Reduced serum levels of vasostatin-2, an anti-inflammatory peptide derived from chromogranin A, are associated with the presence and severity of coronary artery disease

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Aims
We here investigated the endothelial effects of the chromogranin A-derived peptide vasostatin-2 and its relation to coronary artery disease (CAD).

Methods and results
We assessed the impact of recombinant vasostatin-1 and vasostatin-2 on tumour necrosis factor-alpha (TNFα)-, angiotensin II-, and oxidized low-density lipoprotein (oxLDL)-induced expression of adhesion molecules in human arterial endothelial cells. Vasostatin-1 and vasostatin-2 levels were examined in coronary endarterectomy specimens (n = 23), atherosclerotic aortas (n = 16), non-significant-atherosclerotic internal mammary arteries (n = 30), and non-atherosclerotic aortas (n = 10), as well as in peripheral blood mononuclear cells (PBMCs) from severe CAD patients (n = 50) and healthy volunteers (n = 21). Serum levels of vasostatin-2 were analysed in 968 consecutive patients undergoing coronary angiography. Vasostatin-1 and vasostatin-2 concentration-dependent inhibits TNFα-, angiotensin II-, and oxLDL-induced expression of adhesion molecules; and attenuated TNFα-induced adhesion of U937 monocytes to endothelial cells. Vasostatin-2 levels were significantly decreased in endarterectomy samples and atherosclerotic aortas compared with non-atherosclerotic internal mammary arteries and aortas, as well as in PBMCs of severe CAD patients compared with healthy controls (all P < 0.05). Serum vasostatin-2 levels were significantly lower in CAD patients (diameter stenosis ≥ 50%, n = 554) than in controls (normal arteries or diameter stenosis < 30%, n = 281) (P < 0.001). Its concentrations correlated with the number of diseased coronary arteries and Syntax score in CAD patients (all P < 0.05). At multivariable regression analysis, decreased vasostatin-2 levels remained associated with CAD when other variables were taken into account.

Conclusion
Vasostatin-2 has anti-inflammatory properties and is decreased in atherosclerotic plaque specimens and in PBMC of CAD patients. Decreased serum vasostatin-2 levels are associated with the presence and severity of CAD.

Keywords
Vasostatin-2 • Adhesion molecule • Inflammation • Endothelium • Coronary artery disease

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Introduction

Chromogranin A (CgA) is a protein ubiquitously expressed in the secretory cells of endocrine and neuronal tissues, and co-released with catecholamines. Physiologically, CgA acts as a pro-hormone, giving rise to biologically active peptides by proteolytic cleavage, including vasostatin-1 (human CgA1−76), vasostatin-2 (human CgA1−113), chromacin (human CgA173−194), pancreastatin (human CgA250−301), and catestatin (human CgA352−372). Increased circulating CgA levels have important prognostic significance in patients with chronic heart failure and acute coronary syndromes. Among naturally CgA-derived peptides, rat CgA1−64, corresponding to human vasostatin-1, functions to inhibit endothelin-1-stimulated vasoconstriction. Vasostatin-1 suppresses tumour necrosis factor (TNF)α-stimulated gap formation in arterial endothelial cells, and decreases thrombin- or vascular endothelial growth factor-induced permeability of human vascular endothelial cells, thus featuring a protective effect on the endothelial barrier integrity. Moreover, vasostatin-1 decreases leucocyte recruitment, reduces vascular permeability, and inhibits inflammatory neovascularization in a mouse model of ear inflammation. Although vasostatin-1 and vasostatin-2 derive from cleavage within very close pairs of basic amino acid residues (the first and the second, respectively) in the CgA N-terminal domain, functional properties of vasostatin-2 and its relationship with cardiovascular diseases are unknown.

Because of the structural similarities of vasostatin-1 and -2, and because of the known concomitant reduction in vascular protective factors in atherosclerosis or diabetes, we hypothesized that vasostatin-2 plays a role in maintaining vascular homeostasis; and that its levels are decreased in patients with coronary artery disease (CAD) and/or diabetes. We therefore planned the present study to evaluate the relationship of serum levels of vasostatin-2 with CAD in a large cohort of patients. Here, we measured inflammatory markers [high-sensitivity C-reactive protein (hsCRP) and soluble adhesion molecules (sICAM-1, sVCAM-1, sE-selectin)] for comparison. Protein levels of vasostatin-1 and vasostatin-2 were assessed in coronary artery endarterectomy specimens, atherosclerotic aortas, non-atherosclerotic arteries (internal mammary arteries and aortas), and also in peripheral blood mononuclear cells (PBMCs) from patients with coronary artery multi-vessel disease and healthy volunteers. To clarify the biological functions of vasostatin-2 and its relation to atherosclerosis and to compare them with those of vasostatin-1, we examined the influence of recombinant human vasostatin-1 and vasostatin-2 on TNFα-, angiotensin II-, and oxidized low-density lipoprotein (oxLDL) protein-induced effects in human arterial endothelial cells (hAECs), being TNFα, angiotensin II, and oxLDL important putative mediators of atherosclerosis.

