | 0.006 |
| Males | | | | | | |
| Diabetes | 0.78 | 0.22 | 0.34 | 1.21 | 0.31 | 0.001 |
| TPA (mm$^2$) | −0.01 | 0.005 | −0.02 | −0.002 | −0.22 | 0.01 |

Selection model included all atherosclerotic variables (CIMT, MPH, TPA, and GSM), age, sex (if all participants), eGFR, BMI, traditional cardiac risk factors (tobacco use, diabetes, hypertension, and dyslipidemia). Only contributors that remained in the model and that were statistically significant are presented. eGFR: estimated glomerular filtration rate, GSM: grayscale median, CIMT: carotid intima-media thickness, SE: standard error, and Std.: standardized. TPA: total plaque area.

atherosclerotic process.

An exact role for Gas6 in atherosclerosis is undefined, and overall very few studies have been conducted particularly in humans. Murine studies suggest that the main function of Gas6 is to mediate vascular smooth muscle cell survival and alignment within a plaque which could theoretically lead to a strengthened fibrous cap that is more stable and less prone to rupture [18,19]. There is data to suggest that Gas6 promotes apoptotic cell clearance, a function mediated by its interaction with the Mer receptor, which may prevent the accumulation of apoptotic cells in the developing plaque, thereby reducing the size of the necrotic core [18]. Gas6 expression has been shown to be higher for non-complicated human plaques compared to complicated ones that contain abundant inflammatory cells associated with vulnerable plaque [11]. Further, the mRNA, immunoreactivity and protein expression of Gas6 was shown to be considerably higher in the left internal mammary arteries than in the more atherogenic aorta [20]. The authors

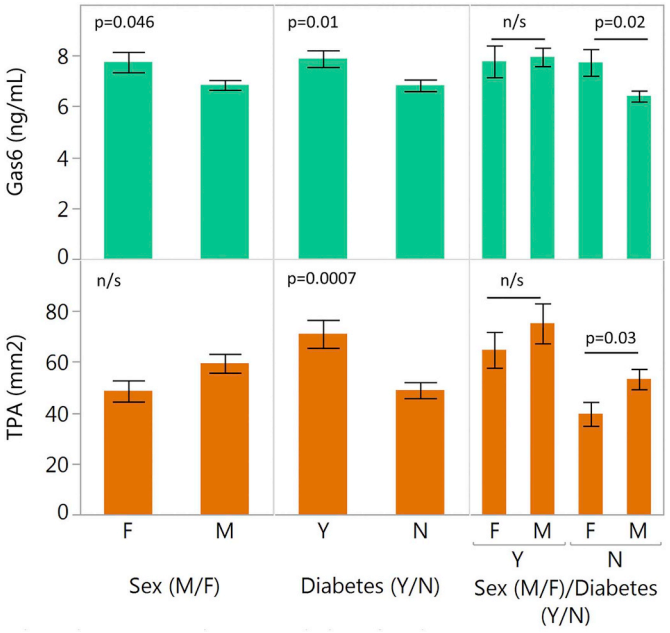


**Fig. 1.** Mean levels of Gas6 and carotid total plaque area in males and females and in relation to diabetes. Gas6 levels were lower in males and this remained significant in non-diabetic participants. Participants with diabetes had increased plaque burden, but decreased Gas6. Bars: standard error, F: female, M: male, n/s: non-significant, TPA: total plaque area, Y/N: yes/no.

hypothesized that this could explain, in part, the infrequent atherosclerotic events involving the LIMA artery when used in coronary artery bypass grafting.

Although the results of these studies suggest that Gas6 may be 'protective', there is conflicting data to indicate the opposite. For example, Gas6 has also been shown to be expressed in macrophages, smooth muscle cells and endothelial cells in all stages of atherosclerosis but most abundantly in the macrophage-rich areas of fibrous cap atheromas [21]. Plaques from Gas6 deficient mice on an apoE−/− background were shown to contain more collagen and fewer inflammatory cells [21]. Thus, there is also evidence to suggest that a role for Gas6 may be to attract leukocytes to the arterial wall and activate endothelial cells and thus, enhance inflammation in the acute phase of atherosclerosis.

