Serum Endostatin and Risk of Mortality in the Elderly
Findings From 2 Community-Based Cohorts

Johan Ärnlöv,* Toralph Ruge,* Erik Ingelsson, Anders Larsson, Johan Sundström,* Lars Lind*

Objective—Experimental data imply that endostatin, a proteolytically cleaved fragment of collagen XVIII, could be involved in the development of cardiovascular disease and cancer. Prospective data concerning the relation between circulating endostatin and mortality are lacking. Accordingly, we aimed to study associations between circulating endostatin and mortality risk.

Approach and Results—Serum endostatin was analyzed in 2 community-based cohorts: the Prospective Investigation of the Vasculature in Uppsala Seniors (PIVUS; women 50%, n=748; mean age, 77 years; median follow-up, 7.9 years) and the Uppsala Longitudinal Study of Adult Men (ULSAM; n=748; mean age, 77 years; median follow-up, 9.7 years). During follow-up, 90 participants died in PIVUS (1.28/100 person-years at risk), and 417 participants died in ULSAM (6.7/100 person-years at risk). In multivariable Cox regression models adjusted for age and established cardiovascular risk factors, 1 SD higher ln(serum endostatin level) was associated with a hazard ratio of mortality of 1.39 and 95% confidence interval, 1.26 to 1.53, on average in both cohorts. In the ULSAM cohort, serum endostatin was also associated with cardiovascular mortality (177 deaths; hazard ratio per SD of ln[endostatin] 1.45, 95% confidence interval [1.25–1.71]) and cancer mortality (115 deaths; hazard ratio per SD of ln[endostatin] 1.35, 95% confidence interval [1.10–1.66]).

Conclusions—High serum endostatin was associated with increased mortality risk in 2 independent community-based cohorts of the elderly. Our observational data support the importance of extracellular matrix remodeling in the underlying pathophysiology of cardiovascular disease and cancer. (Arterioscler Thromb Vasc Biol. 2013;33:00-00.)

Key Words: angiogenesis effects ■ antiangiogenesis effects ■ cardiovascular diseases ■ epidemiology ■ mortality ■ neoplasms ■ vascular stiffness

The importance of extracellular matrix remodeling in human health and disease is currently being unraveled. Collagen XVIII is a heparan sulfate proteoglycan that is localized in most epithelial and endothelial/vascular basement membranes.1 Proteolytic cleavage of the C-terminal domain of collagen XVIII by matrix metalloproteinases, elastases, and cathepsins L and S results in a biologically active fragment called endostatin.2–7

During the last decade, the role of endostatin as a potent endogenous inhibitor of angiogenesis has been well documented.2,8–10 This antiangiogenic action of endostatin has been suggested to be of particular importance in the inhibition of cancer cell proliferation and tumor migration.11–13 Interestingly, elevated endostatin levels have been observed in the circulation in patients with various malignant diseases.14–26 In some malignancies, high endostatin levels in the circulation are associated with a worse prognosis.15,27–29 Experimental data also suggest that there may be a role for endostatin in the development of cardiovascular disease (CVD).20–32 and that circulating levels of endostatin are increased in patients with prevalent CVD.33–38 However, till date no studies have reported associations between endostatin levels and mortality risk.

Based on previous studies suggesting a role for endostatin in the development of cancer and CVD, we hypothesized that increased circulating levels of endostatin would be associated with an increased risk of mortality. Accordingly, we investigated the association between serum levels of endostatin and the risk of total mortality in a community-based cohort of elderly men and women. As a second step, we aimed at replicating the findings in an independent cohort of elderly men and women as a third step, we aimed at investigating the association between endostatin and cause-specific mortality from cancer and CVD.

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Materials and Methods

Materials and methods are available in the online-only supplement.

In brief, serum endostatin was analyzed in 2 community-based cohorts of the elderly: the Prospective Investigation of the Vasculature in Uppsala Seniors (PIVUS; women 50%, n=748; mean age, 77 years) and the Uppsala Longitudinal Study of Adult Men (ULSAM, n=748; mean age, 77 years).\textsuperscript{39,40} Data on cardiovascular risk factors, inflammatory markers, current medication, medical history, and socioeconomic factors were available at baseline in both cohorts. Mortality data were collected from The Swedish Cause-of-Death register.