Methods

Materials

The M200 medium, RPMI-1640, and foetal bovine serum were from Cascade Biologies (Portland, OR, USA) and Invitrogen (Carlsbad, CA, USA). Recombinant human vasostatin-1 and vasostatin-2 were obtained from Adipo Bioscience (Santa Clara, CA, USA) and Abcam (Cambridge, UK), respectively. The primary antibodies [against CgA, VCAM-1, ICAM-1, E-selectin, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH)] and the secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Cell Signalling Technology, respectively (Beverly, MA, USA). Tumour necrosis factor-alpha was from R&D System, Minneapolis, MI, USA; angiotensin II from Sigma Aldrich (St Louis, MO, USA); and oxLDL from Biomedical Technologies (Stoughton, MA, USA).

Study population

Among 968 consecutive patients undergoing coronary angiography from September 2004 to March 2007 for the diagnosis and interventional treatment of CAD, and excluding—for the purpose of this research and to avoid confounding variables—patients with acute coronary syndromes, previous history of myocardial infarction and heart failure, we categorized 554 patients as ‘significant CAD’ (luminal diameter narrowing ≥50%, a severity level previously correlated with reduced coronary flow reserve and according to the clinical standards of the American College of Cardiology/American Heart Association guidelines for coronary angiography). Patients with CAD were further classified according to the number of diseased coronary arteries (1-, 2-, or 3-vessel disease).

The control group consisted of 281 subjects with either normal coronary arteries or non-significant CAD (diameter stenosis <30%) from the same original 968 subjects. There were 133 patients with coronary diameter stenoses ≥30% but <50%. Type 2 diabetes mellitus was defined as fasting serum glucose level >7.0 mmol/L or a 2 h post-prandial glucose >11.1 mmol/L or taking oral hypoglycaemic drugs or parenteral insulin.

To avoid the confounding influence of other diseases, we also here excluded patients with concomitant heart failure, valvular heart disease, congenital heart disease, cardiomyopathy, stroke, chronic viral or bacterial infection, asthma, tumours, or immune system disorders, as well as patients with type 1 diabetes mellitus by C-peptide measurement. Hypertension and hyperlipidaemia were defined according to the published guidelines.

The study protocol was approved by the hospital Ethics Committee, and the written informed consent was obtained from all subjects.

Coronary angiography

Selective coronary angiography was performed through the femoral or radial artery approach by interventional cardiologists blinded to the study protocol. Significant CAD was diagnosed visually if luminal diameter narrowing was estimated as ≥50% diameter stenosis in a major epicardial coronary artery; left main coronary artery narrowing ≥50% was considered as two-vessel disease. An atherosclerosis severity index was defined as the average of the most severe stenoses in the left main, left anterior descending, left circumflex, and right coronary arteries. An atherosclerosis extent index was calculated as the longitudinal percentage of coronary artery segments presenting a stenosis (100 × Σ[stenosis lengths]/Σ[segment lengths]). Additionally, the Syntax score was calculated as previously reported. The total Syntax score was composed of the individual scores for each separate lesion with a diameter stenosis ≥50% in a vessel ≥1.5 mm in diameter by visual assessment.

Biochemical investigations

Blood samples were collected after an overnight fasting in all participants. Serum glucose, liver function, blood urea nitrogen, creatinine,
Vasostatin-2 and coronary artery disease

uric acid, total cholesterol, low-density lipoprotein-cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), lipoprotein (a), apoprotein A, apoprotein B, and triglycerides were measured with standard laboratory techniques on a Hitachi 912 Analyzer (Roche Diagnostics, Germany). Serum levels of hs-C-reactive protein (Biocheck Laboratories, Toledo, OH, USA), sICAM-1, and sVCAM-1 (R&D systems, Minneapolis, MN, USA) were determined by ELISA.22

Serum vasostatin-2 levels were determined with a commercially available human vasostatin-2 ELISA kit (Adipo Bioscience Inc, Santa Clara, CA, USA). For this ELISA, the company produced an anti-vasostatin-2 antibody from a rabbit immunized with recombinant human vasostatin-2/CgA (19–131). According to the manufacturer, this antibody detects serum vasostatin-2, but does not react with other CgA homologues. The detection range was 0.32–1000 ng/mL, with an inter-assay coefficient of variation <14%.