Whether, and to what degree, the function of Gas6, as it relates to atherosclerosis, is modified by vitamin K status is unknown as we have no knowledge of the carboxylation status of the protein. The association between Gas6 and a biomarker of vitamin K status has been examined in one study of hemodialysis patients. These investigators found no relationship between Gas6 level and prothrombin induced by vitamin K absence-II (PIVKA-II), a biomarker that reflects the adequacy of recent dietary vitamin K intake [3]. However, the majority of these patients (73%) exhibited vitamin K deficiency and the assay used for measurement of Gas6 did not assess carboxylation status. Carboxylation of the Gla domain by vitamin K may be critical to the ability of the protein to bind phosphatidylserine residues and therefore an impact of vitamin K status might be expected [2]. Two studies have demonstrated that vitamin K antagonism with warfarin in humans induces a vulnerable plaque phenotype supporting a role for activated vitamin K dependent proteins in plaque remodeling [22,23]. There are several reports linking vitamin K status to coronary atherosclerosis, particularly in patients with reduced kidney function, however these investigations have primarily focused on the role of matrix Gla protein as opposed to Gas6 [24–28]. In this study, Gas6 was inversely associated with eGFR. However, this cohort was restricted largely to people with normal kidney function as the median eGFR was 78 [IQR 65, 89] ml/min/1.73m$^2$. The association of eGFR with carotid atherosclerosis is also established [29].

**Table 5**
Multi-variable linear regression models for plaque height and area.

5A: Dependent variable = Maximum plaque height (mm)

| Pre-selected predictors variables | Individual predictors | | Estimate | SE | 95% Confidence Interval | |
|---|---|---|---|---|---|---|
| | β | p-value | | | Lower bound | Upper bound |
| Age (years) | 0.26 | 0.0002 | 0.04 | 0.01 | 0.02 | 0.06 |
| Sex [Male] | 0.03 | 0.60 | 0.05 | 0.09 | −0.13 | 0.23 |
| eGFR (mL/min/1.73 m$^2$) | 0.04 | 0.51 | 0.003 | 0.004 | −0.01 | 0.01 |
| BMI (kg/m$^2$) | −0.04 | 0.56 | −0.01 | 0.01 | −0.04 | 0.02 |
| Diabetes | 0.22 | 0.002 | 0.32 | 0.10 | 0.12 | 0.52 |
| Tobacco use | 0.08 | 0.25 | 0.14 | 0.12 | −0.10 | 0.38 |
| Hypertension | 0.05 | 0.44 | 0.08 | 0.11 | −0.13 | 0.29 |
| Dyslipidemia | 0.23 | 0.0006 | 0.41 | 0.12 | 0.18 | 0.64 |
| Gas6 (ng/mL) | −0.16 | 0.02 | −0.08 | 0.03 | −0.14 | −0.02 |
| Model summary: | | | | | | |
| Adjusted R-Square | 0.19 | | | | | |
| Model significance | $P < .0001$ | | | | | |

5B: Dependent variable = Total plaque area (mm$^2$)

| Pre-selected predictors variables | Individual predictors | | Estimate | SE | 95% Confidence Interval | |
|---|---|---|---|---|---|---|
| | β | p-value | | | Lower bound | Upper bound |
| Age (years) | 0.25 | 0.0004 | 1.09 | 0.30 | 0.50 | 1.68 |
| Sex [Male] | 0.12 | 0.07 | 5.07 | 2.75 | −0.36 | 10.5 |
| eGFR (mL/min/1.73 m$^2$) | 0.01 | 0.87 | 0.12 | 0.12 | −0.78 | 0.92 |
| BMI (kg/m$^2$) | 0.06 | 0.34 | 0.07 | 0.43 | −0.12 | 0.36 |
| Diabetes | 0.25 | 0.0003 | 11.2 | 3.03 | 5.19 | 17.1 |
| Tobacco use | 0.11 | 0.10 | 6.09 | 3.65 | −1.11 | 13.3 |
| Hypertension | 0.02 | 0.77 | 0.91 | 3.17 | −5.34 | 7.16 |
| Dyslipidemia | 0.20 | 0.004 | 10.3 | 3.50 | 3.37 | 17.2 |
| Gas6 (ng/mL) | −0.16 | 0.01 | −2.43 | 0.98 | −4.36 | −0.49 |
| Model summary: | | | | | | |
| Adjusted R-Square | 0.20 | | | | | |
| Model significance | $P < .0001$ | | | | | |

BMI: body mass index, eGFR: estimated glomerular filtration rate, SE: standard error.

