

**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 (described in detail on [http://www.medsci.uu.se/pivus/pivus.htm](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 (described in detail on [http://www.pubcare.uu.se/ULSAM](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. 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, 6-7). 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. 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
(n=112) 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