Cell cultures and experimental protocol

Human arterial endothelial cells (Cascade Biologics, Portland, OR, USA) were maintained in medium 200 supplemented with growth factors, and cultured at 37°C in a 5% CO₂ humidified atmosphere. U937 monocytoïd cells were grown in RPMI-1640 with 10% foetal bovine serum. We used hAECS at passages 4–5 in all the experiments. Cells were grown to sub-confluence and then stimulated with TNFα (10 ng/mL), angiotensin II (10⁻⁶ mol/L), and oxLDL protein (100 µg/mL) for 24 h, in the presence or absence of recombinant human vasostatin-1 and vasostatin-2 proteins.

Treatment of human vascular samples

We obtained coronary artery endarterectomy samples from patients with severe atherosclerotic coronary arteries (n = 23), and surplus—otherwise discarded—specimens of internal mammary arteries with non-significant atherosclerosis from patients undergoing coronary artery bypass surgery (n = 30). Small pieces of atherosclerotic aorta specimens were collected from patients with severe multi-vessel CAD undergoing coronary artery bypass surgery (n = 16), and atherosclerotic lesions of the aorta were documented in these patients by CT angiography. Normal/non-atherosclerotic aortic tissue was obtained from patients receiving operations because of thoracic aortic aneurysm (n = 10) not associated with obvious genetic disorders or severe atherosclerosis. The intima of the aorta was isolated for molecular experiments.

After wash with PBS, all tissue samples were homogenized and sonicated in a lysis buffer containing 20 mM TRIS (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 0.1% SDS, and a protease inhibitor cocktail. The samples were stored at −80°C until use.

Isolation of human peripheral blood mononuclear cells

Samples (20 mL) of peripheral blood were withdrawn from patients with multi-vessel CAD (n = 50) and healthy volunteers (n = 21). Peripheral blood mononuclear cells were then isolated using dextran sedimentation and a Ficoll-Hypaque density-gradient separation, as previously described.24 Purified PBMCs were lysed in a lysis buffer containing 20 mM TRIS (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 0.1% SDS, and a protease inhibitor cocktail, and stored at −80°C until use.

Western analysis

Human arterial endothelial cells were harvested and sonicated in a lysis buffer containing 20 mM TRIS (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 0.1% SDS, and a protease inhibitor cocktail.25 Protein concentrations of tissue samples and cell lysates were determined according to the method of Bradford. Equal amounts of protein lysates were separated on SDS–polyacrylamide gels, and transferred to nitrocellulose membranes. Membranes were blocked in 5% non-fat milk in TBST buffer, and then incubated with the primary antibody at 4°C overnight. Blots were developed using an electro-luminescence system (Pharmacia, Piscataway, NJ, USA) after incubation with a horseradish peroxidase-linked secondary antibody, using

