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RESEARCH PAPER

Plasma CTGF is independently related to an increased risk of cardiovascular events and mortality in patients with atherosclerotic disease: the SMART study

Karin G. Gerritsen^{1,2}, Lucas L. Falke¹, Stefan H. van Vuuren¹, Jan W. Leeuwis¹, Roel Broekhuizen¹, Tri Q. Nguyen¹, Gert Jan de Borst³, Hendrik M. Nathoe⁴, Marianne C. Verhaar², Robbert J. Kok⁵, Roel Goldschmeding¹, and Frank L. Visseren⁶; for the SMART Study Group

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Abstract

Aims: Connective tissue growth factor (CTGF) plays a key role in tissue fibrogenesis and growing evidence indicates a pathogenic role in cardiovascular disease. Aim of this study is to investigate the association of connective tissue growth factor (CTGF/CCN2) with cardiovascular risk and mortality in patients with manifest vascular disease. **Methods and results:** Plasma CTGF was measured by ELISA in a prospective cohort study of 1227 patients with manifest vascular disease (mean age 59.0 ± 9.9 years). Linear regression analysis was performed to quantify the association between CTGF and cardiovascular risk factors. Results are expressed as beta (β) regression coefficients with 95% confidence intervals (CI). The relation between CTGF and the occurrence of new cardiovascular events and mortality was assessed with Cox proportional hazard analysis. Adjustments were made for potential confounding factors. Plasma CTGF was positively related to total cholesterol (β 0.040; 95%CI 0.013–0.067) and LDL cholesterol (β 0.031; 95%CI 0.000–0.062) and inversely to glomerular filtration rate (β –0.004; 95%CI –0.005 to –0.002). CTGF was significantly lower in patients with cerebrovascular disease. During a median follow-up of 6.5 years (IQR 5.3–7.4) 131 subjects died, 92 experienced an ischemic cardiac complication and 45 an ischemic stroke. CTGF was associated with an increased risk of new vascular events (HR 1.21; 95%CI 1.04–1.42), ischemic cardiac events (HR 1.41; 95%CI 1.18–1.67) and all-cause mortality (HR 1.18; 95%CI 1.00–1.38) for every 1 nmol/L increase in CTGF. No relation was observed between CTGF and the occurrence of ischemic stroke. **Conclusions:** In patients with manifest vascular disease, elevated plasma CTGF confers an increased risk of new cardiovascular events and all-cause mortality.

Keywords

CTGF, cardiovascular disease, atherosclerosis, myocardial infarction, mortality

History

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Introduction

Cardiovascular risk is a growing concern and major health burden. Several management strategies exist, but healthcare could benefit from additional interventional strategies.

Connective tissue growth factor (CTGF/CCN2) is a key mediator of tissue fibrogenesis in various chronic diseases (Dendooven et al., 2011). Many cell types express CTGF, including endothelial cells, vascular smooth muscle cells, fibroblasts and cardiac myocytes (Ohnishi et al., 1998; Van Geest et al., 2014; Yan et al., 2014). CTGF is upregulated by

stimuli involved in cardiovascular damage, including angiotensin II, oxidative stress, endothelin-1, hyperglycemia, advanced glycation end products, transforming growth factor β and mechanical stretch (Cicha & Goppelt-Strube, 2009; Daniels et al., 2009; Lan et al., 2013; Ruiz-Ortega et al., 2007). Depending on the cell type and pathological context, CTGF is involved in various biological processes, including extracellular matrix production, proliferation, apoptosis, chemotaxis and angiogenesis.

Plasma CTGF is elevated in patients with type 1 diabetes mellitus (DM), chronic kidney disease and chronic heart failure (Cheng et al., 2006; Ito et al., 2011; Koitabashi et al., 2008; Nguyen et al., 2008; Roestenberg et al., 2004; Slagman et al., 2011). In patients with type 1 diabetes, plasma CTGF is associated with increased urinary albumin excretion, hypertension and increased carotid intima media thickness (cIMT) and in macroalbuminuric patients also with progression to end

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stage renal disease and increased mortality (Jaffa et al., 2008; Nguyen et al., 2008; Roestenberg et al., 2004). Reduction of vascular stiffness in hypertensive patients is associated with a reduction in CTGF expression levels (Gomez-Garre et al., 2006). In patients with both acute and chronic heart failure plasma CTGF is related to brain natriuretic peptide, NYHA class and echocardiographic parameters of diastolic dysfunction (Behnes et al., 2014; Koitabashi et al., 2008). Emerging evidence also indicates a role of CTGF in the pathogenesis of cardiovascular disease. While being minimally expressed in healthy tissue, CTGF is strongly upregulated in atherosclerotic plaques, in cardiac tissue after myocardial infarction, in cardiac fibrosis and in vascular and cardiac tissues in experimental hypertension (Daniels et al., 2009; Koitabashi et al., 2008; Leeuwis et al., 2010; Ponticos, 2013; Rickard et al., 2014).

Thus far, however, plasma CTGF has not been studied in patients with clinically manifest vascular disease. Considering the role of CTGF in the pathogenesis of cardiovascular fibrosis, we hypothesized that baseline plasma CTGF may reflect cardiovascular disease burden and may identify vascular patients at the highest risk of recurrent cardiovascular events. Therefore, in the present study we aimed to investigate the association of baseline plasma CTGF with future cardiovascular risk and mortality in a high-risk population of patients with manifest atherosclerotic vascular disease.