Results

Baseline characteristics of both cohorts are shown in Table 1. The mean serum level of endostatin was 47±14 ng/mL in both men and women in the PIVUS cohort. During follow-up of the PIVUS study (median 7.9 years, range 0.4–9.8 years), 90 participants died (incidence rate 1.25/100 person-years at risk). During follow-up of the ULSAM cohort (median 9.7 years, range 0.3–12.9 years), 417 participants died (rate 6.7/100 person-years at risk), of which 177 died of CVD (rate 2.8/100 person-years at risk) and 115 of cancer (incidence rate 1.8/100 person-years at risk). Incidence rates by quartiles of serum endostatin are shown in Figure 1, adjusting for age (model A), lifestyle factors (model B), and established cardiovascular risk factors (model C). In tertile models, participants in the highest tertile of serum endostatin, on average, had 61% to 70% higher mortality with increasing serum endostatin level in both cohorts (Figure 2). Spline models suggested a linear increase in mortality with increasing serum endostatin level in both cohorts (Figure 3). Results were affected a little by adding a variable for freezer time to model C (hazard ratio [HR] per SD 1.32, 95% CI [1.26–1.39], P<0.001) or glomerular filtration rate (HR per SD 1.24, 95% CI [1.08–1.42], P<0.001) in model C. There was no statistically significant effect modification by sex or VEGF (P>0.28 for both). Neither VEGF nor the VEGF/endostatin ratio was associated with mortality (data not shown).

Secondary Analyses in the PIVUS Cohort

The risk estimates for ln(endostatin) were similar after additional adjustment for inflammatory markers (interleukin [IL]-1α, IL-2, IL-4, IL-6, IL-8, IL-10, interferon-γ, tissue necrosis factor-α, C-reactive protein and leukocyte count, HR per SD 1.44, 95% CI [1.16–1.80], P<0.001), vascular endothelial growth factor (VEGF; HR per SD 1.54, 95% CI [1.26–1.89], P<0.001), cathepsins L and S, matrix metalloproteinase-9, and tissue inhibitor of matrix metalloproteinase-1 (HR per SD 1.44, 95% CI [1.16–1.79], P<0.001) or glomerular filtration rate (HR per SD 1.61, 95% CI [1.27–2.05], P<0.001) in model C. There was no statistically significant effect modification by sex or VEGF (P>0.28 for both). Neither VEGF nor the VEGF/endostatin ratio was associated with mortality (data not shown).

Secondary Analyses in the ULSAM Cohort

Risk estimates for ln(endostatin) were similar after adjustment for inflammatory markers (C-reactive protein, IL-6, SAA, 15-keto-dihydro-prostaglandin factor\textsubscript{2}, and F\textsubscript{2}-isoprostanes [HR per SD 1.27, 95% CI [1.14–1.44], P<0.001]) or glomerular filtration rate (HR per SD 1.24, 95% CI [1.08–1.42], P