<table>
<thead>
<tr>
<th>Table I</th>
<th>Baseline clinical characteristics and biochemical assessments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variable</td>
<td>Control group (n = 21)</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>141 (50.2)</td>
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<tr>
<td>Age (years)</td>
<td>61.8 (10.4)</td>
</tr>
<tr>
<td>Cigarette smoking, n (%)</td>
<td>45 (16.0)</td>
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<tr>
<td>Hypertension, n (%)</td>
<td>157 (55.9)</td>
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<tr>
<td>Systolic BP (mmHg)</td>
<td>128 (17)</td>
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<tr>
<td>Diastolic BP (mmHg)</td>
<td>78 (10)</td>
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<tr>
<td>Hyperlipidaemia, n (%)</td>
<td>107 (38.1)</td>
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<tr>
<td>Type 2 diabetes mellitus</td>
<td>100 (35.6)</td>
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<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.63 (0.91)</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>4.26 (1.06)</td>
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<tr>
<td>HDL-C (mmol/L)</td>
<td>1.20 (0.32)</td>
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<tr>
<td>LDL-C (mmol/L)</td>
<td>2.51 (0.84)</td>
</tr>
<tr>
<td>Apoptosis A (g/L)</td>
<td>1.25 (0.3)</td>
</tr>
<tr>
<td>Apoptosis B (g/L)</td>
<td>0.86 (0.24)</td>
</tr>
<tr>
<td>Lipoprotein (a) (g/L)</td>
<td>0.18 (0.15)</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>100 (27.2)</td>
</tr>
<tr>
<td>Blood urea nitrogen (mmol/L)</td>
<td>5.16 (1.44)</td>
</tr>
<tr>
<td>Creatinine (µmol/L)</td>
<td>76.6 (20.9)</td>
</tr>
<tr>
<td>Uric acid (µmol/L)</td>
<td>328.1 (83.4)</td>
</tr>
<tr>
<td>ACEI or ARB, n (%)</td>
<td>135 (48.0)</td>
</tr>
<tr>
<td>Beta-blockers, n (%)</td>
<td>118 (42.0)</td>
</tr>
<tr>
<td>Antipla telet agents, n (%)</td>
<td>85 (30.2)</td>
</tr>
<tr>
<td>Statins, n (%)</td>
<td>98 (34.9)</td>
</tr>
<tr>
<td>Insulin, n (%)</td>
<td>28 (10)</td>
</tr>
<tr>
<td>Vasostatin-2 (ng/mL)</td>
<td>5.82 (3.22)</td>
</tr>
<tr>
<td>hs-C-reactive protein (mg/L)</td>
<td>6.13 (4.41)</td>
</tr>
<tr>
<td>sICAM-1 (ng/mL)</td>
<td>269.4 (109.1)</td>
</tr>
<tr>
<td>sVCAM-1 (ng/mL)</td>
<td>522.7 (252.3)</td>
</tr>
</tbody>
</table>

Data are number (%) and mean (SD).

CAD, coronary artery disease; BP, blood pressure; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; sICAM-1, soluble inter-cellular adhesion molecule 1; sVCAM-1, soluble vascular cell adhesion molecule 1.
GAPDH as an internal control. Band intensity was quantified by scanning densitometry.

**Quantitative real-time polymerase chain reaction analysis**

Total RNA was isolated using the Trizol reagent (Invitrogen, San Diego, CA, USA). For reverse transcription, 1 μg of the total RNA was converted to first-strand cDNA using a reverse transcription kit (Promega, Madison, WI, USA). Quantitative real-time polymerase chain reaction (PCR) analysis was performed (StepOne, Applied Biosystems, Foster City, CA, USA) using SYBR Green (Takara, Otsu, Japan). The thermal cycling program was 10 s at 95°C for enzyme activation; 40 cycles of denaturation at 95°C for 5 s; and annealing and extension at 60°C for 31 s. The comparative cycle threshold method was used to determine relative mRNA expression of genes as normalized by the GAPDH housekeeping gene. Primer sequences are detailed as follows: VCAM-1: sense: 5′-TGTTTGTCAGGCTAAG-3′; anti-sense: 5′-GACCAAGACGTTGTATC-3′; ICAM-1: sense: 5′-AGGTGTATGAACGTGAC-3′; anti-sense: 5′-TGGGACGTAGGTAAG-3′; and E-selectin: sense: 5′-GTTAGGAACCCAGAAACC-3′; anti-sense: 5′-TGTAAGCATAGGGCAAG-3′.

**Endothelial cell adhesion assays**

Endothelial cell adhesion assays were performed using a commercially available kit (ECM645, Millipore). Briefly, hAECs were seeded in black fluorescence tissue culture plates and grown for 48–72 h or until confluence. Cells were treated with the control pro-inflammatory factor TNFa (with or without cycloheximide); then calcein AM-labelled U937 cells were added to the wells. The plate was briefly incubated to allow for cell binding, washing out non-specific cell binding. Finally, the plate was read at 485 nm/530 nm wavelengths in a fluorescence plate reader.

