Increased urinary cystatin C indicated higher risk of cardiovascular death in a community cohort

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Running title: Urine Cystatin C and mortality

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Abstract

Objectives: Urinary Cystatin C (u-CysC) is a new biomarker for acute tubular kidney dysfunction and may also indicate chronic tubular dysfunction. Chronic kidney disease is an important cardiovascular risk factor, however it is not known if u-CysC is a risk marker for cardiovascular death.

Methods: The association between u-CysC and cardiovascular mortality was investigated in a Swedish community-based cohort of 604 men aged 78 years. During follow-up (mean 6.7 years), 203 participants died, of which 90 due to cardiovascular causes.

Results: High u-CysC (>0.029 mg/mmol Cr) was associated with a more than 2-fold risk of cardiovascular death (multivariable hazard ratio for quintile 5 vs. 1: 2.5, 95% CI 1.2-5.2, $p<0.05$) in Cox regression models independent of cardiovascular risk factors, glomerular filtration rate (eGFR) and urinary Albumin. Participants with low eGFR (≤60 mL/min), albuminuria (≥ 3 mg/mmol Cr) and high u-CysC (>0.029 mg/mmol Cr) combined had a significantly higher cardiovascular mortality risk compared to participants with one or two of these biomarkers normal (hazard ratio 15, 95% CI: 6.7-36, $P<0.001$, compared to all three biomarkers normal).

Conclusions: This study is the first to show that increased concentrations of the tubular kidney biomarker u-CysC indicated risk of cardiovascular death independently of other cardiovascular risk factors, glomerular filtration and albuminuria. Additional research is needed to further establish the usefulness of u-CysC in clinical practice.

Key Words: chronic kidney disease, risk factors, mortality, epidemiology, atherosclerosis

Abbreviations: U-CysC = urinary cystatin C; Cr = creatinine; $eGFR_{CysC}$ = estimated glomerular filtration rate (by S-CystatinC); u-Alb = urinary Albumin; HR = hazard ratio; u-

NGAL = urinary neutrophil gelatinase-associated lipocalin; u-KIM-1 = urinary kidney injury molecule-1

1. Introduction

Cystatin C (CysC) is a small protein produced at a constant rate by all nucleated cells [1]. It has important protective extracellular functions by inhibiting cysteine proteinases, such as papain, ficin, and cathepsins [2], which otherwise cause uncontrolled proteolysis and tissue damage. Serum concentrations of CysC are seemingly independent of sex and muscular mass and constant up to 60 years of age [3, 4]. The low molecular weight and positive charge at physiological pH allows CysC to filter freely in the kidney glomerulus with no retrieval back to the circulation, thus serum CysC is a clinically established biomarker for estimating glomerular filtration rate (GFR) [5-7]. Following glomerular filtration, CysC is reabsorbed in proximal tubule and subsequently almost completely catabolized [8, 9]. The remaining CysC is eliminated in the urine and urinary CysC (u-CysC) concentrations are thus normally very low [9-11]. Tubular dysfunction reduce the degradation leading to a subsequent increase in u-CysC concentrations and several studies have suggested that u-CysC is a reliable biomarker of acute tubular damage [5, 12-20] and worse prognosis [16, 19] in critically ill patients. Furthermore, US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) accept u-CysC as one of seven qualified biomarkers of renal injury that can be used by drug companies to evaluate kidney damage during animal studies of new drugs [21].

Kidney damage of chronic nature represents a trait closely related to increased risk of atherogenesis. Even mildly impaired glomerular filtration, estimated by serum creatinine or serum CysC [6] or urinary albumin, predicts cardiovascular and all-cause mortality [22-25]. Chronic kidney disease is considered a significant fatal cardiovascular risk factor [26, 27], however whether specific tubular damage is associated with an increased cardiovascular risk is less studied. We hypothesized that increases in u-CysC may indicate chronic low-grade tubular dysfunction, thus signalling an increased cardiovascular risk. Accordingly, the aim of

this study was to explore the associations between u-CysC and cardiovascular mortality in a community-based cohort of elderly men with up to 10 years of follow-up.