| Table 1. Baseline Characteristics in the PIVUS and ULSAM Cohorts |
|------------------|------------------|
|                  | PIVUS            | ULSAM            |
| Number of subjects | 931              | 748              |
| Female, n (%)     | 463 (50)         | 0 (0)            |
| Age, y            | 70±0.2           | 77±0.8           |
| Serum endostatin, ng/mL | 47.2±13.9       | 54.9±17.4        |
| Body mass index, kg/m² | 27.0±4.4         | 26.3±3.5         |
| Serum total cholesterol, mg/dL | 5.4±1.0         | 5.4±1.0          |
| Serum HDL-cholesterol, mg/dL | 1.5±0.4         | 1.3±0.3          |
| Fasting plasma glucose, mg/dL | 5.3±1.6         | 5.9±1.4          |
| Systolic blood pressure, mm Hg | 150±22         | 150±21           |
| Glomerular filtration rate | 92±21           | 73±17            |
| Serum VEGF, ng/L  | 238±180          | n/a              |
| Serum cathepsin S, μg/L | 19±6            | 14±4             |
| Serum cathepsin L, μg/L | 5±3             | 5±3              |
| Serum MMP-9, ng/L | 213±115          | n/a              |
| Serum TIMP-1, ng/L | 179±41           | n/a              |
| Smoking, n (%)    | 97 (10)          | 57 (8)           |
| Diabetes mellitus, n (%) | 80 (9)         | 107 (14)         |
| Previous cardiovascular disease, n (%) | 149 (16)       | 204 (27)         |
| Lipid-lowering treatment, n (%) | 145 (16)       | 127 (17)         |
| Antihypertensive treatment, n (%) | 284 (30)      | 356 (48)         |
| Leisure time physical activity, n (%) | 103 (11)      | 58 (8)           |
| Sedentary         | 103 (11)         | 58 (8)           |
| Moderate          | 552 (59)         | 254 (35)         |
| Regular           | 212 (23)         | 373 (52)         |
| Athletic          | 64 (7)           | 34 (5)           |
| Education/social status, n (%) | 518 (56)     | 416 (56)         |
| High              | 173 (18)         | 218 (29)         |
| Middle            | 240 (26)         | 114 (15)         |

Data are mean±SD for continuous variables and n (%) for categorical variables. PIVUS indicates Prospective Investigation of the Vasculature in Uppsala Seniors; ULSAM, Uppsala Longitudinal Study of Adult Men; and VEGF, vascular endothelial growth factor.
P=0.002), or cathepsins S and L (HR per SD 1.35, 95% CI [1.20–1.51], P<0.001) compared with those without prevalent CVD at baseline (HR for 1 SD increase of endostatin 1.30, 95% CI [1.04–1.61], P=0.02), but there was no significant effect modification by CVD status at baseline (P=0.15).

Cancer Mortality

One SD higher ln(endostatin) was associated with 24% to 35% higher cancer mortality rate (Table 2, models A–C). In tertile models, participants in the highest tertile of endostatin were at up to a doubled risk of cancer mortality compared with participants in the lowest tertile (Table 2, models A–C). The results were attenuated after exclusion of participants with history of cancer at baseline and of those who developed cancer during the first 2 years of follow-up (model C, HR per SD 1.24, 95% CI [0.97–1.59], P=0.08).

Discussion

Principal Findings

In 2 independent cohorts of elderly men and women, higher serum levels of endostatin were associated with an increased mortality risk. This association was independent of age, lifestyle factors, and cardiovascular risk factors. Moreover, higher levels of endostatin were associated with cause-specific mortality both from cancer and CVD. The association was not influenced by further adjustment for markers reflecting kidney dysfunction, systemic inflammation, angiogenic activity, or proteases that generate endostatin fragments from collagen XVIII. Our data indicate that endostatin is involved in, or a marker for, pathological processes leading to the progression of cancer and CVD, and that it may be a promising biomarker for further validation and clinical application.

Figure 1. Associations of serum endostatin levels with mortality rate. CI indicates confidence interval; HR, hazard ratio; and T, tertile.

Figure 2. Cumulative incidence of total mortality by tertiles of serum endostatin. T indicates tertile.
study. We are aware of no previous study that has reported the prospective association between circulating endostatin and the risk for mortality in the community-based setting.

Comparison With the Literature
Elevated endostatin levels have been observed in the circulation from patients with different malignant diseases and have also been associated with the severity of disease. Moreover, higher endostatin levels have been reported in patients with prevalent CVD such as stroke and coronary atherosclerosis. However, prospective data on the association between circulating endostatin and adverse events are scarce and partly conflicting. In patients with non-Hodgkin lymphoma and renal carcinoma, high levels of circulating endostatin were associated with a decreased expected survival time. In addition, in patients with intracranial atherosclerosis, higher circulating endostatin has been shown to be an independent predictor of cerebral ischemic events, and in patients with ischemic stroke, higher endostatin was associated with a poorer long-term functional outcome. Interestingly, Seko et al. suggested that circulating levels of endostatin are increased in patients with acute myocardial infarction, but