Methods

Study design and patients

We used data from patients enrolled in the Second Manifestations of ARterial disease (SMART) study, an ongoing prospective single-center cohort study in patients with manifest atherosclerotic disease or cardiovascular risk factors that started in September 1996 (Simons et al., 1999). Patients aged 18–80 years, newly referred to the University Medical Center (UMC) Utrecht with manifest atherosclerotic disease or a cardiovascular risk factor, underwent a vascular screening program including a questionnaire, laboratory assessments and noninvasive screening for manifestations of atherosclerotic disease and cardiovascular risk factors other than the qualifying diagnosis. Patients with terminal malignant disease, those not independent in daily activities (Rankin scale >3) or not sufficiently fluent in Dutch were excluded. The rationale and design of the study, including the criteria for the various manifestations of atherosclerotic disease, have been described in detail (Simons et al., 1999). The Medical Ethics Committee approved the study, and all the participants gave their written informed consent.

For the current study, data of 1227 participants with clinically manifest atherosclerotic disease (coronary heart disease, cerebrovascular disease, peripheral arterial disease or abdominal aortic aneurysm), included between June 2001 and January 2006, were analyzed.

Data acquisition

Baseline measurements were performed on a single day at the UMC Utrecht. Medical history, use of current medication and

current and past cigarette smoking behavior were derived from a standardized questionnaire described previously (Simons et al., 1999). Height, weight and blood pressure were measured. Glomerular filtration rate (GFR) was estimated by the abbreviated Modification of Diet in Renal Disease equation (Levey et al., 2006). The Framingham 10-year cardiovascular risk score (%) was calculated using gender, age, smoking behavior, blood levels of HDL and total cholesterol, and systolic blood pressure as parameters as described (Wilson et al., 1998). Ultrasound measurements of cIMT and abdominal adipose tissue were performed as described (Simons et al., 1999; Stolk et al., 2003).

Electrocardiographic left ventricular hypertrophy (LVH) was assessed by the Sokolow–Lyon voltage criterion ($SV_1 + RV_{5/6} > 3.5$ mV) (Sokolow & Lyon, 2001) and the Cornell voltage criterion ($RaVL + SV_3 > 2.0$ mV in women and > 2.8 mV in men) (Casale et al., 1985). The patients meeting either criterion were considered to have LVH. For 46 patients no valid electrocardiogram was available.

Blood samples were collected after an overnight fast. Plasma total cholesterol, low-density lipoprotein (LDL)-cholesterol, high-density lipoprotein (HDL)-cholesterol, triglycerides, homocysteine and creatinine were determined as described (Simons et al., 1999). High sensitivity C-reactive protein (hsCRP) was measured by immunonephelometry (Nephelometer Analyzer BN II, Dade-Behring, Marburg, Germany). HsCRP measurements below the lower limit of detection of 0.2 mg/L were set at 0.2 mg/L.

Plasma CTGF levels were determined by sandwich ELISA, using two humanized monoclonal antibodies, one for capture and one for detection (FibroGen Inc., San Francisco, CA) against two distinct epitopes on the aminoterminal part of CTGF, detecting both full length CTGF and the N-fragment, as described (Roestenberg et al., 2004). The detection limit of the assay was 0.02 nmol/L and intra- and interassay variations were <1 and 10%, respectively.

Urine albumin and creatinine were determined as described (Simons et al., 1999). The albumin-to-creatinine ratio (ACR) was used to estimate albuminuria. Microalbuminuria was defined as $ACR \geq 3.5$ and < 25 mg/mmol (female) or ≥ 2.5 and < 25 mg/mmol (male) and macroalbuminuria as $ACR \geq 25$ mg/mmol (Tapp et al., 2004).

Follow-up

Patients received a questionnaire every 6 months to provide information on hospitalization and outpatient clinic visits. Outcomes of interest for this study were a composite endpoint of vascular death, ischemic stroke and ischemic cardiac complications (Table 1). All-cause mortality was recorded as well. If a possible event was reported, original source documents were retrieved and reviewed. All possible events were audited by three independent physicians of the End Point Committee. If a patient had multiple events, the first event was used for analysis. Follow-up duration (years) was defined as the period between study inclusion and date of first cardiovascular event, date of death, date of loss to follow-up or the preselected date of 1 March 2010. From 1996 until 1 March 2010, 52 (4%) patients were lost to follow-up due to migration or discontinuation of the study.

Table 1. Definition of study outcome events.

Ischemic cardiac complication	Myocardial infarction, sudden death or fatal congestive heart failure
Ischemic stroke	Relevant clinical features that caused an increase in impairment of at least one grade on the modified Rankin scale, with or without a new relevant ischemic lesion at brain imaging
Vascular death	Death caused by myocardial infarction, stroke, sudden death (unexpected cardiac death occurring within 1 hour after onset of symptoms, or within 24 hours given convincing circumstantial evidence), congestive heart failure, rupture of abdominal aortic aneurysm or death from another vascular cause
Composite vascular outcome event/Vascular event	A composite of stroke, ischemic cardiac complication, vascular mortality, retinal infarction or bleeding or fatal rupture of abdominal aortic aneurysm
Other vascular event	A composite of hemorrhagic stroke, retinal infarction or bleeding, sudden death, fatal rupture of abdominal aortic aneurysm and other vascular complications or death.
Nonvascular death	Death caused by infection, cancer, unnatural death, or death from another nonvascular cause.
All-cause mortality	Death of a vascular or non-vascular cause

Data analysis

Continuous variables are expressed as mean \pm standard deviation (SD) when normal distributed or as median (interquartile range (IQR)) in case of skewed distribution. Categorical variables are expressed as numbers (percentage).