We found significant associations between Gas6 and two clinical measures of obesity (BMI and waist circumference [WC]), as well as diabetes. Two other observational studies in humans have similarly shown that higher levels of circulating Gas6 are associated with obesity, insulin resistance and altered glucose tolerance whilst one study reported lower levels in people with diabetes [30]. Gas6 has also been implicated in obesity in pre-clinical studies [8,30] whilst one study reported lower levels in people with diabetes [30]. Gas6 has also been implicated in obesity in pre-clinical studies [31]. Mice fed a high-fat diet with over-expression of Gas6/TAM had enhanced body fat accumulation whereas Gas6 deficient mice had less fat mass. Thus, at least in animal models, it appears that Gas6 could be involved in reversible growth arrest of pre-adipocytes allowing them to respond to adipogenic stimuli such as a high fat diet.

In this study, females had significantly higher levels of Gas6. The presence of a SHBG-like domain suggests a close association with sex hormones that could potentially modify cardiovascular risk. In one previous study of 278 middle-aged adults, a higher level of Gas6 was associated with obesity and markers of endothelial dysfunction and insulin resistance however this relationship was observed in women only [8]. In our study, the relationship between Gas6 with diabetes remained significant, after adjustment, when examined separately in

males. The direction of association was similar in females but the absence of statistical significance likely reflects the smaller sample size. In a study examining testosterone levels in relation to Gas6 in cardiac patients, it was found that Gas6 and testosterone levels were much lower in male patients with coronary heart disease than in control subjects [32].

## 5. Limitations

There are limitations to our study. Given that this is a cross-sectional study, the relationship we have found between Gas6 and atherosclerosis suggests that an association exists. Further studies are required to determine mechanisms and causality. The absence of carboxylation status of Gas6 limits the interpretation of this study as it relates to the activity of Gas6 and/or a potential role for vitamin K in atherosclerosis.

## 6. Conclusions

Although a higher level of the vitamin K dependent protein Gas6 was associated with adverse cardiovascular risk factors such as diabetes and obesity, it was independently associated with reduced carotid plaque height and reduced total plaque area in this study of people undergoing elective coronary angiography. This interesting finding, as of now suggested by only a few other investigators, points to the potential role of Gas6 as a protective factor in atherosclerosis and plaque remodeling [11]. There is now a critical need to understand the mechanism of this potential protective role, not only to further our understanding of the atherosclerotic process, but to contribute to potential future therapeutic target development.

## Declarations of interest

None.