Table 2. Associations of Serum Endostatin to Cardiovascular and Cancer Mortality in the ULSAM Cohort

<table>
<thead>
<tr>
<th>Continuous models: per SD higher ln(endostatin)</th>
<th>CVD Mortality</th>
<th>Cancer Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hazard Ratio (95% CI)</td>
<td>P Value</td>
<td>Hazard Ratio (95% CI)</td>
</tr>
<tr>
<td>Model A</td>
<td>1.55 (1.34–1.79)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Model B</td>
<td>1.52 (1.31–1.77)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Model C</td>
<td>1.45 (1.25–1.71)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Tertile models (T2 and T3 vs T1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1 (Endo &lt;47.2 μg/L)</td>
<td>Referent</td>
<td>Referent</td>
</tr>
<tr>
<td>T2 (Endo 47.3–58.3 μg/L)</td>
<td>0.93 (0.62–1.38)</td>
<td>0.72</td>
</tr>
<tr>
<td>T3 (Endo 58.4–213.0 μg/L)</td>
<td>2.05 (1.4–2.9)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Model B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1 (Endo &lt;47.2 μg/L)</td>
<td>Referent</td>
<td>Referent</td>
</tr>
<tr>
<td>T2 (Endo 47.3–58.3 μg/L)</td>
<td>1.00 (0.66–1.50)</td>
<td>0.99</td>
</tr>
<tr>
<td>T3 (Endo 58.4–213.0 μg/L)</td>
<td>1.96 (1.35–2.84)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Model C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1 (Endo &lt;47.2 μg/L)</td>
<td>Referent</td>
<td>Referent</td>
</tr>
<tr>
<td>T2 (Endo 47.3–58.3 μg/L)</td>
<td>0.65 (0.56–1.26)</td>
<td>0.42</td>
</tr>
<tr>
<td>T3 (Endo 58.4–213.0 μg/L)</td>
<td>1.70 (1.17–2.48)</td>
<td>0.006</td>
</tr>
</tbody>
</table>

Model A adjusted for age; model B adjusted for lifestyle (age, body mass index [BMI], smoking, leisure time physical activity, and education level); model C adjusted for cardiovascular risk factors (age, systolic blood pressure, diabetes mellitus, smoking, BMI, total cholesterol, HDL-cholesterol, antihypertensive treatment, lipid-lowering treatment, and history of cardiovascular disease [CVD]). CI indicates confidence interval; and ULSAM, Uppsala Longitudinal Study of Adult Men.
It is also possible that the results may be confounded by some other causal factor. For instance, it is possible that higher circulating levels of endostatin reflect a reduced renal clearance of endostatin. Moreover, circulating levels of endostatin have been shown to be associated with other important risk factors for mortality such as age, physical activity, body mass index, and glucose intolerance. However, the fact that the results were essentially unaltered in all multivariable models would argue against confounding by these factors as a major explanation of our findings. However, we cannot rule out confounding by an unmeasured factor.

**Clinical Implications**
Angiogenesis-modulating treatment of malignant and CVD has gained much interest in the past years, and several of such compounds are now included in the pharmaceutical arsenal. Endostar, an antiangiogenic agent based on endostatin, is currently used in the arsenal treatment against cancer. It should be worthwhile to investigate whether endostatin can be used to monitor such treatment or to identify patients who would benefit from that treatment.

**Strengths and Limitations**
The strengths of our investigation include the homogenous, community-based study samples with longitudinal data, the detailed characterization of the study participants pertaining to lifestyle and cardiovascular risk factors, and the replication of the results in an independent cohort. To our knowledge, the ULSAM and PIVUS cohorts are the largest cohorts that have analyzed circulating levels of endostatin. Limitations include the unknown generalizability to other age and ethnic groups.

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**Disclosures**
None.

