



University of Southern Denmark

Circulating endostatin as a risk factor for cardiovascular events in patients with stable coronary heart disease: A CLARICOR trial sub-study

Ruge, Toralph; Carlsson, Axel; Kjøller, Erik; Hilden, Jørgen; Kolmos, Hans Jørn; Sajadieh, Ahmed; Kastrup, Jens; Jensen, Gorm Boje; Larsson, Anders; Nowak, Christoph; Christian Jakobsen, Janus; Winkel, Per; Gluud, Christian Nyfeldt; Årnlöv, Johan

Published in:
Atherosclerosis

DOI:
10.1016/j.atherosclerosis.2019.02.031

Publication date:
2019

Document version:
Accepted manuscript

Document license:
CC BY-NC-ND

Citation for published version (APA):

Ruge, T., Carlsson, A., Kjøller, E., Hilden, J., Kolmos, H. J., Sajadieh, A., Kastrup, J., Jensen, G. B., Larsson, A., Nowak, C., Christian Jakobsen, J., Winkel, P., Gluud, C. N., & Årnlöv, J. (2019). Circulating endostatin as a risk factor for cardiovascular events in patients with stable coronary heart disease: A CLARICOR trial sub-study. *Atherosclerosis*, 284, 202-208. <https://doi.org/10.1016/j.atherosclerosis.2019.02.031>

Go to publication entry in University of Southern Denmark's Research Portal

Terms of use

This work is brought to you by the University of Southern Denmark.
Unless otherwise specified it has been shared according to the terms for self-archiving.
If no other license is stated, these terms apply:

- You may download this work for personal use only.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying this open access version

If you believe that this document breaches copyright please contact us providing details and we will investigate your claim.
Please direct all enquiries to puresupport@bib.sdu.dk

Accepted Manuscript

Circulating endostatin as a risk factor for cardiovascular events in patients with stable coronary heart disease – a CLARICOR trial sub-study

Toralph Ruge, Axel C. Carlsson, Erik Kjølner, Jørgen Hilden, Hans Jørn Kolmos, Ahmad Sajadieh, Jens Kastrup, Gorm Boje Jensen, Anders Larsson, Christoph Nowak, Janus Christian Jakobsen, Per Winkel, Christian Gluud, Johan Ärnlöv

PII: S0021-9150(19)30118-2

DOI: <https://doi.org/10.1016/j.atherosclerosis.2019.02.031>

Reference: ATH 15898

To appear in: *Atherosclerosis*

Received Date: 17 September 2018

Revised Date: 19 February 2019

Accepted Date: 27 February 2019

Please cite this article as: Ruge T, Carlsson AC, Kjølner E, Hilden Jø, Kolmos HansJø, Sajadieh A, Kastrup J, Jensen GB, Larsson A, Nowak C, Jakobsen JC, Winkel P, Gluud C, Ärnlöv J, Circulating endostatin as a risk factor for cardiovascular events in patients with stable coronary heart disease – a CLARICOR trial sub-study, *Atherosclerosis* (2019), doi: <https://doi.org/10.1016/j.atherosclerosis.2019.02.031>.

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

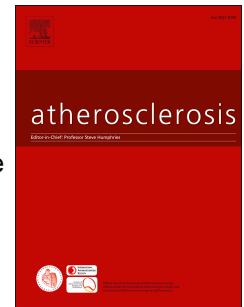
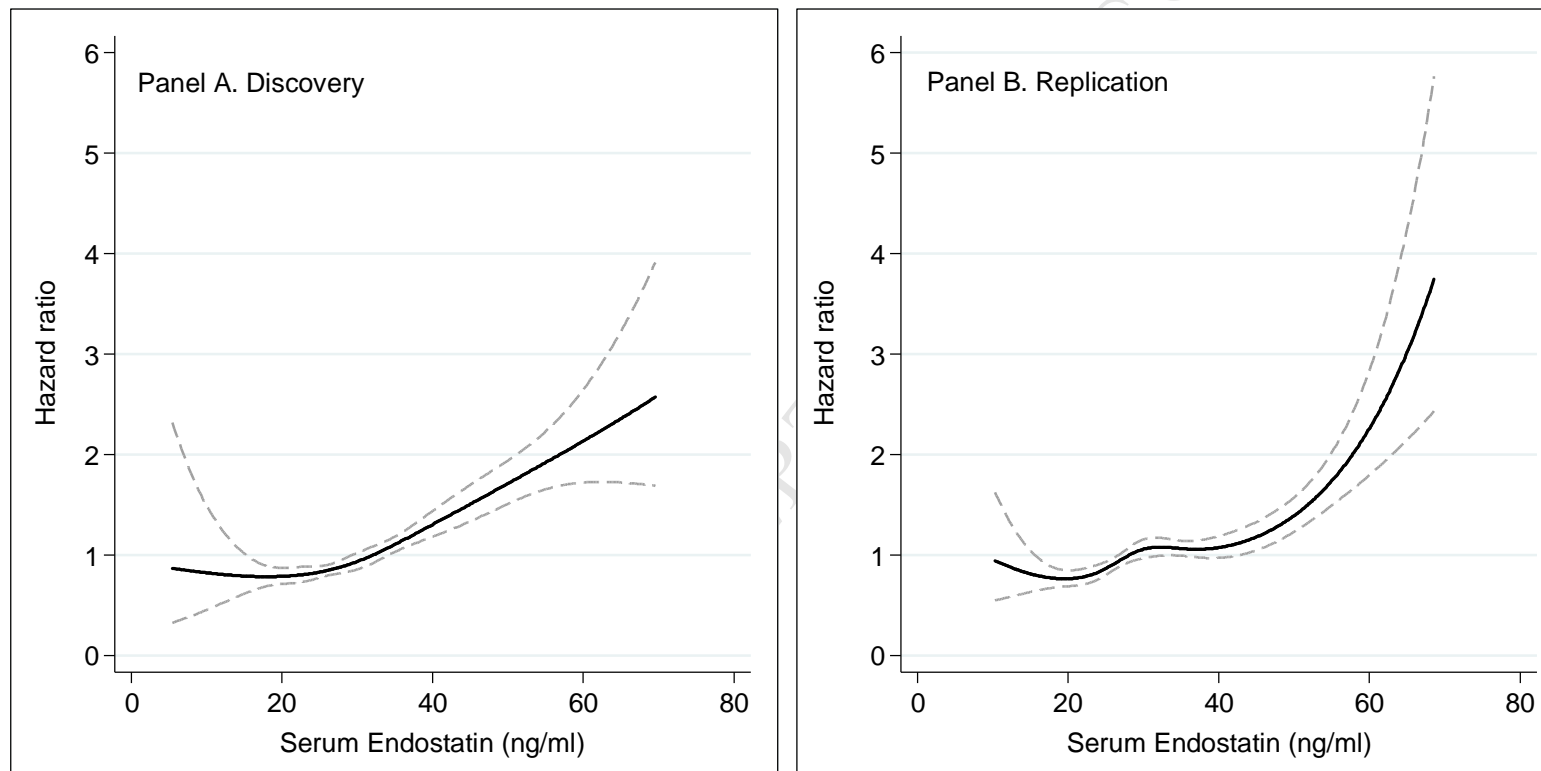