The relation between various patient characteristics and plasma CTGF was quantified with linear regression analysis with CTGF as the dependent variable, and adjustments were made for age, gender and estimated GFR (eGFR). The natural logarithm of CTGF was used to get a normal distribution that allowed parametric analysis. Results are expressed as beta (β) regression coefficients with 95% confidence intervals (95%CI) denoting the change in plasma CTGF for every change in each individual patient characteristic.

Cox proportional hazards analysis was performed to estimate hazard ratios (HRs) with 95% confidence intervals for the occurrence of a new vascular event and all-cause mortality associated with every 1 nmol/L increase in plasma CTGF. Three models were used. In model 1 the unadjusted association between plasma CTGF, cardiovascular events and mortality was examined. In model 2 adjustments were made for age, gender and eGFR, since eGFR was independently associated with plasma CTGF and is also related to cardiovascular outcome. In model 3 additional adjustments were made for the Framingham risk score (%), diabetes mellitus, systolic and diastolic blood pressure, total cholesterol, LDL cholesterol, use of renin angiotensin aldosterone system (RAAS) blockers and use of statins, which are all considered as potentially confounding factors in the relation between CTGF and vascular events. To investigate whether the relation between CTGF and new vascular events and between CTGF and all-cause mortality was modified by gender, we included these interaction terms in the Cox model. If the p -value of the interaction term was <0.05 effect-modification was considered to be present. Next, the study population was divided into tertiles of plasma CTGF and HRs for the occurrence of cardiovascular events and mortality were estimated for each tertile using the lowest plasma CTGF tertile as reference. In addition, one minus survival plots based on Cox regression analysis were made for each CTGF tertile with adjustment for the same confounding variables as included in model 3 (gender, age, eGFR, DM, blood pressure, total cholesterol, use of RAAS blockers and use of statins).

Single imputation methods were used to reduce missing covariate data for smoking ($n = 8$ ($<1\%$)), use of statins ($n = 48$ (4%)), systolic blood pressure ($n = 1$ ($<1\%$)), diastolic blood pressure ($n = 1$ ($<1\%$)), body mass index ($n = 1$ ($<1\%$)), abdominal adipose tissue ($n = 8$ ($<1\%$)), intima media thickness ($n = 25$ (2%)), eGFR ($n = 14$ (1%)), total cholesterol ($n = 16$ (1%)), LDL-cholesterol ($n = 21$ (2%)), HDL-cholesterol ($n = 21$ (2%)), triglycerides ($n = 19$ (2%)), homocysteine ($n = 18$ (1%)) and albuminuria ($n = 107$ (9%)), since incomplete case analysis leads to loss of statistical power and possibly bias. $p < 0.05$ was considered significant (two-tailed). All the analyses were performed with SPSS software (version 16.0; SPSS Inc., Chicago, IL).

Results

Baseline characteristics

In Table 2 the baseline characteristics of the study population are presented. Mean age was 59.0 ± 9.9 years and 80% was male. The majority of the patients had an eGFR >60 mL/min/ 1.73m^2 ($n = 1074$ (88%)). Median plasma CTGF was 1.18 nmol/L with a range of 0.18–6.47 nmol/L.

Relationship between plasma CTGF and patient characteristics

Compared to patients with other vascular disease localizations, plasma CTGF was lowest in patients with cerebrovascular disease. Plasma CTGF was inversely related to eGFR, adjusted for age and gender ($\beta -0.004$ using the natural logarithm of CTGF; 95%CI -0.005 to -0.002), which implies that for every 1 mL/min/ 1.73m^2 increase in eGFR plasma CTGF decreased with 0.4% (95%CI 0.2 to 0.5) (Table 3). Univariate analysis revealed that plasma CTGF relates to total cholesterol and LDL cholesterol ($R^2 = 0.0061$, $p < 0.01$ and $R^2 = 0.0031$, $p < 0.01$ respectively; Supplementary Figure 1). After the adjustment for age, gender and eGFR, plasma CTGF remained positively associated with total cholesterol ($\beta 0.040$; 95%CI 0.013 to 0.067) and LDL cholesterol ($\beta 0.031$; 95%CI 0.000 to 0.062). Plasma CTGF was not related to DM, blood pressure or pulse pressure, BMI or abdominal adipose tissue, left ventricular hypertrophy, cIMT, smoking, use of RAAS blockers, hsCRP or albuminuria. There were no

Table 2. Baseline characteristics of the study population ($n = 1227$).