**References**


Significance

In the present study, an association between serum endostatin and mortality risk was found and validated in an independent cohort. Our observational data provide further support for the importance of extracellular matrix remodeling in the underlying pathophysiology of cancer and cardiovascular disease.
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Supplement Material

Supplementary Table I. Incidence rates in tertiles of serum endostatin for total mortality in the PIVUS cohort, and for total mortality, cardiovascular mortality, cancer mortality and non-cardiovascular/non-cancer mortality in the ULSAM cohort

<table>
<thead>
<tr>
<th>Tertile</th>
<th>The PIVUS cohort</th>
<th>The ULSAM cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total mortality</td>
<td>Total mortality</td>
</tr>
<tr>
<td>NE/NR</td>
<td>IR (95% CI)</td>
<td>NE/NR (95% CI)</td>
</tr>
<tr>
<td>T1</td>
<td>24/287 1.0 (0.7-1.5)</td>
<td>120/258 5.4 (4.5-6.4)</td>
</tr>
<tr>
<td>T2</td>
<td>25/286 1.0 (0.7-1.6)</td>
<td>132/250 6.0 (5.0-7.1)</td>
</tr>
<tr>
<td>T3</td>
<td>41/270 1.7 (1.2-2.3)</td>
<td>165/240 9.2 (7.9-10.7)</td>
</tr>
</tbody>
</table>

T=Tertiles, IR= incidence rates per 100 person years follow-up, NE/NR= Number of events/ Numbers at risk.

Tertile cutpoints in the PIVUS cohort: T1 <40.2 µg/L, T2 40.3-50.6 µg/L, T3 50.7 – 139.2 µg/L

Tertile cutpoints in the ULSAM cohort: T1 < 47.2 µg/L, T2 µg/L 47.3-58.3 µg/L, T3 58.4-213.0 µg/L
Methods and Material

Study samples
The Prospective Investigation of the Vasculature in Uppsala Seniors (PIVUS)
All 70-year old men and women living in Uppsala, Sweden, between 2001-2004 were eligible for the PIVUS study ¹ (described in detail on http://www.medsci.uu.se/pivus/pivus.htm). Of 2025 invited individuals, 1016 agreed to participate. Of these, 85 participants were excluded due to missing data on endostatin (n=13) or missing covariates (n=72), leaving 931 participants as the present study sample.

The Uppsala Longitudinal Study of Adult Men (ULSAM)
The ULSAM study was initiated in 1970. All 50-year-old men, born in 1920-24 and living in Uppsala, Sweden, were invited to a health survey, focusing at identifying cardiovascular risk factors ² (described in detail on http://www.pubcare.uu.se/ULSAM). These analyses are based on the fourth examination cycle, when participants were approximately 77 years old (1998-2001). Of 1398 invited men, 838 (60%) participated. Of these, 90 were excluded due to missing data on endostatin (n=53) or covariates (n=37), leaving 748 participants as the present study sample.

All participants in both studies gave written informed consent and the Ethics Committee of Uppsala University approved the study protocols.

Baseline investigations
The investigations in PIVUS and ULSAM were performed using similar standardized methods, including anthropometrical measurements, blood pressure, blood sampling, and questionnaires regarding socioeconomic status, medical history, smoking habits, medication and physical activity level ¹⁻². Venous blood samples were drawn in the morning after an overnight fast and stored at –70°C until analysis. Serum levels of endostatin were analyzed 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%. In PIVUS, the serum samples had been frozen for a median of 7.3 years (range 5.7-9.0 years) until analysis of endostatin in March 2010 and in ULSAM for 10.8 years (range 9.6-13.0 years) until analysis in February 2011. The intraassay CV was < 6%.

Inflammatory markers, cystatin C-based glomerular filtration rate, Cathepsin S, MMP-9, TIMP-1 were measured as previously described (ULSAM ³⁻⁵, PIVUS ⁴⁻⁷). Cathepsin L was analyzed by ELISA kits (DY952, R&D Systems, Minneapolis, MN.), serum VEGF quantification was performed by Randox Ltd (Crumlin, UK), using a biochip array analyzer (Evidence®).