Figure 1 Spline curve of the association between endostatin and the composite outcome in the discovery and replication sample (panel A and B, respectively)



Circulating endostatin as a risk factor for cardiovascular events in patients with stable coronary heart disease – a CLARICOR trial sub-study

Toralph Ruge¹, Axel C. Carlsson^{2,3}, Erik Kjølner⁴, Jørgen Hilden⁵, Hans Jørn Kolmos⁶, Ahmad Sajadieh⁷, Jens Kastrup⁸, Gorm Boje Jensen⁹, Anders Larsson¹⁰, Christoph Nowak², Janus Christian Jakobsen^{11,12}, Per Winkel¹¹, Christian Glud¹¹, and Johan Ärnlöv^{2,13*}

¹ Department of Emergency Medicine, Karolinska University Hospital, Huddinge, Stockholm, Sweden

² Division of Family Medicine and Primary Care, Department of Neurobiology, Care Sciences and Society, Karolinska Institutet, Huddinge, Sweden

³ Department of Medical Sciences, Cardiovascular Epidemiology, Uppsala University, Uppsala, Sweden

⁴ Department of Cardiology, Herlev Hospital, Copenhagen University Hospital, Copenhagen, Denmark

⁵ Section of Biostatistics, University of Copenhagen, Copenhagen, Denmark

⁶ Department of Clinical Microbiology, Odense University Hospital, Denmark

⁷ Copenhagen University Hospital of Bispebjerg & Frederiksberg, Denmark

⁸ Rigshospitalet of Copenhagen University, Denmark

⁹ Copenhagen University Hospital Hvidovre, Denmark

¹⁰ Department of Medical Sciences, Uppsala University, Uppsala, Sweden

¹¹ Copenhagen Trial Unit, Centre for Clinical Intervention Research, Rigshospitalet, Copenhagen University Hospital, Copenhagen, Denmark

¹² Department of Cardiology, Holbæk Hospital, Holbæk, Denmark

¹³ School of Health and Social Studies, Dalarna University, Falun, Sweden.

*Corresponding author: Johan Ärnlöv, Division of Family Medicine, Department of Neurobiology, Care Sciences and Society, Karolinska Institutet, Huddinge, Sweden phone: +46-70-6077703
E-mail: johan.arnlov@ki.se

Running title: Endostatin and risk for cardiovascular events

TOC Category – Clinical and Population Studies

TOC Subcategory – Vascular Biology

Words in abstract: 249, Number of words in main text: 3808, Tables:4, Figures:1

Keywords: Endostatin, extracellular matrix, cardiovascular, mortality, epidemiology

Subject codes: Myocardial Biology, Cardiovascular Disease, Vascular Disease

Abstract

Background and aim: Raised levels of serum endostatin, a biologically active fragment of collagen XVIII, have been observed in patients with ischemic heart disease but associations with incident cardiovascular events in patients with stable coronary heart disease is uncertain.

Methods: The CLARICOR-trial is a randomised, placebo-controlled trial of stable coronary heart disease patients evaluating 14-day treatment with clarithromycin. The primary outcome was a composite of acute myocardial infarction, unstable angina pectoris, cerebrovascular disease or all-cause mortality. In the present sub-study using 10-year follow-up data, we investigated associations between serum endostatin at entry (randomization) and the composite outcome and its components during follow-up. The placebo group was used as a discovery sample (1204 events, n=1998) and the clarithromycin-treated group as replication sample (1220 events, n=1979).

Results: In Cox regression models adjusting for cardiovascular risk factors, glomerular filtration rate, and current pharmacological treatment, higher serum endostatin was associated with an increased risk of the composite outcome in the discovery sample (hazard ratio per standard deviation increase 1.11, 95% CI 1.03-1.19, p=0.004), but slightly weaker and not statistically significant in the replication sample (hazard ratio 1.06, 95 % CI 1.00-1.14, p=0.06). In contrast, strong and consistent associations were found between endostatin and cardiovascular and all-cause mortality in all multivariable models and sub-samples. Addition of endostatin to a model with established cardiovascular risk factors provided no substantial improvement of risk prediction (<1%).

Conclusion: Raised levels of serum endostatin might be associated with cardiovascular events in patients with stable coronary heart disease. The clinical utility of endostatin measurements remains to be established.

Abbreviations

CLARICOR: effect of clarithromycin versus placebo on mortality and morbidity in patients with ischemic heart disease

Introduction

Collagen XVIII, a major component of the basal membranes, is a heparin sulphate proteoglycan with multifunctional activity. Cleavage of collagen XVIII during extracellular matrix (ECM) remodelling gives rise to endostatin, a biologically active fragment with anti-angiogenic and anti-fibrotic activity¹⁻³. Endostatin has been thoroughly studied in the field of malignant diseases, and has been suggested to reflect extracellular matrix turnover during tumour growth⁴.

Apart from its role in cancer growth, endostatin has been shown to contribute to atherosclerosis, and raised levels of serum endostatin are associated with the risk of various cardiovascular diseases⁵⁻¹⁰ as well as with cardiovascular mortality¹¹. Despite the importance of endostatin in the development of cardiovascular disease, few studies have evaluated serum endostatin as a biomarker for coronary heart disease and cardiovascular prognosis, particularly few in patients with prevalent cardiovascular disease.

Here, we aimed to assess the association between serum endostatin and future risk of adverse cardiovascular events in persons with stable coronary heart disease at baseline. We hypothesized that elevated levels of serum endostatin reflect cardiovascular damage that predisposes to a cardiovascular event.