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## References

[1] J.H. van der Meer, T. van der Poll, C. van't Veer, TAM receptors, Gas6, and protein S: roles in inflammation and hemostasis, Blood 123 (2014) 2460–2469.
[2] I. Hasanbasic, I. Rajotte, M. Blostein, The role of gamma-carboxylation in the anti-apoptotic function of gas6, J. Thromb. Haemost. 3 (2005) 2790–2797.
[3] I.J. Lee, B. Hilliard, A. Swami, J.C. Madara, S. Rao, T. Patel, J.P. Gaughan, J. Lee, C.A. Gadegbeku, E.T. Choi, P.L. Cohen, Growth arrest-specific gene 6 (Gas6) levels are elevated in patients with chronic renal failure, Nephrol. Dial. Transplant. 27 (2012) 4166–4172.
[4] C. Ekman, A. Linder, P. Akesson, B. Dahlback, Plasma concentrations of Gas6 (growth arrest specific protein 6) and its soluble tyrosine kinase receptor sAxl in sepsis and systemic inflammatory response syndromes, Crit. Care 14 (2010) R158.
[5] C. Ekman, A. Gottsater, B. Lindblad, B. Dahlback, Plasma concentrations of Gas6 and soluble Axl correlate with disease and predict mortality in patients with critical limb ischemia, Clin. Biochem. 43 (2010) 873–876.
[6] C. Ekman, A. Jonsen, G. Sturfelt, A.A. Bengtsson, B. Dahlback, Plasma concentrations of Gas6 and sAxl correlate with disease activity in systemic lupus erythematosus, Rheumatology (Oxford) 50 (2011) 1064–1069.
[7] H. Stepan, J. Richter, K. Kley, S. Kralisch, A. Jank, W. Schaarschmidt, T. Ebert, U. Lossner, B. Jessnitzer, J. Kratzsch, M. Bluher, M. Stumvoll, M. Fasshauer, Serum levels of growth arrest specific protein 6 are increased in preeclampsia, Regul. Pept. 182 (2013) 7–11.
[8] F.C. Hsiao, Y.F. Lin, P.S. Hsieh, N.F. Chu, Y.S. Shieh, C.H. Hsieh, C.H. Lee, Y.J. Hung, Circulating growth arrest-specific 6 protein is associated with adiposity, systemic inflammation, and insulin resistance among overweight and obese adolescents, J. Clin. Endocrinol. Metab. 98 (2013) E267–E274.
[9] F.C. Kuo, Y.J. Hung, Y.S. Shieh, C.H. Hsieh, F.C. Hsiao, C.H. Lee, The levels of plasma growth arrest-specific protein 6 is associated with insulin sensitivity and inflammation in women, Diabetes Res. Clin. Pract. 103 (2014) 304–309.
[10] A.M. Johri, J.E. Herr, T.Y. Li, O. Yau, V. Nambi, Novel ultrasound methods to investigate carotid artery plaque vulnerability, J. Am. Soc. Echocardiogr. 30 (2017) 139–148.
[11] S. Clauser, O. Meilhac, I. Bieche, P. Raynal, P. Bruneval, J.B. Michel, D. Borgel, Increased secretion of Gas6 by smooth muscle cells in human atherosclerotic carotid plaques, Thromb. Haemost. 107 (2012) 140–149.
[12] A.M. Johri, C.M. Calnan, M.F. Matangi, J. MacHaalany, M.F. Hetu, Focused vascular ultrasound for the assessment of atherosclerosis: a proof-of-concept study, J. Am. Soc. Echocardiogr. 29 (2016) 842–849.
[13] A.M. Johri, P. Behl, M.F. Hetu, M. Haqqi, P. Ewart, A.G. Day, B. Parfrey, M.F. Matangi, Carotid ultrasound maximum plaque height-a sensitive imaging biomarker for the assessment of significant coronary artery disease, Echocardiography 33 (2016) 281–289.
[14] G.M. Biasi, A. Froio, E.B. Diethrich, G. Deleo, S. Galimberti, P. Mingazzini, A.N. Nicolaides, M. Griffin, D. Raithel, D.B. Reid, M.G. Valsecchi, Carotid plaque echolucency increases the risk of stroke in carotid stenting: the imaging in Carotid Angioplasty and risk of Stroke (ICAROS) study, Circulation 110 (2004) 756–762.
[15] M.M. Sabetai, T.J. Tegos, A.N. Nicolaides, S. Dhanjil, G.J. Pare, J.M. Stevens, Reproducibility of computer-quantified carotid plaque echogenicity: can we overcome the subjectivity? Stroke 31 (2000) 2189–2196.
[16] Z. Cagman, O.O. Bingol, Z. Cirakli, A. Gedikbasi, P. Ay, D. Colantonio, A.R. Uras, K. Adeli, F. Uras, Reference intervals for growth arrest-specific 6 protein in adults, Scand. J. Clin. Lab. Invest. 77 (2017) 109–114.