**Statistical analysis**

Continuous variables are presented as mean (SD or SEM, as indicated), and categorical data were summarized as frequencies or percentages. For categorical clinical variables, differences were evaluated by the Chi-square test. Normal distribution of continuous variable was assessed with the Kolmogorov–Smirnov test, and logarithmic or square root transformations were performed on continuous variables with the non-normal distribution. Differences among groups were analysed by one-way analysis of variance (ANOVA), followed by a post hoc least significance difference analysis for between-group comparisons. Unpaired Student’s t-test was performed to assess difference in parameters between the CAD and non-CAD groups, in diabetic or non-diabetic patients. The correlations of serum vasostatin-2 (dependent variable) with other biomarkers and the number of diseased coronary arteries (independent variables) were assessed with the Pearson or Spearman rank test as appropriate. In the multivariable stepwise logistic regression analysis, we adopted two models: in model 1, we introduced conventional risk factors (including those listed in Table 1, see below) for the assessment of CAD determinants. In model 2, we additionally adjusted the analysis for the novel factor vasostatin-2. Receiver operating characteristic analysis of risk factors was performed including and excluding vasostatin-2. Using C statistics, we compared the discriminatory capability of model 2 and model 1. We also evaluated risk reclassification with the addition of vasostatin-2, according to the method developed by Pencina et al., for determining the net reclassification improvement (NRI) and category-less NRI. Model calibration was assessed using the Hosmer–Lemeshow χ² test.

![Figure 1](https://academic.oup.com/eurheartj/article-abstract/33/18/2297/425326/3388327) Chromogranin A (CgA, 55 kDa) and chromogranin A-derived vasostatin-2 and vasostatin-1 (17 and 10 kDa) levels in endarterectomy specimens and control tissue. Chromogranin A and chromogranin A-derived peptide levels were examined by western blot (left panel) and quantitated by densitometry (right panels) in endarterectomy samples of coronary atherosclerotic arteries (n = 23) and in non-atherosclerotic internal mammary arteries (n = 30). *P < 0.05 vs. non-atherosclerotic vessels.
For cell experiments, data represent the average of six experiments. All analyses used two-sided tests, with an overall significance level of alpha = 0.05. Statistical analyses were performed with the SPSS 13.0 for Windows (SPSS, Inc., Chicago, IL, USA) and SAS Version 9.1 (SAS Institute, Cary, NC, USA).

Results

Ex vivo studies: protein levels of vasostatin-2 in vascular samples and in peripheral blood mononuclear cells

To evaluate vasostatin levels in atherosclerotic and control vessels, we examined protein expression in endarterectomy samples of severe atherosclerotic coronary arteries (n = 23) and in internal mammary arteries with non-significant atherosclerosis (n = 30) by western blot. We found significantly higher protein expression of CgA and, in contrast, remarkably lower expression of both vasostatin-1 and vasostatin-2 in atherosclerotic vessels when compared with control tissue (all P < 0.05) (Figure 1). To exclude the influence of intrinsic disparity of protein levels across different arteries, we analysed vasostatin-2 levels in atherosclerotic (n = 16) and non-atherosclerotic aortic intimas (n = 10). Consistent results of increased CgA and decreased vasostatin-2 levels were observed in atherosclerotic aorta compared with controls (Figure 2).

In addition, we asked the question of whether vasostatin levels differ in PBMCs between CAD patients and healthy controls. We isolated PBMCs from patients with multi-vessel CAD (n = 50) and healthy volunteers (n = 21), and analysed vasostatin levels. Notably, a significant difference was observed in vasostatin-2 levels between CAD patients and controls (P < 0.05) (Figure 3).

In vivo study: baseline characteristics of study patients

Baseline demographic and clinical characteristics of all participants are listed in Table 1. Compared with the control group, patients in the CAD group were older, more frequently male and cigarette smokers, and with higher blood pressure. Serum levels of HDL and apoA were lower, while apoB and lipoprotein (a) were higher in the CAD group than in the control group (all P < 0.05). Serum creatinine and blood urea nitrogen levels were

Figure 2 Vasostatin-2 in peripheral blood mononuclear cells. Vasostatin-2 levels were assessed by western blot in peripheral blood mononuclear cells from severe atherosclerotic patients (n = 50) and healthy controls (n = 21). *P < 0.05 vs. healthy controls.

Figure 3 Chromogranin A (CgA, 55 kDa) and chromogranin A-derived vasostatin-2 (17 kDa) levels in atherosclerotic and non-atherosclerotic aortic intima specimens. Chromogranin A and chromogranin A-derived vasostatin-2 levels were examined by western blot (left panel) and quantitated by densitometry (right panels) in atherosclerotic aorta (n = 16) and in non-atherosclerotic aorta specimens (n = 10). *P < 0.05 vs. non-atherosclerotic aorta.
significantly increased in patients with CAD (both $P < 0.001$). The rate of type 2 diabetes was similar in the two groups. In patients with CAD, those with diabetes had more multi-vessel diseases and higher disease extent and severity index (all $P < 0.05$ vs. non-diabetic subjects).