Methods

Study design and patients

In 1999, all residents of Copenhagen, Denmark, with a hospital diagnosis of myocardial infarction or angina pectoris (International Statistical Classification of Diseases (ICD) codes I20.9-I21.9) between 1993-1999 were invited to participate in the randomized, placebo-controlled, multi-centre CLARICOR (*effect of clarithromycin versus placebo on mortality and morbidity in patients with ischemic heart disease*) trial. After providing informed consent, eligible participants with stable coronary heart disease (excluding persons who had suffered from acute myocardial infarction or unstable angina pectoris episode during the previous 3 months and those that had been subjected to percutaneous transluminal coronary angioplasty and coronary bypass surgery during the last 6 months) were randomly assigned to 14-days of treatment with either oral clarithromycin 500 mg once daily (Klacid Uno®, Abbott, UK) or placebo. Other exclusion criteria included New York Heart Association class IV heart failure, impaired renal or hepatic function, or cancer. In total, 4,372 patients with stable coronary heart disease were randomized, 4,350 patients donated blood and serum samples were available for analysis in 4,298 patients. Participants were followed for a maximum of ten years from the end of treatment in April 2000 until 31st of December 2009. The results on the effects of clarithromycin on cardiovascular outcomes and mortality as well as on adverse events have previously been reported¹²⁻¹⁸. In the present sub-study of CLARICOR, we used the placebo arm as a discovery sample for the investigating the association between serum endostatin and outcome, and the clarithromycin arm as a replication sample. We excluded participants with missing data in any of the variables, leaving n=1,998 participants in the discovery sample, and n=1,979 in the replication sample.

Outcomes

Outcome information about fatal and non-fatal hospital episodes were extracted from the Danish National Hospital Register that records hospital admissions for all registered Danish residents via linkage to the 10-digit central person registration number. Information about dates and causes of death were obtained from the Danish Central Civil Register and the National Register of Causes of Death. For each recorded main diagnosis and for each underlying cause of death, we classified the outcomes into: acute myocardial infarction (ICD codes I21.0–23.9); unstable angina pectoris (I20.0, I24.8–24.9); cerebrovascular disease (I60.0–64.9 and G45.0–46.8); peripheral vascular disease (I70.2–70.9); other cardiovascular diseases (I00.0–99.9 unless already covered); and non-

cardiovascular disease (A00.0-T98.3 unless already covered)¹⁶. The a-priori defined composite outcome comprised of acute myocardial infarction, unstable angina pectoris, cerebrovascular disease, or all-cause death during follow-up.

Laboratory analyses

Laboratory analyses were performed on Mindray BS380 (Mindray, Shenzhen, China) with reagents from Abbott Laboratories (Abbott Park, IL, USA) according to the manufacturer's instructions (Supplementary table 1). Estimated glomerular filtration rate (eGFR) was estimated using the creatinine based CKD-EPI formula¹⁹ Blood samples were obtained at entry (randomization), prior to the initiation of treatment, and immediately frozen and stored at -70° C or lower. Serum levels of endostatin were analysed using a commercially available ELISA kit for endostatin (DY1098, R&D Systems, Minneapolis, MN). The assays had a total coefficient of variation (CV) of approximately 7%. Laboratory technicians were blinded to participant assignment.

Ethics

CLARICOR complies with the Declaration of Helsinki and has been approved by the local ethics committees (ClinicalTrials.gov NCT00121550; Regional Ethics Committee KF 01-076/99 and HB 2009/015; the Danish Data Protection Agency 1999-1200-174 and 2012-41-0757; and the Danish Medicines Agency 2612–975). All participants gave written informed consent.

Statistical analyses

For all analyses, endostatin serum levels were natural logarithm-transformed in order to promote a normal distribution and expressed per standard deviation increase. Log-transformation was also applied to CRP and apolipoprotein B. The following adjusted Cox proportional hazards regression models were used to assess the association between endostatin and outcomes:

Model A was adjusted for age, sex, and CRP. Model B was adjusted for factors in model A and established cardiovascular risk factors (hypertension, diabetes, smoking, apolipoprotein A1, and apolipoprotein B). Model C was adjusted for factors in model B and eGFR. Model D was adjusted for established risk factors, co-morbidities and cardiovascular pharmacotherapies, and standard

biochemical predictors as shown in Table 1. Model D was considered the primary model in accordance with the pre-specified analyses plan²⁰.

We used penalized spline curves to depict the potential nonlinearity of the association between endostatin and the composite outcome.

For the composite outcome and for all-cause mortality, the proportional hazards assumption was violated for age at entry (Bonferroni adjusted $p < 0.00056$ for the composite outcome, and $p < 0.0044$ for all-cause mortality). Therefore, we excluded age from all multivariable models for those two outcomes. In order to provide additional insights into the potential influence of age on these associations we conducted multivariable logistic regression models (including age as a covariate as the proportional hazard assumption are not a prerequisite for these analyses).

In accordance with the published statistical analysis plan²⁰, improvement in risk prediction when adding endostatin to multivariable Model D was calculated (please see as explained in note to table).

Analyses were conducted in STATA, version 14.2 (College Station, Texas, USA) and SAS 9.4 (SAS Institute, Inc.100 SAS Campus Drive, Cary, NC 27513-2414).

Results

The baseline demographic data of the discovery and replication cohorts are described in Table 2. There were no obvious differences in these variables between the two samples.

The mean follow-up was 6.5 years in the discovery and 6.4 years in the replication cohort sample; maximum follow-up was 10.2 years in both samples. Table 3 shows the number of events and incidence rates per 100 person-years for the primary composite outcome and the secondary outcomes. The two groups have very similar rates ($p > 0.60$ in all rows).

The crude association between serum endostatin and the composite outcome in the discovery and replication sample is graphically depicted by spline curves in Figure 1. Visual examination of the curves suggests a linear increase in hazard for higher endostatin levels in both samples.

As seen in Table 4, higher serum endostatin was associated with an increased risk of the composite outcome in the both the discovery and replication sample in models adjusted for sex, CRP, established cardiovascular risk factors, and eGFR (Models A-C). In the fully-adjusted model (model D) endostatin levels were not quite so strongly associated with the composite outcome, reaching significance in the discovery cohort only (hazard ratio per standard deviation increase of endostatin 1.11, 95 % CI 1.03 – 1.19, $p=0.004$) vs. replication HR = 1.06, 95 % CI 1.00 – 1.14, $p=0.064$). However, in the multivariable logistic regression analyses that also included age as a co-variate, higher endostatin was associated with an increased risk of the composite outcome in both the discovery and replication sample (Model D, Supplementary table 2).

As regards the secondary Cox analyses in Table 4, one observes that myocardial infarction closely follows the hazard ratio pattern just described for the composite outcome. Cardiovascular as well as total mortality was more strongly associated with higher endostatin levels in all multivariable models in both cohorts, whereas the outcomes stroke, unstable angina show no consistent association with endostatin.