[17] H. Jaksch-Bogensperger, A. Hammerschmid, L. Aigner, E. Trinka, R. Gehwolf, Y. Ebner, M. Hutterer, S. Couillard-Despres, Proseek single-plex protein assay kit system to detect sAxl and Gas6 in serological material of brain tumor patients, Biotechnol Rep (Amst) 18 (2018) e00252.
[18] Y. Ishimoto, K. Ohashi, K. Mizuno, T. Nakano, Promotion of the uptake of PS liposomes and apoptotic cells by a product of growth arrest-specific gene, gas6, J. Biochem. 127 (2000) 411–417.
[19] T. Nakano, K. Kawamoto, K. Higashino, H. Arita, Prevention of growth arrest-induced cell death of vascular smooth muscle cells by a product of growth arrest-specific gene, gas6, FEBS Lett. 387 (1996) 78–80.
[20] C.H. Lee, Y.S. Shieh, C.S. Tsai, Y.J. Hung, Y.T. Tsai, C.Y. Lin, Expression of growth arrest-specific protein 6 and Axl molecules in the left internal mammary artery of patients undergoing coronary artery bypass grafting, J. Clin. Pathol. 67 (2014) 506–511.
[21] E. Lutgens, M. Tjwa, F.P. Garcia De, E. Wijnands, L. Beckers, B. Dahlback, M.J. Daemen, P. Carmeliet, L. Moons, Genetic loss of Gas6 induces plaque stability in experimental atherosclerosis, J. Pathol. 216 (2008) 55–63.
[22] F. Plank, C. Beyer, G. Friedrich, M. Stuhlinger, F. Hintringer, W. Dichtl, M. Wildauer, G. Feuchtner, Influence of vitamin K antagonists and direct oral anticoagulation on coronary artery disease: a CTA analysis, Int. J. Cardiol. 260 (2018) 11–15.
[23] L.J. Schurgers, I.A. Joosen, E.M. Laufer, M.L. Chatrou, M. Herfs, M.H. Winkens, R. Westenfeld, V. Veulemans, T. Krueger, C.M. Shanahan, W. Jahnen-Dechent, E. Biessen, J. Narula, C. Vermeer, L. Hofstra, C.P. Reutelingsperger, Vitamin K-antagonists accelerate atherosclerotic calcification and induce a vulnerable plaque phenotype, PLoS One 7 (2012) e43229.
[24] M.D. Crosier, S.L. Booth, I. Peter, B. Dawson-Hughes, P.A. Price, C.J. O'Donnell, U. Hoffmann, M.K. Williamson, J.M. Ordovas, Matrix Gla protein polymorphisms are associated with coronary artery calcification in men, J. Nutr. Sci. Vitaminol. (Tokyo) 55 (2009) 59–65.
[25] K.A. Lomashvili, X. Wang, R. Wallin, W.C. O'Neill, Matrix Gla protein metabolism in vascular smooth muscle and role in uremic vascular calcification, J. Biol. Chem. 286 (2011) 28715–28722.
[26] G. Schlieper, R. Westenfeld, T. Kruger, E.C. Cranenburg, E.J. Magdeleyns, V.M. Brandenburg, Z. Djuric, T. Damjanovic, M. Ketteler, C. Vermeer, N. Dimkovic, J. Floege, L.J. Schurgers, Circulating nonphosphorylated carboxylated matrix gla protein predicts survival in ESRD, J. Am. Soc. Nephrol. 22 (2011) 387–395.
[27] L.J. Schurgers, E.C. Cranenburg, C. Vermeer, Matrix Gla-protein: the calcification inhibitor in need of vitamin K, Thromb. Haemost. 100 (2008) 593–603.
[28] L.J. Schurgers, D.V. Barreto, F.C. Barreto, S. Liabeuf, C. Renard, E.J. Magdeleyns, C. Vermeer, G. Choukroun, Z.A. Massy, The circulating inactive form of matrix gla protein is a surrogate marker for vascular calcification in chronic kidney disease: a preliminary report, Clin. J. Am. Soc. Nephrol. 5 (2010) 568–575.
[29] S.W. Choi, H.Y. Kim, Y.H. Lee, S.Y. Ryu, S.S. Kweon, J.A. Rhee, J.S. Choi, M.H. Shin, eGFR is associated with subclinical atherosclerosis independent of albuminuria: the Dong-gu Study, Atherosclerosis 212 (2010) 661–667.
[30] Y.J. Hung, C.H. Lee, N.F. Chu, Y.S. Shieh, Plasma protein growth arrest-specific 6 levels are associated with altered glucose tolerance, inflammation, and endothelial dysfunction, Diabetes Care 33 (2010) 1840–1844.
[31] E. Maquoi, G. Voros, P. Carmeliet, D. Collen, H.R. Lijnen, Role of Gas-6 in adipogenesis and nutritionally induced adipose tissue development in mice, Arterioscler. Thromb. Vasc. Biol. 25 (2005) 1002–1007.
[32] R. Zhao, Y. Li, W. Dai, Serum sex hormone and growth arrest-specific protein 6 levels in male patients with coronary heart disease, Asian J Androl 18 (2016) 644–649.