**Serum levels of vasostatin-2, high-sensitivity-C-reactive protein, and soluble adhesion molecules**

Serum vasostatin-2 levels were significantly lower in the CAD group than in the control group ($P < 0.01$). In contrast, serum levels of hs-C-reactive protein, sICAM-1, and sVCAM-1 were higher in the CAD group than in the control group (for all such comparisons, $P < 0.001$) (Table 1).

Serum vasostatin-2 levels correlated significantly, and always negatively, with the number of diseased coronary arteries (Pearson's $r = -0.206, P < 0.001$), the severity (Pearson's $r = -0.178, P < 0.01$), the extent index (Pearson's $r = -0.192, P < 0.01$), as well as the Syntax score (Pearson's $r = -0.248, P < 0.001$). There were progressively lower levels of vasostatin-2 across 1-, 2-, and 3-vessel disease subgroups (4.62 ± 3.09, 4.36 ± 3.26, and 4.25 ± 2.15 ng/mL, respectively), but such differences did not achieve statistical significance (ANOVA $P = 0.451$; with difference between 1- and 2-vessel disease subgroups, $P = 0.297$; and with difference between 1- and 3-vessel disease subgroups, $P = 0.282$). Furthermore, vasostatin-2 levels were negatively correlated with sVCAM-1 concentrations (Pearson’s $r = -0.088, P = 0.025$).

For patients with coronary artery diameter stenosis ≥30% but <50%, serum levels of vasostatin-2 were 4.76 ± 3.03 ng/mL, higher than in the control group ($P < 0.01$), but not significantly different from those of the CAD group (Supplementary material online, Table 1).

To assess the influence of type 2 diabetes mellitus on serum vasostatin-2 levels, we further subgrouped the subjects of the control and the CAD group according to the presence or absence of diabetes. Serum vasostatin-2 levels were significantly lower in diabetic than in non-diabetic patients both in the control and the CAD groups (both $P < 0.001$) (Figure 4).

**Multivariable logistic regression analysis**

Multivariable logistic regression analysis was performed to ascertain the risk of CAD as a function of conventional risk factors and biochemical variables as listed in Table 1 (Table 2). In Model 1, adjusted for conventional cardiovascular risk factor and established inflammatory factors, male gender, age, smoking, hypertension, hs-C-reactive protein, sICAM-1, and sVCAM-1 were independent risk factors for CAD. When vasostatin-2 was included in the multivariable analysis (Model 2), they all (including vasostatin-2) remained significantly associated with CAD (Table 2). In the study subjects, the addition of vasostatin-2 marginally, yet significantly, improved risk prediction (C statistic, from 0.754 to 0.78; $P = 0.03$ (Supplementary material online, Figure 51)). Net reclassification improvement was also improved significantly with vasostatin-2 (by 5.94%; $P < 0.001$) with the cut-point for categories of risk set at 0.5. Similarly, category-less NRI was also improved (by 29.97%, $P < 0.001$). The calibration of Models 1 (0.789) and 2 (0.439) was

![Figure 4](https://academic.oup.com/eurheartj/article-abstract/33/18/2297/425326/1822201220120528)  
**Figure 4** Vasostatin-2 serum levels in diabetic and non-diabetic patients, in the control and the coronary artery disease groups.

<table>
<thead>
<tr>
<th>Variable</th>
<th>OR (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Model 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>2.3 (1.6–3.4)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Age (SD)</td>
<td>2.3 (1.6–3.4)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Smoking</td>
<td>4.5 (2.7–7.6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hypertension</td>
<td>1.5 (1.0–2.3)</td>
<td>0.031</td>
</tr>
<tr>
<td>Hyperlipidaemia</td>
<td>1.4 (1.0–2.1)</td>
<td>0.068</td>
</tr>
<tr>
<td>Diabetes</td>
<td>1.4 (0.9–2.1)</td>
<td>0.094</td>
</tr>
<tr>
<td>hs-C-reactive protein (SD)</td>
<td>1.5 (1.0–2.2)</td>
<td>0.033</td>
</tr>
<tr>
<td>sICAM-1 (SD)</td>
<td>1.8 (1.2–2.6)</td>
<td>0.004</td>
</tr>
<tr>
<td>sVCAM-1 (SD)</td>
<td>1.9 (1.3–2.7)</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>Model 2</strong></td>
<td></td>
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<tr>
<td>Male</td>
<td>2.3 (1.5–3.4)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Age (SD)</td>
<td>2.5 (1.7–3.7)</td>
<td>&lt;0.001</td>
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<tr>
<td>Smoking</td>
<td>3.5 (2.1–6.0)</td>
<td>&lt;0.001</td>
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<tr>
<td>Hypertension</td>
<td>1.6 (1.0–2.4)</td>
<td>0.03</td>
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<tr>
<td>Hyperlipidaemia</td>
<td>1.3 (0.9–2.0)</td>
<td>0.141</td>
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<tr>
<td>Diabetes</td>
<td>1.1 (0.7–1.7)</td>
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<td>hs-C-reactive protein (SD)</td>
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<td>sICAM-1 (SD)</td>
<td>1.5 (1.0–2.2)</td>
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<td>sVCAM-1 (SD)</td>
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<td>&lt;0.001</td>
</tr>
<tr>
<td>Vasostatin-2 (SD)</td>
<td>0.3 (0.2–0.5)</td>
<td>&lt;0.001</td>
</tr>
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</table>