2. Methods and materials

2.1 Study population

This study is based on participants from the third reinvestigation of the Swedish cohort Uppsala Longitudinal Study of Adult Men (ULSAM) performed in 1997-2001. The cohort initially started in 1970 when all 50-year-old men living in Uppsala county (N =2841) were invited to participate in a health survey [28]. Out of the 839 men participating in the third reinvestigation 27 years later, 199 were excluded at baseline because of missing urine samples and 36 because of missing covariate data, thus 604 men constituted the study population. The Ethics Committee at Uppsala University approved the study, and all participants gave informed consent.

2.2 Baseline investigations

24-hour urine was collected, aliquoted and stored at -70° until analysis. U-CysC was analysed on a Mindray BS-380 (Shenzhen Mindray Bio-medical Electronics, Shenzhen, China) with reagents from Gentian (Moss, Norway). The total coefficient of variation (CV) for the u-CysC method was 2.5% at 0.48 mg/L and 1.5% at 0.8 mg/L [10]. Concentrations of u-CysC were adjusted for urinary creatinine concentrations (IL Test creatinine 181672-00, Monarch 2000 analyser, Instrumental Laboratories, Lexington, MA) and the ratio given in mg/mmol creatinine (Cr). Blood sampling, anthropometrical and blood pressure measurement, questionnaires regarding medication and smoking habits and diabetes definition were performed using the same standardized methods as described previously [28, 29]. Serum cystatin C and cholesterol and fasting plasma glucose were measured as previously described [30] and GFR in mL/min/1.73m^2 was calculated from serum cystatin C results in mg/L by the validated formula y = 77.24x-1.2623 ($eGFR_{cysC}$) [7]. Information regarding present cardiovascular or cerebrovascular disease at baseline was collected from the Swedish Hospital

Discharge Registry using the International Statistical Classification of Diseases, Ninth Revision (ICD-9) codes 410-413, 428, 433-436 and ICD-10 codes I20-I25, I50, I63-I66.

2.3 Endpoint definitions

Data on mortality due to cardiovascular causes (ICD-10 I00-99) was obtained from the Swedish Cause of Death register.

2.4 Statistics

The baseline characteristics in the u-CysC/mmol Cr quintiles were compared with analysis of variance or chi-square tests. The associations of u-CysC concentrations and mortality was analysed with Cox proportional hazard regression in a univariable and in three multivariable models (A, B and C). Model A was adjusted for established cardiovascular risk factors including systolic blood pressure, body mass index, smoking status, diabetes, and prevalent cardiovascular disease. Model B was adjusted for the same as model A and additionally for $eGFR_{cysC}$. Model C was adjusted for the same as model A , $eGFR_{cysC}$ and urinary albumin. U-CysC was entered into the models in quintiles and as a standardised continous variable (expressed as 1 SD increase). U-CysC was entered both as absolut values (in mg/L) and as a ratio to Cr (in mg/mmol Cr) in separate analyses. To study potential nonlinearity of the associations we examined Cox regression models using penalized splines with four degrees of freedom.

The participants were also divided into four groups according to; all three kidney biomarkers normal (normal u-CysC here defined as <0.029 mg/mmol Cr [below highest quintile], normal $eGFR_{CysC}$ >60 mL/min and normal u-Alb <3 mg/mmol Cr); 1 pathological biomarker; 2 pathological biomarkers; all 3 biomarkers pathological. Additionally the study participants

were divided into four groups according to; u-CysC above and below 0.029 mg/mmol Cr and u-Alb above and below 3 mg/mmol Cr or $eGFR_{cysC}$ below and above 60 mL/min, respectively. We also performed analyses after exclusion of participants with present or previous diagnosis of cardiovascular disease at baseline (excluded n = 162) to limit the possibility of reverse causation as an explanation of our findings.

We performed tests for effect modification by prevalent cardiovascular disease, diabetes, smoking and $eGFR_{CysC}$ by including multiplicative interaction terms of these variables and u-CysC. P-values <0.05 were regarded as statistically significant. Calculations were performed with Stata 13.0 (College Station, TX).