Prediction improvement

The number and percent of correct predictions obtained for the composite outcome and all-cause mortality when endostatin was added to standard predictors are shown in Table 5. Only small improvements were seen (<1%).

ACCEPTED MANUSCRIPT

Discussion

Main findings

In a large study of patients with stable coronary heart disease, higher levels of serum endostatin might be related with an increased 10-year risk of cardiovascular events in patients with stable coronary heart disease. These associations were found in both the discovery and replication sample of the study, with statistically compatible hazard ratios, although the replication data were also formally compatible with there being no association when examined under the conditions of the pre-defined fully adjusted model ($p=0.06$). The strongest associations, however, were seen between higher serum endostatin and cardiovascular and all-cause mortality.

Strengths and limitations

Major strengths of the present study include the discovery replication approach in a large study sample with detailed characterization of the participants, and the longitudinal study design with up to 10 years of follow-up. The present cohort study is to our knowledge the largest study that has measured circulating levels of endostatin, and the first in patients with stable coronary heart disease. Moreover, the National Danish Registers are known to be of high completeness and accuracy¹⁷. Limitations include the unknown generalizability to other ethnic groups. In addition, diabetes and hypertension were only self-reported which may have led to an increased misclassification of these cardiovascular risk factors. As we have no repeated blood sampling for endostatin analyses, it was not possible to evaluate the effects of clarithromycin treatment on circulating endostatin levels.

Comparison with previous studies

In two previous studies, serum endostatin was increased in patients with acute myocardial infarction^{21,22}. Also, higher serum endostatin in the coronary circulation of patients with ischemic heart disease were associated with poorer collateral formation²³ and expression of endostatin is increased in human heart biopsies from patients with non-ST elevation myocardial infarction²². Moreover, our results are in accordance with a previous community-based study, in which associations between endostatin and cardiovascular death were particularly strong in individuals with a history of cardiovascular disease compared to those without¹¹. Still, to our knowledge, this

study is the first to report the association between circulating endostatin and adverse cardiovascular outcomes in patients with stable coronary heart disease.

Possible mechanisms for observed associations

One interpretation of the association between higher serum endostatin levels and cardiovascular events, could be that higher circulating levels of endostatin in patients with stable ischemic heart disease mirror an increased systemic active extracellular matrix re-modulation driven by an increased inflammatory stress (inflammatory cytokines, hypoxia, ischemia) as seen in patient with atherosclerosis and cardiovascular diseases²⁴. Specifically, this state of increased inflammation will stimulate elastase, matrix-derived metalloproteinases (MMP), as well as the cathepsins, all known to be involved in the release of endostatin from the basal membrane^{1, 3, 25, 26}. Additionally, inflammation may not only initiate the reactions leading to extracellular matrix remodelling and atherosclerotic lesions; but may also trigger plaque rupture, the final event behind most thrombotic and atherosclerotic manifestations events²⁴.

It is also possible that the association between endostatin and cardiovascular mortality reflects systemically increased angiogenic activity initiated by an angiogenic stimulus involving extracellular matrix remodelling and release of endostatin. Vascular endothelial growth factor (VEGF), one of the most potent endogenous stimulators of angiogenesis, is regulated by hypoxia, inflammatory cytokines as well as oncogenes^{27, 28}. Initiation of angiogenesis by VEGF involves extracellular matrix degradation and leads to an imbalance in the local angiogenic milieu favouring angiogenesis. As a consequence to the extracellular matrix breakdown, endostatin is released in order to maintain homeostasis in the angiogenic milieu. Alteration in the systemic balance of pro and anti-angiogenic homeostasis has been shown to be a key factor in the destabilization of atherosclerotic plaques²⁹.

ECM remodelling as observed in ischemic heart disease leads to substantial pathological deposition of extracellular matrix proteins in the myocardium and cardiac fibrosis^{24, 30}. The extracellular matrix remodelling process can be initiated for example by hypertension, cardiac stress, valve dysfunction, hypertrophy of the myocardium and by myocardial infarction³⁰. Cardiac fibrosis has

been suggested to be associated with a reduction in local capillary perfusion leading to tissue hypoxia and a subsequent activation of the angiogenic milieu³¹. It has been shown that the expression of endostatin is increased in rat cardiomyocytes exposed to hypoxia as well as after induction of a myocardial infarction³². Moreover, an association between endostatin and reduced collateral circulation has been shown repeatedly in previous studies^{7, 23, 33}. Thus, there is evidence that local cardiac fibrosis and hypoxia following atherosclerotic changes in the coronary circulation may promote an increased expression of endostatin, which can lead to a subsequent systemic increase of circulating endostatin.

Also, endostatin has been put forward as a relevant marker for kidney damage and dysfunction in patients with diabetes³⁴, and in the general population³⁵. Moreover, endostatin levels have been shown to be associated with underlying factors that predispose to both chronic kidney disease and ischemic heart disease such as diabetes^{33, 36, 37} and the duration of hypertension⁵. Therefore, it is possible that individuals with elevated circulating endostatin are more likely to develop chronic kidney disease, which in turn substantially increases the risk of cardiovascular events. However, the associations remained robust after adjustment for baseline eGFR, indicating that other pathways are important as well.

Clinical implications

With the development of novel (and costly) treatment regimens such as proprotein-convertase subtilisin kexin type 9 (PCSK9) inhibitors³⁹ or canakinumab, that target the interleukin-1 β innate immunity pathway⁴⁰ an improved identification of high risk patients with prevalent cardiovascular disease that would benefit of such treatments will be increasingly important. Even though endostatin predicted adverse cardiovascular events in the present study, the improvement in risk prediction was low and our data do not support a clinical utility for endostatin measurements in patients with stable coronary heart disease.

Conclusions

Raised levels of serum endostatin might be associated with cardiovascular events in patients with stable coronary heart disease. Yet, the clinical utility of our findings remains to be established.

Conflict of interest

The authors declares that there is no conflict of interest’.

Financial support

This study was supported by The Swedish Research Council, Swedish Heart-Lung Foundation, the Marianne and Marcus Wallenberg Foundation, the Thuréus foundation, Dalarna University, Uppsala University, and The Copenhagen Trial Unit. The CLARICOR trial is investigator initiated and controlled. This trial was supported by grants from nonprofit funds (The Danish Heart Foundation, The Copenhagen Hospital Corporation, The Danish Research Council, and The 1991 Pharmacy Foundation) as well as from The Copenhagen Trial Unit. Abbott Laboratories, IDC, Queensborough, UK supplied the clarithromycin and placebo tablets. The funding sources did not play any role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; and preparation, review, or approval of the manuscript.