Abbreviations as in Table 1. OR denotes the odds ratio for significant coronary artery disease (CAD). CI, confidence interval.

For age, hs-C-reactive protein, sICAM-1, sVCAM-1, and vasostatin-2, the units of change were specified as 10 (SD), 4.41 (SD), 109.1 (SD), 252.3 (SD), and 3.22 (SD), respectively. The values of SD are from those of the Control group in Table 1, adjusted for conventional cardiovascular risk factor and established inflammatory factors.
The inclusion of vasostatin-2 in Table 2 resulted in a statistically significant 5.2% improvement (Nagelkerke R square for Models 1 and 2: 0.246 and 0.298, respectively; \( P < 0.05 \)) in explaining the variation in the dependent variable.

**Effects of vasostatin-1 and vasostatin-2 in vitro in cell experiments**

To clarify the biological functions of vasostatin-2 and to compare them with those of vasostatin-1, we added recombinant vasostatin-1 and vasostatin-2 at increasing concentrations (3, 4.5, and 6 \( \mu g/mL \)) to the medium of hAECs, and then stimulated the cells with TNF-\( \alpha \) (10 ng/mL), Ang II (10\(^{-6}\) mol/L), or oxLDL (100 \( \mu g/mL \)). Concentrations of vasostatin-1 and -2 in these experiments were chosen according to the previous literature.\(^{30,31}\) Although higher than concentrations measured in vivo, the biological significance of such concentrations is in the possibility that tissue concentrations of these peptides are higher than plasma concentrations, and in the possibly lower biological activity of prokaryotic recombinant proteins here used (in this case from *E. coli* BL21) compared with eukaryotic proteins. In control cells, these pro-inflammatory factors induced a significant increase in VCAM-1, ICAM-1, and E-selectin expression (Figure 5 and Supplementary material online, Figures S2 and S3). Notably, the addition of vasostatin-1 or vasostatin-2 inhibited the TNF-\( \alpha \)-, Ang II-, and oxLDL-induced increased adhesion molecule protein expression in a concentration-dependent manner. Similar results were obtained in characterizing adhesion molecule steady-state mRNA levels by real-time RT–PCR analysis (Supplementary material online, Figures 4–6).

Consistent with western analysis data, both vasostatin-1 and vasostatin-2 concentration-dependently reduced TNF-\( \alpha \)-induced adhesion of U937 monocyteid cells to hAECs (Figure 6), indicating functional anti-adhesive/anti-inflammatory properties of both vasostatins.

**Discussion**

Our study has unravelled that decreased levels of vasostatin-2 protein occurring in serum, PBMCs, and specimens of atherosclerotic plaques are modestly but significantly associated with the presence, extent, and severity of CAD. Vasostatin-2 also restrained TNF-\( \alpha \)-, Ang II-, and oxLDL-induced expression of adhesion molecules, and attenuated the adhesion of monocytes to endothelial...
cells. These results thus demonstrate a possible causal link between reduced CgA-derived vasostatins and atherosclerosis.