Characteristic	
Age (years)	59.0 \pm 9.9
Male gender, n (%)	986 (80)
Hypertension (ever or current), n (%)	638 (52)
Diabetes mellitus, n (%)	199 (16)
Type 1, n (%)	6 (1)
Type 2, n (%)	193 (16)
Smoking	
Former, n (%); current, n (%)	578 (47); 438 (36)
Pack years	20.2 (6.1–34.2)
Framingham risk score (average 10-year risk in %)	13.4 \pm 9.27
<i>Medication</i>	
Blood pressure lowering agents, n (%)	914 (75)
RAAS blocker, n (%)	409 (33)
Statin, n (%)	786 (64)
Anti-platelet agents, n (%)	933 (76)
<i>Measurements</i>	
Systolic blood pressure (mmHg)	142 \pm 22
Diastolic blood pressure (mmHg)	82 \pm 12
Pulse pressure (mmHg)	58 (50–69)
Body mass index (kg/m ²)	26.9 \pm 3.8
Abdominal adipose tissue (cm)	9.5 \pm 2.6
Intima media thickness (mm)	0.90 (0.75–1.07)
eGFR (mL/min/1.73m ²)	78.1 (67.3–88.8)
Left ventricular hypertrophy*, n (%)	294 (25)
<i>Laboratory measurements</i>	
<i>Lipids</i>	
Total cholesterol (mmol/L)	4.9 \pm 1.0
LDL cholesterol (mmol/L)	2.9 \pm 0.9
HDL cholesterol (mmol/L)	1.3 \pm 0.4
Triglycerides (mmol/L)	1.50 (1.10–2.11)
HsCRP (mg/L)	2.0 (0.9–4.1)
Homocysteine (μ mol/L)	12.8 (10.5–15.5)
<i>Albuminuria</i>	
Microalbuminuria, n (%)	233 (19)
Macroalbuminuria, n (%)	39 (3)
Plasma CTGF (nmol/L)	1.18 (0.89–1.64)
<i>Localization of vascular disease†</i>	
Cerebrovascular disease, n (%)	293 (24)
Coronary artery disease, n (%)	755 (62)
Aneurysm abdominal aorta, n (%)	112 (9)
Peripheral arterial disease, n (%)	279 (23)

Data are expressed as mean \pm SD, median (interquartile range) or absolute number (percentage). CTGF = connective tissue growth factor; eGFR = estimated glomerular filtration rate; HDL = high-density lipoprotein; hsCRP = high-sensitivity C-reactive protein; LDL = low-density lipoprotein; RAAS = renin-angiotensin-aldosterone system.

*A valid ECG was available in 1181 patients.

†Patients could be classified into one or more categories.

indications for presence of a J-shaped relationship between these variables and plasma CTGF.

CTGF and risk of cardiovascular events and mortality

During a median follow-up of 6.5 years (IQR 5.3–7.4) (total number of follow-up years 7637), 131 subjects died (of whom 73 due to a cardiovascular cause), 92 experienced an ischemic cardiac complication and 45 an ischemic stroke. After adjustment for age, gender and eGFR, every 1 nmol/L increase in plasma CTGF was associated with an increased risk of all vascular events (HR 1.21; 95%CI 1.04–1.41), ischemic cardiac events (HR 1.39; 95%CI 1.17–1.65) and all-cause mortality (HR 1.19; 95%CI 1.02–1.39) (Table 4). RAAS activation, diabetes mellitus and changes in cholesterol

metabolism are phenomena known to influence plasma CTGF levels (Watts & Spiteri, 2004; Wolf, 2006; Yang et al., 2013). Therefore, additional adjustment for DM, blood pressure, total cholesterol, LDL cholesterol, use of RAAS blockers and use of statins were made. However, none substantially changed the relation between CTGF and cardiovascular events (model 3). Additional analyses adjusting for factors that were possibly in the causal pathway (Framingham risk score, cIMT, pulse pressure, hsCRP, left ventricular hypertrophy, LDL and albuminuria) also did not markedly alter the HRs (data not shown). The risk of ischemic stroke associated with plasma CTGF was not significantly increased (HR 0.88; 95%CI 0.61–1.27). The relation between CTGF and subsequent vascular events and between CTGF and all-cause mortality was not modified by gender (p -values for interaction 0.335 for all vascular events and 0.223 for all-cause mortality).

We further assessed the relationship between plasma CTGF and cardiovascular events and mortality by dividing the study population into tertiles of plasma CTGF (Figure 1, Table 5). Compared to the patients in the lowest tertile of plasma CTGF, patients in the highest tertile had a 61% higher risk of developing a new cardiovascular event and a 75% higher risk of dying from any cause, after adjustment for age, gender and eGFR (Table 5). HR for nonvascular death in the highest tertile did not reach significance when the lowest tertile was used as reference, but was significantly higher when the lowest and middle tertile were combined into a single reference group (HR 1.79; 95%CI 1.06–3.03).

Discussion

The main finding of the present study is that baseline plasma CTGF is associated with an increased risk of new cardiovascular events and mortality in patients with clinically manifest atherosclerotic vascular disease in a large population of patients with manifest vascular disease. This was independent of established cardiovascular risk factors

Plasma CTGF has been studied in patients with type I DM and was found to be an independent predictor of all-cause mortality in patients with diabetic nephropathy (Nguyen et al., 2008). We recently found a positive association between plasma CTGF and risk of all-cause mortality in end stage renal disease patients on hemodialysis (Den Hoedt et al., 2012). In line with these findings, we observed a robust relationship between plasma CTGF and risk of death in the current study population of patients with manifest vascular disease. CTGF was associated with both vascular and nonvascular death. This might be explained by CTGF as a key determinant of activity of tissue fibrogenesis, and fibrosis as the common final pathway of chronic diseases of diverse etiology. Besides fibrosis, CTGF has been implicated in various other pathological processes, such as ischemia, inflammation and metabolic derangements (Ohnishi et al., 1998; Roestenberg et al., 2006; Sanchez-Lopez et al., 2009).