Author contributions

T.R. drafted the manuscript and researched data. A.C.C. researched data, edited the manuscript, and contributed to the discussion. EK was chairman of the outcome committee and critically revised. J.H. critically revised the manuscript, contributed to data maintenance and statistics. HJK had the original idea to do the CLARICOR trial and critically revised. AS, JK, JCJ, and GBJ recruited patients and critically revised. A.L. revised manuscript, contributed to discussion and measured serum endostatin. C.N. critically revised the manuscript and contributed to discussion. PW critically revised the manuscript, conducted the necessary data management to produce the data file used and contributed to statistical analyses CG is the coordinating investigator of the trial and critically revised, J.Ä. researched data, edited manuscript, contributed to discussion, and provided funding.

Acknowledgements

We thank the participants of the CLARICOR trial, the investigators and research personnel of the CLARICOR trial (please see Jespersen et al. 2006¹², Gluud et al. 2008¹⁴ and Winkel et al. 2015¹⁵, and our funders (see below).

Table 1. Standard predictors adjusted for in Model D

Clinical predictors	Current medical treatment	Standard biochemical predictors
Sex, age, smoking history, history of myocardial infarction compared to angina only, hypertension, and diabetes.	The current medical treatment was included as proxy predictors because information about post infarction heart failure and post-infarction angina pectoris are not available to us. Aspirin (Yes/No), beta-blocker (Yes/No), calcium-antagonist (Yes/No), ACE-inhibitor (Yes/No), long lasting nitrate (Yes/No), diuretic (Yes/No), digoxin (Yes/No), statin (Yes/No), and anti-arrhythmic drugs (Yes/No).	Log transformed high-sensitivity-reactive protein (CRP), glomerular filtration rate (GFR) estimated by creatinine, triglycerides and lipoproteins (total cholesterol, HDL cholesterol, LDL cholesterol, apoprotein A1, and apoprotein B).

Table 2 Baseline characteristics in the discovery (placebo) and replication (clarithromycin) cohorts

Variable	Discovery cohort	Replication cohort
Number of participants	1998	1979
Female	624 (31)	603 (30)
Age (years)	65 ±10	65 ±10
Endostatin (ng/ml)	31.4 ±1.2	32.0 ±1.2
CRP (mg/L)	5.25 ±7.7	5.76 ±9.3
Apolipoprotein A1 (mg/dL)	1.70 ±0.34	1.70 ±0.36
Apolipoprotein B (mg/dL)	1.21 ±0.32	1.21 ±0.33
Diabetes	300 (15)	301 (15)
Hypertension	805 (40)	790 (40)
eGFR (ml/min)	76.3 ±20	76.5 ±19
Never smoked	395 (20)	338 (17)
Former smoker	925 (46)	906 (46)
Current smoker	678 (34)	735 (37)
Previous myocardial infarction	636 (32)	640 (32)
Statin treatment	822 (41)	814 (41)
Aspirin treatment	1764 (88)	1737 (88)
Beta blocker treatment	619 (31)	591 (30)
Calcium antagonist treatment	702 (35)	681 (34)
ACE inhibitor treatment	523 (26)	553 (28)
Long acting nitrate treatment	412 (21)	411 (21)
Diuretics	691 (37)	702 (35)
Digoxine treatment	117 (7)	140 (7)
Antiarrhythmic treatment	42 (2)	46 (2)

Data are mean ± standard deviation for continuous variables and n (%) for categorical variables

Table 3. Number of events and incidence rates with 95% confidence intervals

Outcomes		Discovery cohort	Replication cohort
Composite outcome	NE (%)	1204 (60)	1220 (62)
	IR per 100 years	9.23	9.67
	95% CI	8.72 - 9.76	9.15 - 10.23
Acute myocardial infarction	NE (%)	446 (22)	422 (21)
	IR per 100 years	2.97	2.90
	95% CI	2.71 – 3.26	2.63 – 3.19
Unstable angina pectoris	NE (%)	356 (18)	356 (18)
	IR per 100 years	2.40	2.50
	95% CI	2.16-2.66	2.25 – 2.77
Stroke	NE (%)	298 (15)	324 (16)
	IR per 100 years	1.91	2.18
	95% CI	1.71 – 2.14	1.96 – 2.43
Cardiovascular mortality	NE (%)	382 (19)	348 (17)
	IR per 100 years	2.39	2.10
	95% CI	2.15 – 2.64	1.88 – 2.32
Non-cardiovascular mortality	NE (%)	390	406
	IR per 100 years	2.34	2.54
	95% CI	2.12 – 2.59	2.30 – 2.80
All-cause mortality	NE (%)	738 (37)	788 (40)
	IR per 100 years	4.44	4.92
	95% CI	4.13 – 4.77	4.59 – 5.28

NE= Number of events (% of participants at risk). IR= incidence rate: Number of events per 100 person years follow-up, lower/upper bounds of 95% confidence intervals.

Table 4. The association between serum endostatin and endpoints in Cox regression models; hazard ratios are per 1SD increment.

		Discovery cohort				Replication cohort			
		Model A	Model B	Model C	Model D	Model A	Model B	Model C	Model D
Composite outcome*	Hazard ratio	1.30	1.31	1.14	1.11	1.27	1.26	1.10	1.06
	95% CI	1.23-1.38	1.23-1.39	1.06-1.21	1.03-1.19	1.20-1.35	1.19-1.34	1.03-1.17	1.00-1.14
	p-value	<0.001	<0.001	<0.001	0.004	<0.001	<0.001	0.004	0.064
Acute myocardial infarction	Hazard ratio	1.29	1.26	1.19	1.19	1.18	1.16	1.12	1.07
	95% CI	1.17-1.42	1.14-1.39	1.07-1.33	1.06-1.33	1.07-1.30	1.05-1.28	1.00-1.25	0.96-1.20
	p-value	<0.001	<0.001	0.002	0.004	0.001	0.003	0.050	0.22
Unstable angina pectoris	Hazard ratio	1.13	1.14	1.14	1.06	1.10	1.10	1.09	1.08
	95% CI	1.01-1.26	1.02-1.27	1.00-1.29	0.93-1.20	0.99-1.23	0.98-1.22	0.97-1.24	0.96-1.23
	p-value	0.032	0.024	0.045	0.39	0.077	0.097	0.16	0.20
Stroke	Hazard ratio	1.06	1.04	1.00	0.97	1.05	1.02	0.97	0.93
	95% CI	0.94-1.20	0.92-1.18	0.88-1.15	0.84-1.11	0.94-1.18	0.91-1.15	0.85-1.10	0.82-1.07
	p-value	0.34	0.52	0.95	0.63	0.38	0.14	0.62	0.31
Cardiovascular mortality	Hazard ratio	1.46	1.48	1.39	1.33	1.38	1.33	1.25	1.14
	95% CI	1.31-1.63	1.33-1.65	1.23-1.58	1.17 – 1.52	1.24-1.52	1.20 – 1.47	1.11 – 1.41	1.01 – 1.30
	p-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.030
Non-cardiovascular mortality	Hazard ratio	1.17	1.22	1.14	1.11	1.30	1.31	1.23	1.21
	95% CI	1.06-1.30	1.10-1.36	1.01-1.28	0.98-1.26	1.17-1.43	1.19-1.45	1.10-1.38	1.07-1.36
	p-value	<0.001	<0.001	<0.036	0.086	<0.001	<0.001	<0.001	0.002