Chromogranin A is a secretory adipokine. Around 10 multi-functional regulatory peptides are generated by proteolytic cleavage of the CgA precursor, including vasostatin-1 and vasostatin-2.1,2 Vasostatin-1 exerts a spectrum of inhibitory influences: it counteracts endothelin-1-stimulated vasoconstriction, reduces TNFα- or VEGF-induced gap formation in endothelial

Figure 6 Vasostatin-1 and vasostatin-2 inhibit monocytoid cell adhesion to tumour necrosis factor-alpha (TNFα)-activated human arterial endothelial cells (hAECs). Upper panel: hAECs were seeded in black fluorescence tissue culture plates and grown for 48–72 h or until confluency. Cells were then treated with TNFα (with or without cycloheximide, CHX), and subsequently calcein AM-labelled U937 cells were added to the wells. Each plate was incubated briefly to allow for cell binding, and non-specifically bound cell were washed away. Finally, the plate was read at 485 nm/530 nm in a fluorescent plate reader, as described in Methods. Lower panel: quantitative analysis of binding of U937 cells to hAECs is presented by bar graphs of fluorescence values, *P < 0.05; **P < 0.01 vs. TNFα induction.
cells, and—probably by this mechanism—reduces vascular permeability in a mouse model of ear inflammation.\textsuperscript{6–9} Vascular properties and effects of vasostatin-2 at the vascular level have been, conversely, so far largely unknown. Because of their structural similarities, we postulated that vasostatin-2 might elicit vascular effects similar to vasostatin-1. Our \textit{in vitro} cell experimental results indeed support this hypothesis, documenting anti-inflammatory properties of vasostatin-2 in inhibiting adhesion molecule expression and monocoytoid cell adhesion to the endothelium after endothelial activation induced by three atherosclerosis-relevant and unrelated pro-inflammatory stimuli, such as the cytokine TNF-\alpha, the peptide Ang II, and oxLDL.

In atherosclerotic vascular tissue samples, we observed an elevation of CgA, but decreased levels of both vasostatin-1 and vasostatin-2 compared with control vessels. This information is consistent with the notion that CgA is closely involved in the development of major cardiovascular diseases.\textsuperscript{3–5} However, it also suggests that disturbed processing of CgA occurs in the atherosclerosis milieu, reflecting the impairment of vascular homeostasis. Chromogranin A is physiologically cleaved by tissue-specific proteases, such as plasmin, as a result of tissue-derived plasminogen activator-induced proteolytic activation of plasminogen to plasmin.\textsuperscript{32} However, levels and activities of tissue plasminogen activator are decreased in inflammatory and diabetic vascular tissues, and in smoking status.\textsuperscript{33–35} Such reduction might thus contribute to the disturbed CgA turnover here documented in atherosclerosis.

In the present study, serum vasostatin-2 levels were decreased in patients with CAD and/or diabetes. At multivariable regression analysis, vasostatin-2 was an independent risk factor for CAD, beyond the prediction allowed by traditional risk factors. Thus, such data suggest the possible usefulness of this molecule as a marker of atherosclerosis risk. Such data will need to be confirmed in other data setting and also in prospective cohort data.

The data on serum from peripheral blood are substantially supported by the findings of vascular tissues, although the difference between the CAD and the control groups was here not large. The reason for this is unclear. Nevertheless, these results suggest that vascular-protective activities of vasostatin-2 are reduced in atherosclerosis or in the presence of diabetes due to its reduced protein levels. Moreover, our study also shows decreased vasostatin-2 levels in PBMCs of patients with severe CAD. Previous reports had suggested that vasostatin-1 exerts the above-mentioned anti-inflammatory properties both extracellularly and intracellularly.\textsuperscript{32} Whether vasostatin-2 exerts anti-inflammatory properties by similar mechanisms and whether decreased vasostatin-2 levels in PBMCs relate to increased inflammation remain unclear.

Although the causes of reduced levels of vasostatin-2 in atherosclerosis and diabetes require further investigations, the plausibility of a causal role for such reduced levels in promoting diabetic atherosclerosis prompts the hypothesis that restoring such levels might reverse these pro-inflammatory changes. Thus, our study also paves the road to a possible new intervention target.

We recognize limitations in our findings. The most relevant one is that the study presented here is cross-sectional, thereby allowing to detect associations, but not to infer causality or to formulate predictions. Furthermore, the several exclusion criteria and the selection of the study population introduce several selection biases that may attenuate or even abrogate the prognostic significance here found for vasostatin-2. Larger-scale, long-term prospective studies are needed to confirm our results and to assess the prognostic significance of vasostatin-2 and of possible medications that change vasostatin-2 levels. However, the demonstration, as given in the present study, that decreased serum levels of vasostatin-2 are negatively associated with the presence and severity of CAD, together with the initial documentation of its anti-inflammatory vascular properties establish a rationale for such further investigations. Further data are also needed to explore the mechanisms underlying the anti-inflammatory profile of vasostatin-2.

Supplementary material
Supplementary material is available at \textit{European Heart Journal} online.

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Conflict of interest
none declared.

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