In addition to the association with mortality, we found a clear association between CTGF and risk of new cardiovascular events and, in particular, of ischemic cardiac complications. This finding raises the question whether CTGF has a causal role in atherosclerosis and ischemic heart disease or merely reflects a large cardiovascular disease burden.

Table 3. Relation between various patient characteristics and plasma CTGF ($n = 1227$).

Characteristic	β (95% CI)*	Change in CTGF (%) (95% CI)†
Age (years)	0.003 (0.000 to 0.006)**	0.3 (0.0–0.6)
Gender (male = 1)	−0.053 (−0.125 to 0.018)	−5.2 (−11.8 to 1.8)
Hypertension (ever or current) (yes = 1)	0.009 (−0.049 to 0.066)	0.9 (−4.8 to 6.8)
Diabetes mellitus (yes = 1)	0.039 (−0.116 to 0.037)	4.0 (−11.0 to 3.8)
Type 1 (yes = 1)	0.019 (−0.386 to 0.423)	1.9 (−32.0 to 52.7)
Type 2 (yes = 1)	−0.041 (−0.118 to 0.037)	−4.0 (−11.1 to 3.7)
Smoking		
Smoking status‡	0.019 (−0.006 to 0.044)	1.9 (−0.6 to 4.5)
Pack years	0.001 (−0.001 to 0.002)	0.1 (0.0 to 0.2)
Framingham Risk score (%)	0.002 (−0.001 to 0.006)	1.0 (0.99 to 1.01)
Medication		
RAAS blocker (yes = 1)	−0.004 (−0.067 to 0.059)	−0.4 (−6.5 to 6.1)
Statin (yes = 1)	−0.031 (−0.091 to 0.029)	−3.1 (−8.7 to 2.9)
Measurements		
Systolic blood pressure (per 10 mmHg)	−0.001 (−0.015 to 0.012)	−0.1 (−1.5 to 1.2)
Diastolic blood pressure (per 10 mmHg)	−0.010 (−0.034 to 0.014)	−1.0 (−3.3 to 1.4)
Pulse pressure (per 10 mmHg)	0.009 (−0.011 to 0.030)	0.9 (−1.1 to 3.0)
Body mass index (kg/m ²)	−0.003 (−0.011 to 0.004)	−0.3 (−1.1 to 0.4)
Abdominal adipose tissue (cm)	0.000 (−0.011 to 0.012)	0.0 (−1.1 to 1.2)
Intima media thickness (mm)	−0.004 (−0.097 to 0.090)	−0.4 (−9.2 to 9.4)
eGFR (mL/min/1.73m ²)	−0.004 (−0.005 to −0.002)**	−0.4 (−0.5 to −0.2)
Left ventricular hypertrophy§(yes = 1)	−0.027 (−0.095 to 0.041)	−2.7 (−9.1 to 4.2)
Laboratory measurements		
Lipids		
Total cholesterol (mmol/L)	0.040 (0.013 to 0.067)**	4.1 (1.3 to 6.9)
LDL cholesterol (mmol/L)	0.031 (0.000 to 0.062)**	3.1 (0.0 to 6.4)
HDL cholesterol (mmol/L)	0.051 (−0.029 to 0.130)	5.2 (−2.9 to 13.9)
Triglycerides (mmol/L)	0.023 (−0.002 to 0.048)	2.3 (−0.2 to 4.9)
HsCRP (mg/L)	0.002 (−0.002 to 0.005)	0.2 (−0.2 to 0.5)
Homocysteine (μmol/L)	0.003 (−0.002 to 0.008)	0.3 (−0.2 to 0.8)
Albuminuria	0.020 (−0.036 to 0.077)	2.0 (−3.5 to 8.0)

Adjustment for age, gender and eGFR.

*Data are expressed as beta regression coefficient (β) with 95% CI calculated with multiple linear regression. The natural logarithm of CTGF will increase with β and CTGF will be multiplied by e^β if the variable increases by 1 unit or changes from 0 to 1 for a dichotomous variable.

†Relative change in CTGF (with CI) if the patient variable increases by 1 unit or changes from 0 to 1 for a dichotomous variable.

‡Never = 0, former = 1, current = 3.

§ $N = 1181$.

||Normoalbuminuria = 0, microalbuminuria = 1, macroalbuminuria = 2. CI = confidence interval; CTGF = connective tissue growth factor; eGFR = estimated glomerular filtration rate; HDL = high-density lipoprotein; hsCRP = high-sensitivity C-reactive protein; LDL = low-density lipoprotein; RAAS = renin-angiotensin-aldosterone system.

Table 4. Hazard ratios for new vascular events and all-cause mortality for each increase of plasma CTGF with 1 nmol/L ($n = 1227$).