All-cause mortality*	Hazard ratio	1.54	1.57	1.20	1.18	1.47	1.46	1.15	1.09
	95% CI	1.43-1.65	1.46-1.69	1.10-1.31	1.08-1.28	1.37-1.58	1.36-1.56	1.06-1.24	1.00-1.18
	p-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.001	<0.05

CI confidence interval. Model A was adjusted for age, sex and C-reactive protein. Model B was adjusted for factors in model A and established cardiovascular risk factors (hypertension, diabetes, smoking, apolipoprotein a1, and apolipoprotein B). Model C was adjusted for factors in model B and estimated glomerular filtration rate (eGFR). Model D was adjusted for established risk factors and co-morbidities, standard biochemical predictors and treatments as shown in Supplementary Table 1.

* Proportional hazards assumption was violated for age; all models in this row is shown without adjustments for age.

Table 5. Improvement of outcome prediction by adding endostatin to the standard predictor full model

Type of predictions	The composite outcome		
	Standard predictors included (SP)	SP plus Biomarker included	Total predictions*
True favorable predictions N (%)*	2910 (48.7)	2927(49.0)	
True unfavorable predictions N (%)	1174 (19.7)	1169(19.6)	
Total number of true predictions N (%)	4084 (68.4)	4096(68.6)	5972
	All-cause mortality		
True favorable predictions N (%)	4585 (76.8)	4592(76.9)	
True unfavorable predictions N (%)	392(6.57)	401 (6.72)	
Total number of true predictions N (%)	4977 (83.4)	4993 (83.6)	5971

*A favorable (*or* unfavorable) prediction assigns $> 50\%$ ($< 50\%$) probability to being alive on a given day; the prediction is ‘true’ when the patient was in fact alive (*or* no longer alive). Three timepoints were examined, *viz.* the participant’s 3, 6 and 9-year date after randomization. Thus, the total number of predictions is 3*number of participants minus losses to follow-up. – We show the increase in the number of true predictions when we use endostatin plus the standard predictors instead of using only the standard predictors for the outcome all-cause mortality. This amounts to $4993 - 4977 = 16$. In percent of the total number of predictions made at the three time points (5971) this amounts to $16/5971$ (0.27%).

References

1. Seppinen L, Pihlajaniemi T. The multiple functions of collagen xviii in development and disease. *Matrix Biol.* 2011;30:83-92
2. Boehm T, Folkman J, Browder T, O'Reilly MS. Antiangiogenic therapy of experimental cancer does not induce acquired drug resistance. *Nature.* 1997;390:404-407
3. O'Reilly MS, Boehm T, Shing Y, Fukai N, Vasios G, Lane WS, Flynn E, Birkhead JR, Olsen BR, Folkman J. Endostatin: An endogenous inhibitor of angiogenesis and tumor growth. *Cell.* 1997;88:277-285
4. Sund M, Kalluri R. Tumor stroma derived biomarkers in cancer. *Cancer Metastasis Rev.* 2009;28:177-183
5. Carlsson AC, Ruge T, Sundstrom J, Ingelsson E, Larsson A, Lind L, Arnlov J. Association between circulating endostatin, hypertension duration, and hypertensive target-organ damage. *Hypertension.* 2013;62:1146-1151
6. Liou JY, Shyu KG, Lu MJ, Chao HH, Wang BW, Kuan PL. Pericardial fluid and serum levels of vascular endothelial growth factor and endostatin in patients with or without coronary artery disease. *J Formos Med Assoc.* 2006;105:377-383
7. Panchal VR, Rehman J, Nguyen AT, Brown JW, Turrentine MW, Mahomed Y, March KL. Reduced pericardial levels of endostatin correlate with collateral development in patients with ischemic heart disease. *J Am Coll Cardiol.* 2004;43:1383-1387
8. Hou Q, Ling L, Wang F, Xing S, Pei Z, Zeng J. Endostatin expression in neurons during the early stage of cerebral ischemia is associated with neuronal apoptotic cell death in adult hypertensive rat model of stroke. *Brain Res.* 2010;1311:182-188
9. Arenillas JF, Alvarez-Sabin J. Basic mechanisms in intracranial large-artery atherosclerosis: Advances and challenges. *Cerebrovasc Dis.* 2005;20 Suppl 2:75-83