Diabetes mellitus was diagnosed as fasting plasma glucose ≥7.0 mmol/l (≥126mg/dl), or use of anti-diabetic medication. Prevalent cardiovascular disease at baseline was defined as a history of ischemic heart disease or cerebrovascular disease, or Q-, QS-complexes or left bundle-branch block in baseline ECG. Leisure time physical activity was assessed by a questionnaire as previously described ⁸. Education level was stratified as low (elementary school, 6-7 years), medium (high school), or high (college studies).

End-point definitions
The Swedish Cause-of-Death register was used to define total mortality, cardiovascular mortality (death from ischemic heart disease or cerebrovascular disease [ICD-9] codes 410-414, 430-438,
or [ICD-10] codes I20-I25, I60-I69/G45) and cancer mortality ([ICD-9] 140-239 and [ICD-10] C00-D48). Data on cause-specific mortality was not available in the PIVUS cohort.

### Statistical analysis

#### Primary analyses

We initially investigated distributions of all variables. Serum endostatin was logarithmically transformed for use in all analyses. We thereafter investigated cohort-specific associations of serum endostatin (modelled both as a continuous variable, per standard deviation; and by tertiles, lowest tertile as reference) with total mortality using Cox proportional hazards regression in the following multivariable models:

A) Age- and sex-adjusted;

B) Lifestyle model (age, sex, BMI, smoking, leisure time physical activity and education level);

C) Cardiovascular risk factor model (age, sex, systolic blood pressure, diabetes, smoking, BMI, total cholesterol, HDL-cholesterol, antihypertensive treatment, lipid-lowering treatment and prevalent cardiovascular disease).

Results were then summarized across cohorts using fixed effects models, as the number of cohorts (two) was too small to allow precise estimation of the between-studies variance ($\tau^2$) needed for random-effects models. We evaluated heterogeneity of effects using the $I^2$ statistic. Proportional hazards assumptions were confirmed by Schoenfeld’s tests. We investigated potential nonlinearity of the associations using penalized splines. As the effects of long-term freezing on endostatin levels are uncertain, we also added freezer time as a covariate in separate models. In secondary analyses, multiple imputation methods were used to account for the potential influence of missing data.

A two-sided p-value <0.05 was regarded as significant in all analyses. Stata 12.1 (Stata Corp College Station, TX, USA) was used for all analyses.

#### Secondary analyses in the PIVUS cohort

We performed secondary analyses in the PIVUS cohort in which inflammatory markers (serum interleukin (IL)-1a, IL-1b, IL-2, IL-4, IL-6, IL-8 IL-10, interferon-gamma, TNF-alpha, C-reactive protein (CRP) and leukocyte count), kidney function, cathepsin L, cathepsin S, MMP-9, TIMP-1 or VEGF were added to model C. We investigated effect modification by gender and VEGF levels by including multiplicative interaction terms in Model C. In secondary analyses, we also investigated the association between VEGF, VEGF/endostatin-ratio and mortality. Tertile limits for serum endostatin in the PIVUS cohort were 40.2 and 50.6 μg/L.

#### Secondary analyses in the ULSAM cohort

In the ULSAM cohort, we also investigated the association between serum endostatin and cause-specific mortality from cardiovascular causes or cancer. In secondary analyses, we further added the following markers of inflammation and oxidative stress to multivariable model C (CRP, IL-6, plasma serum amyloid A [SAA], urinary 15-keto-dihydro-PGF$_{2\alpha}$ [reflecting COX-mediated inflammation] and F$_2$-isoprostanes [reflecting oxidative stress]. We investigated effect modification by prevalent cardiovascular disease by including a multiplicative interaction term in Model C, and by separately studying substrata with and without prevalent cardiovascular disease. We also investigated the association between serum endostatin and cancer mortality after exclusion of participants with cancer at baseline or during the first 2 years of follow-up.
to limit the possibility of reverse causation as an explanation of our findings. Tertile limits for serum endostatin in the ULSAM cohort were 47.2 and 58.3 μg/L.

References