	# Events	Hazard ratio (95% CI)		
		Model I	Model II	Model III
Ischemic cardiac complication	92	1.44 (1.22–1.71)	1.39 (1.17–1.65)	1.41 (1.18–1.67)
Ischemic stroke	45	1.01 (0.72–1.42)	0.88 (0.61–1.27)	0.88 (0.61–1.28)
Vascular death	73*	1.31 (1.07–1.60)	1.13 (0.91–1.40)	1.11 (0.89–1.38)
All vascular events	153	1.30 (1.12–1.50)	1.21 (1.04–1.41)	1.21 (1.04–1.42)
Other vascular events	61	1.38 (1.12–1.72)	1.24 (0.99–1.55)	1.21 (0.96–1.54)
Nonvascular death	57*	1.31 (1.04–1.64)	1.25 (0.99–1.57)	1.26 (1.00–1.60)
All cause mortality	131	1.31 (1.13–1.52)	1.19 (1.02–1.39)	1.18 (1.00–1.38)

Uni/Multivariate Cox proportional hazards regression analysis Model I = univariate; model II = adjustment for age, gender and estimated glomerular filtration rate; model III = model II with additional adjustment for type I and type II diabetes mellitus, systolic and diastolic blood pressure, total cholesterol, use of RAAS blockers and use of statins. CI = confidence interval; CTGF = connective tissue growth factor; RAAS = renin-angiotensin-aldosterone system.

*One deceased patient was not classified as vascular or nonvascular death.

In atherosclerotic plaques and fibrotic myocardium CTGF expression is strongly upregulated (Daniels et al., 2009) and high plasma CTGF levels may result from high CTGF release into the circulation. In the current study, however, plasma

CTGF was not associated with surrogate markers of atherosclerotic burden such as cIMT (Poredos, 2004) and pulse pressure (Syeda et al., 2003), nor with left ventricular hypertrophy which is linked to cardiac fibrosis (Weber &