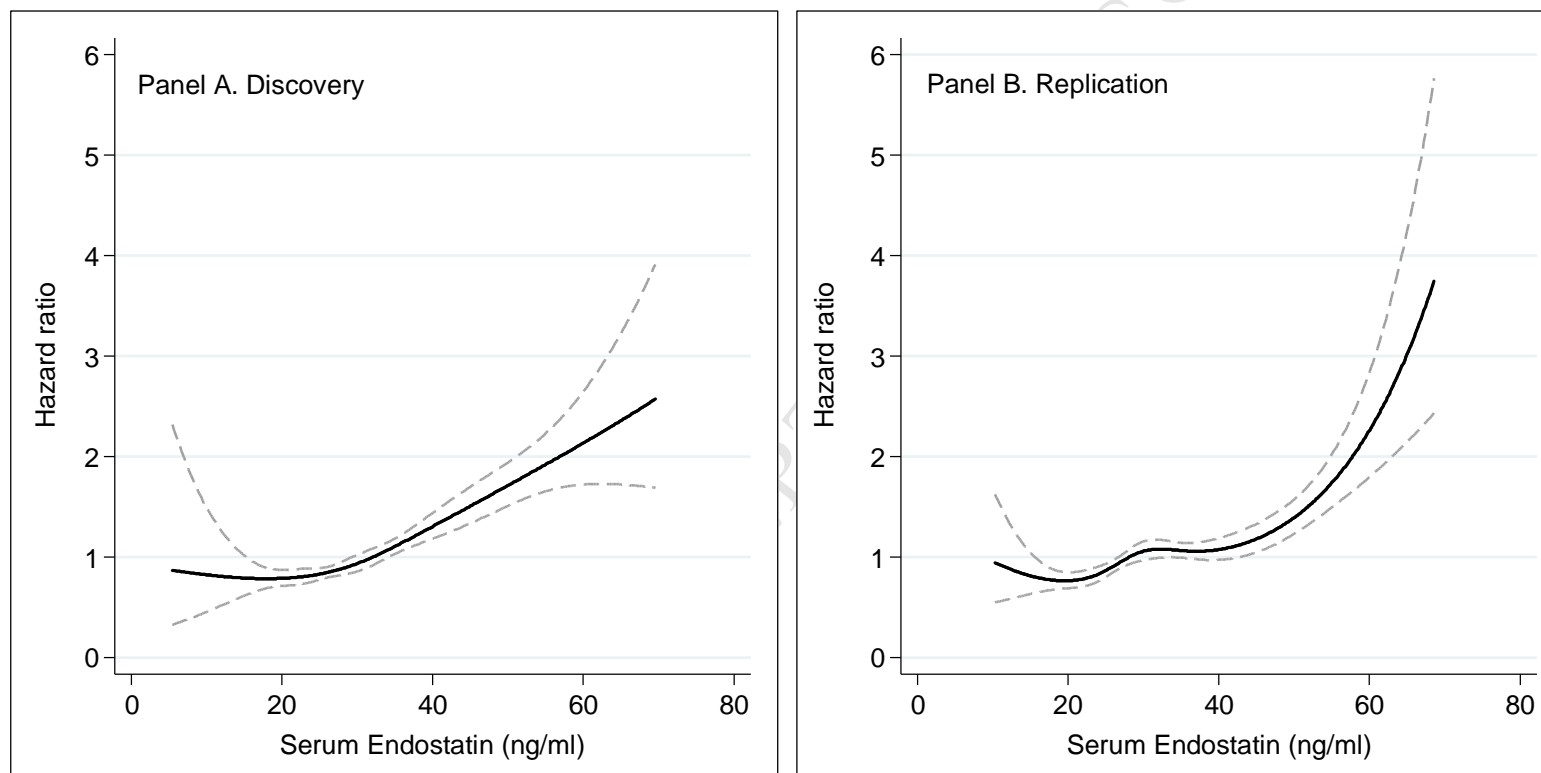
10. Golledge J, Clancy P, Hankey GJ, Yeap BB, Norman PE. Serum endostatin concentrations are higher in men with symptoms of intermittent claudication. *Dis Markers*. 2014;2014:298239
11. Arnlov J, Ruge T, Ingelsson E, Larsson A, Sundstrom J, Lind L. Serum endostatin and risk of mortality in the elderly: Findings from 2 community-based cohorts. *Arterioscler Thromb Vasc Biol*. 2013;33:2689-2695
12. Jespersen CM, Als-Nielsen B, Damgaard M, Hansen JF, Hansen S, Helo OH, Hildebrandt P, Hilden J, Jensen GB, Kastrup J, Kolmos HJ, Kjølner E, Lind I, Nielsen H, Petersen L, Glud C, Group CT. Randomised placebo controlled multicentre trial to assess short term clarithromycin for patients with stable coronary heart disease: Claricor trial. *BMJ*. 2006;332:22-27
13. Jespersen CM, Kolmos HJ, Frydendall N, Hilden J, Glud C, Hansen JF, Group CT. Compliance with and short-term adverse events from clarithromycin versus placebo in patients with stable coronary heart disease: The claricor trial. *J Antimicrob Chemother*. 2009;64:411-415
14. Glud C, Als-Nielsen B, Damgaard M, Fischer Hansen J, Hansen S, Helo OH, Hildebrandt P, Hilden J, Jensen GB, Kastrup J, Kolmos HJ, Kjølner E, Lind I, Nielsen H, Petersen L, Jespersen CM, Group CT. Clarithromycin for 2 weeks for stable coronary heart disease: 6-year follow-up of the claricor randomized trial and updated meta-analysis of antibiotics for coronary heart disease. *Cardiology*. 2008;111:280-287
15. Winkel P, Hilden J, Hansen JF, Kastrup J, Kolmos HJ, Kjølner E. Clarithromycin for stable coronary heart disease increases all-cause and cardiovascular mortality and cerebrovascular morbidity over 10 years in the claricor randomised, blinded clinical trial. *Int J Cardiol*. 2015;182
16. Kjølner E, Hilden J, Winkel P, Frandsen NJ, Galatius S, Jensen G, Kastrup J, Hansen JF, Kolmos HJ, Jespersen CM, Hildebrandt P, Glud C, Group CT. Good interobserver agreement was attainable on outcome adjudication in patients with stable coronary heart disease. *J Clin Epidemiol*. 2012;65:444-453

17. Kjøller E, Hilden J, Winkel P, Galatius S, Frandsen NJ, Jensen GB, Fischer Hansen J, Kastrup J, Jespersen CM, Hildebrandt P, Kolmos HJ, Gluud C, Group CT. Agreement between public register and adjudication committee outcome in a cardiovascular randomized clinical trial. *Am Heart J*. 2014;168:197-204 e191-194
18. Winkel P, Hilden J, Fischer Hansen J, Hildebrandt P, Kastrup J, Kolmos HJ, Kjøller E, Jespersen CM, Gluud C, Jensen GB, Group CT. Excess sudden cardiac deaths after short-term clarithromycin administration in the claricor trial: Why is this so, and why are statins protective? *Cardiology*. 2011;118:63-67
19. Levey AS, Stevens LA, Schmid CH, Zhang YL, Castro AF, 3rd, Feldman HI, Kusek JW, Eggers P, Van Lente F, Greene T, Coresh J, Ckd EPI. A new equation to estimate glomerular filtration rate. *Ann Intern Med*. 2009;150:604-612
20. Winkel P, Jakobsen J, Hilden J, Lange T, Boje J, Kjøller E, Sajadieh A, Kastrup J, Kolmos H, Larsson A, Ärnlöv J, Gluud C. Predictors for major cardiovascular outcomes in stable ischaemic heart disease (premac): Statistical analysis plan for data originating from the claricor (clarithromycin for patients with stable coronary heart disease) trial. *Diagnostic and Prognostic Research (2017)* DOI 10.1186/s41512-017-0009-y
. 2017;1:10
21. Seko Y, Fukuda S, Nagai R. Serum levels of endostatin, vascular endothelial growth factor (vegf) and hepatocyte growth factor (hgf) in patients with acute myocardial infarction undergoing early reperfusion therapy. *Clin Sci (Lond)*. 2004;106:439-442
22. Campbell DJ, Somaratne JB, Jenkins AJ, Prior DL, Yii M, Kenny JF, Newcomb AE, Kelly DJ, Black MJ. Reduced microvascular density in non-ischemic myocardium of patients with recent non-st-segment-elevation myocardial infarction. *Int J Cardiol*. 2013;167:1027-1037
23. Mitsuma W, Kodama M, Hanawa H, Ito M, Ramadan MM, Hirono S, Obata H, Okada S, Sanada F, Yanagawa T, Kashimura T, Fuse K, Tanabe N, Aizawa Y. Serum endostatin in the coronary circulation of patients with coronary heart disease and its relation to coronary collateral formation. *Am J Cardiol*. 2007;99:494-498