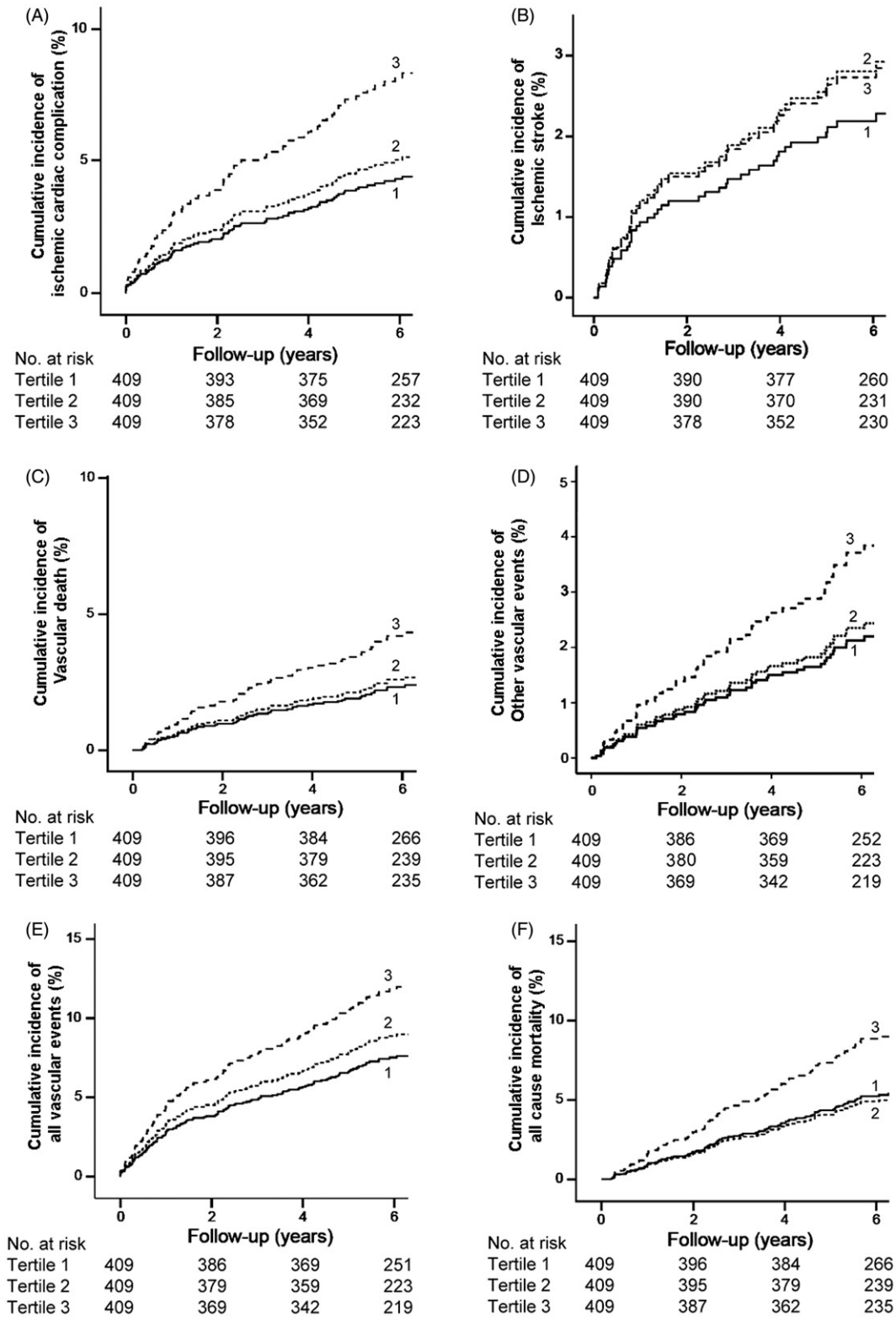


Figure 1. Cumulative percentage of study population with a new cardiovascular event or death according to tertiles of plasma CTGF. One minus survival plots based on Cox regressions using multivariate model III (age, gender, estimated glomerular filtration rate, diabetes mellitus, blood pressure, use of RAAS blockers, total cholesterol and use of statins). (A) Ischemic cardiac complication. (B) Ischemic stroke. (C) Vascular death. (D) Other vascular events. (E) All vascular events. (F) All-cause mortality.

Brilla, 1993). Pre-clinical studies suggest a role for CTGF in atherogenesis. *In vitro*, CTGF increases vascular smooth muscle cell (VSMC) proliferation, migration and extracellular matrix production, which may contribute to neointima formation (Fan et al., 2000). *In vitro* it has been shown that

CTGF stimulates osteogenic differentiation of VSMC (Huang et al., 2013). Furthermore, CTGF promoted adherence and migration of monocytes and activated platelets to VSMCs (Cicha et al., 2005; Jedsadayanmata et al., 1999; Schober et al., 2002). Mesenchymal stem cells (MSCs) are circulating

Table 5. Hazard ratios of plasma CTGF tertiles for new vascular events and all-cause mortality.

		Hazard ratio (95% CI)					
Plasma CTGF		Tertile 1 0.78 (0.18–0.98) <i>n</i> = 409		Tertile 2 1.18 (0.98–1.48) <i>n</i> = 409		Tertile 3 1.85 (1.48–6.47) <i>n</i> = 409	
Median (range) (nmol/L)	Model	# Events		# Events		# Events	
Ischemic cardiac complication	I	23	1.00 (reference)	27	1.22 (0.70–2.13)	42	2.02 (1.22–3.37)
	II		1.00 (reference)		1.19 (0.68–2.08)		1.89 (1.13–3.17)
	III		1.00 (reference)		1.17 (0.67–2.05)		1.93 (1.15–3.23)
Ischemic stroke	I	11	1.00 (reference)	17	1.57 (0.74–3.36)	17	1.63 (0.77–3.49)
	II		1.00 (reference)		1.37 (0.64–2.95)		1.28 (0.59–2.77)
	III		1.00 (reference)		1.29 (0.60–2.78)		1.25 (0.57–2.73)
Vascular death	I	17	1.00 (reference)	21	1.30 (0.68–2.46)	35*	2.32 (1.30–4.15)
	II		1.00 (reference)		1.15 (0.60–2.18)		1.81 (1.00–3.27)
	III		1.00 (reference)		1.12 (0.59–2.13)		1.83 (1.00–3.34)
All vascular events	I	39	1.00 (reference)	49	1.31 (0.86–2.00)	65	1.83 (1.23–2.73)
	II		1.00 (reference)		1.22 (0.80–1.87)		1.61 (1.08–2.41)
	III		1.00 (reference)		1.19 (0.78–1.82)		1.61 (1.08–2.42)
Other vascular events	I	15	1.00 (reference)	18	1.28 (0.64–2.53)	28	2.13 (1.13–3.99)
	II		1.00 (reference)		1.14 (0.57–2.28)		1.79 (0.94–3.39)
	III		1.00 (reference)		1.11 (0.56–2.22)		1.76 (0.92–3.37)
Nonvascular death	I	17	1.00 (reference)	14	0.86 (0.42–1.74)	26*	1.73 (0.94–3.19)
	II		1.00 (reference)		0.81 (0.40–1.65)		1.62 (0.87–3.00)
	III		1.00 (reference)		0.79 (0.39–1.61)		1.58 (0.85–2.95)
All cause mortality	I	34	1.00 (reference)	35	1.08 (0.67–1.72)	62*	2.06 (1.35–3.13)
	II		1.00 (reference)		0.98 (0.61–1.58)		1.75 (1.15–2.69)
	III		1.00 (reference)		0.94 (0.58–1.51)		1.72 (1.21–2.65)

Uni/Multivariate Cox proportional hazards regression analysis. Model I = univariate; model II = adjustment for age, gender and estimated glomerular filtration rate; model III = model II with additional adjustment for type I and type II diabetes mellitus, systolic and diastolic blood pressure, total cholesterol, use of RAAS blockers and use of statins. CI = confidence interval; CTGF = connective tissue growth factor; RAAS = renin-angiotensin-aldosterone system.

*One deceased patient was not classified as vascular or nonvascular death.

cells involved in arterial repair by mesenchymal to endothelial transdifferentiation (Wan et al., 2012). Stimulation of MSCs with CTGF leads to fibroblastic differentiation and increased extracellular matrix production (Lee et al., 2010; Li et al., 2016). Pericytes are the major vascular supportive cell type involved in the maintenance of vascular homeostasis and integrity. Under pathological conditions pericytes transdifferentiate to myofibroblasts, thus compromising their role in vascular support (Humphreys et al., 2010; van Dijk et al., 2015). Culturing pericytes with CTGF under pathological conditions increased expression of extracellular matrix genes α Smooth Muscle Actin (Supplementary Figure 2), which are all markers associated with neointima hyperplasia and myofibroblast accumulation. Additionally, CTGF-mediated modulation results in reduced VEGF-A signaling (Inoki et al., 2002). Taken together, this suggests a major role for CTGF in vascular disease by switching angiogenesis and vascular repair towards neointima formation and atherogenesis.

In a murine model, CTGF injection leads to increased oxidative stress and a vascular inflammatory response associated with endothelial dysfunction (Rodrigues-Diez et al., 2015). CTGF has been implicated in hypertension induced organ damage via mechanical stress induced CTGF gene expression in endothelial cells, vascular smooth muscle cells and cardiomyocytes (Finckenberg, 2003; Lee et al., 2005; Yoshisue et al., 2002). The role of CTGF in the etiology of cardiomyopathy is controversial. Cardiac CTGF expression

is increased after ischemic-reperfusion injury and is associated with replacement and reactive fibrosis following myocardial infarction (Daniels et al., 2009). Myocardial overexpression of CTGF in transgenic mice promoted age-dependent development of cardiac hypertrophy (Panek et al., 2009) and enhanced pressure-overload-induced cardiac fibrosis (Yoon et al., 2010). In line with this, dilating cardiomyopathy appears to be CTGF regulated (Koshman et al., 2015), and CTGF reduction attenuates left ventricular remodeling and dysfunction in models of pressure overload (Szabo et al., 2014). However, in other animal models of chronic pressure overload it has been reported that alteration of CTGF levels are of little consequence to the phenotype (Accornero et al., 2015; Fontes et al., 2015). In stark contrast, it has also been reported that CTGF exerts protective effects during experimental cardiac pressure overload (Gravning et al., 2013). Additional evidence for a pathogenic role of CTGF in cardiovascular disease comes from a study in experimental diabetes, which showed that neutralizing anti-CTGF antibody therapy prevented and reversed arterial stiffening, cardiac dysfunction and hypertension (Langsetmo et al., 2006). Future studies should clarify the role of CTGF in the pathogenesis of cardiovascular disease and should evaluate whether anti-CTGF therapies are beneficial.

An interesting question is what the source is of increased plasma CTGF in patients with manifest cardiovascular disease. The N-terminal CTGF cleavage fragment is the predominant form of CTGF in plasma which is largely cleared by the kidney (Gerritsen et al., 2012). Indeed in our study,

plasma CTGF was negatively related to eGFR. However, multivariable survival analysis adjusting for eGFR showed CTGF to be independent of renal clearance, indicating an additional contribution of locoregional *de novo* production. Under physiological circumstances CTGF is produced at low levels. As discussed above, increased CTGF expression has been shown in many tissues and cells, including atherosclerotic plaques and fibrotic myocardium (Daniels et al., 2009; Ponticos, 2013), cardiomyocytes and fibroblasts upon myocardial infarction (Daniels et al., 2009) and endothelial cells, vascular smooth muscle cells and cardiomyocytes exposed to mechanical stress as in hypertension (Finckenberg, 2003; Lee et al., 2005; Yoshisue et al., 2002). However, given the wide variety of tissues that overexpress CTGF during disease that are also involved in cardiovascular disease, the source of the excessive plasma CTGF is probably even more diverse.

Our finding that CTGF is associated with total and LDL cholesterol may suggest that CTGF is involved in the pathway through which lipoproteins promote the development of atherosclerotic vascular disease. This is supported by previous observations that treatment of human aortic endothelial cells with LDL induced CTGF gene expression (Sohn et al., 2006) and that HMG-CoA reductase inhibitors inhibited CTGF induction in human umbilical cord endothelial cells exposed to non-uniform shear stress (Cicha et al., 2008).

Remarkably, while the association with the composite endpoint of vascular events was evident, we did not observe an increased risk of ischemic stroke in patients with higher baseline plasma CTGF. Plasma CTGF was lower in patients with previous cerebrovascular disease, but stratification for prior cerebrovascular disease did not substantially change the results. Although our study provides no proper explanation for the discrepancy between cerebrovascular and other vascular events, it is interesting to note that in a previous study we observed an association between CTGF levels in carotid plaques from patients undergoing carotid endarterectomy and stable plaque characteristics (Leeuwis et al., 2010).

Study limitations

We acknowledge several limitations of this study. First, we measured CTGF only once at baseline. However, it is not known to what extent CTGF levels vary over time in the individual patient. Second, recruitment was conducted at a single center and the study population included mainly Caucasian patients. Both facts limit the generalization of our results to a wider-ranging population with manifest vascular disease.

Strengths of the present study are the large number of well-described patients with various manifestations of atherosclerotic vascular disease and the virtually complete follow-up.

Conclusions

Elevated plasma CTGF increases the risk of cardiovascular events and all-cause mortality in patients with manifest vascular disease. CTGF may therefore be regarded as a novel marker to identify vascular patients at the highest risk of recurrent cardiovascular events and mortality.

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Supplementary material available online
Supplementary Figures 1 and 2