24. Shami A, Goncalves I, Hultgardh-Nilsson A. Collagen and related extracellular matrix proteins in atherosclerotic plaque development. *Curr Opin Lipidol.* 2014;25:394-399
25. Felbor U, Dreier L, Bryant RA, Ploegh HL, Olsen BR, Mothes W. Secreted cathepsin I generates endostatin from collagen xviii. *EMBO J.* 2000;19:1187-1194
26. Heljasvaara R, Nyberg P, Luostarinen J, Parikka M, Heikkila P, Rehn M, Sorsa T, Salo T, Pihlajaniemi T. Generation of biologically active endostatin fragments from human collagen xviii by distinct matrix metalloproteases. *Exp Cell Res.* 2005;307:292-304
27. Neufeld G, Cohen T, Gengrinovitch S, Poltorak Z. Vascular endothelial growth factor (vegf) and its receptors. *FASEB J.* 1999;13:9-22
28. Carmeliet P, Jain RK. Molecular mechanisms and clinical applications of angiogenesis. *Nature.* 2011;473:298-307
29. Virmani R, Kolodgie FD, Burke AP, Finn AV, Gold HK, Tulenko TN, Wrenn SP, Narula J. Atherosclerotic plaque progression and vulnerability to rupture: Angiogenesis as a source of intraplaque hemorrhage. *Arterioscler Thromb Vasc Biol.* 2005;25:2054-2061
30. Abou-Raya S, Naim A, Marzouk S. Cardiac matrix remodelling in congestive heart failure: The role of matrix metalloproteinases. *Clin Invest Med.* 2004;27:93-100
31. Oka T, Komuro I. Molecular mechanisms underlying the transition of cardiac hypertrophy to heart failure. *Circ J.* 2008;72 Suppl A:A13-16
32. Isobe K, Kuba K, Maejima Y, Suzuki J, Kubota S, Isobe M. Inhibition of endostatin/collagen xviii deteriorates left ventricular remodeling and heart failure in rat myocardial infarction model. *Circ J.* 2010;74:109-119
33. Sodha NR, Clements RT, Boodhwani M, Xu SH, Laham RJ, Bianchi C, Sellke FW. Endostatin and angiostatin are increased in diabetic patients with coronary artery disease and associated with impaired coronary collateral formation. *Am J Physiol Heart Circ Physiol.* 2009;296:H428-434

34. Carlsson AC, Ostgren CJ, Lanne T, Larsson A, Nystrom FH, Arnlov J. The association between endostatin and kidney disease and mortality in patients with type 2 diabetes. *Diabetes Metab.* 2016
35. Ruge T, Carlsson AC, Larsson TE, Carrero JJ, Larsson A, Lind L, Arnlov J. Endostatin level is associated with kidney injury in the elderly: Findings from two community-based cohorts. *Am J Nephrol.* 2014;40:417-424
36. Sodha NR, Boodhwani M, Clements RT, Xu SH, Khabbaz KR, Sellke FW. Increased antiangiogenic protein expression in the skeletal muscle of diabetic swine and patients. *Arch Surg.* 2008;143:463-470
37. Matyal R, Mahmood F, Robich M, Glazer H, Khabbaz K, Hess P, Bianchi C, Hagberg R, Hu SX, Sellke FW. Chronic type ii diabetes mellitus leads to changes in neuropeptide y receptor expression and distribution in human myocardial tissue. *Eur J Pharmacol.* 2011;665:19-28
38. Ogawa M, Suzuki J, Takayama K, Isobe M. Matrix metalloproteinase suppression induced by clarithromycin in murine cardiac allografts. *Transplant Proc.* 2009;41:395-397
39. Sabatine MS, Giugliano RP, Wiviott SD, Raal FJ, Blom DJ, Robinson J, Ballantyne CM, Somaratne R, Legg J, Wasserman SM, Scott R, Koren MJ, Stein EA, Open-Label Study of Long-Term Evaluation against LDLCI. Efficacy and safety of evolocumab in reducing lipids and cardiovascular events. *N Engl J Med.* 2015;372:1500-1509
40. Ridker PM, Everett BM, Thuren T, MacFadyen JG, Chang WH, Ballantyne C, Fonseca F, Nicolau J, Koenig W, Anker SD, Kastelein JJP, Cornel JH, Pais P, Pella D, Genest J, Cifkova R, Lorenzatti A, Forster T, Kobalava Z, Vida-Simiti L, Flather M, Shimokawa H, Ogawa H, Dellborg M, Rossi PRF, Troquay RPT, Libby P, Glynn RJ, Group CT. Antiinflammatory therapy with canakinumab for atherosclerotic disease. *N Engl J Med.* 2017;377:1119-1131

Figure 1 Spline curve of the association between endostatin and the composite outcome in the discovery and replication sample (panel A and B, respectively)



Highlights

- In Cox regression models adjusting for cardiovascular risk factors, glomerular filtration rate, and current pharmacological treatment, higher serum endostatin was associated with an increased risk of the composite outcome (acute myocardial infarction, unstable angina pectoris, cerebrovascular disease or all-cause mortality) in the discovery sample but not in the replication sample.
- Strong and consistent associations were found between endostatin and cardiovascular and all-cause mortality in all multivariable models and sub-samples.
- We believe that these results imply that endostatin might be involved in the development of cardiovascular events in patients with stable coronary heart disease.