3. Results

3.1 Baseline characteristics

Baseline characteristics of the study population in quintiles of u-CysC are shown in Table 1. A majority of the study participants (80 %) had u-Alb < 3 mg/mmol Cr, 18 % had microalbuminuria (3-29 mg/mmol Cr) and 2 % macroalbuminuria (> 30 mg/mmol Cr). During follow-up (of mean [SD] 6.7 [2.0] years corresponding to 4031 person-years in total) 203 participants died of which 90 died of cardiovascular causes. Total mortality incidence rate (95% CI) was 50 (44-58) and cardiovascular mortality incidence rate 22 (18-27) per 1000 person-years.

3.2 Cox regression models for associations between u-CysC and mortality

A 1-SD increase of u-CysC, expressed as both absolute u-CysC concentrations and u-CysC/Cr ratio, was significantly associated with total mortality in the univariable models and in the models adjusting for established cardiovascular risk factors, $eGFR_{CysC}$ and u-Alb, multivariable HR (95% CI) 1.1 (1.0-1.2).

Participants in the highest quintile of u-CysC had a 2- to 2.5-fold higher cardiovascular mortality risk compared to quintile 1 which was significant in the univariable models and in all other models, see Table 2. The hazard ratios tended to be overall slightly higher in the models with u-CysC expressed as a ratio to Cr than in the models with absolute u-CysC concentrations. The cumulative incidence of cardiovascular mortality in quintile 1-4 and quintile 5 of u-CysC is shown in Figure 1. In the subgroup with exclusion of participants with present or previous diagnosis of cardiovascular disease at baseline participants in the highest quintile of u-CysC also had a significantly higher cardiovascular mortality risk in all models (multivariable HR [95% CI] 3.1 [1.2-7.9] compared to the lowest quintile).

A 1-SD increase of u-CysC and u-CysC /Cr ratio, was significantly associated with a 1.2 HR of cardiovascular mortality in the univariable models and in the models adjusting for established cardiovascular risk factors and $eGFR_{CysC}$ (model A and B), see Table 2. However, the associations were weakened and no longer significant when u-Alb was added to the models. The regression spline showed a linear increase in hazard for cardiovascular mortality with increasing u-CysC /Cr ratio (Figure 2). No significant effect modification of diabetes, previous cardiovascular disease, $eGFR_{CysC}$ or smoking status was observed (P>0.05 for all).

3.3 Cox regression models for associations of u-CysC, $eGFR_{CysC}$ and u-Alb combined and cardiovascular mortality

The group of participants with high u-CysC concentrations (>0.029 mg/mmol Cr), low $eGFR_{CysC}$ (≤60 mL/min) and high u-Alb (≥ 3 mg/mmol Cr) combined had a 16-fold increased risk in cardiovascular mortality compared to participants with all three biomarkers normal. Participants with two pathological biomarkers (high u-CysC, low $eGFR_{CysC}$ or high u-Alb) or only one pathological biomarker had also increased risk (HR [95% CI] 3.7 [2.0-6.7] and 2.3 [1.4-3.8], respectively) but the risk was significantly lower than in the group with three pathological biomarkers combined (HR 15 [6.7-36]).

When analysing the additive cardiovascular mortality risk of u-CysC on u-Alb and eGFR separately, an additive mortality risk by high u-CysC (>0.029 mg/mmol Cr) was seen both in participants with normal eGFR and low eGFR, Fig 3b. High u-CysC did not add mortality risk in the group without albuminuria but the combination of albuminuria and high CysC was associated with a 5-fold increased mortality risk compared to the referent group without albuminuria and with u-CysC <0.029 mg/mmol Cr, Fig 3a.

4. Discussion

This is the first study to report that increased concentrations of the new tubular kidney biomarker u-CysC indicated cardiovascular mortality risk up to 10 years later. Other established cardiovascular risk factors, glomerular filtration (eGFR) or u-Alb alone could primarily not explain these associations in this community-based study. Further, the association seen between the combination of high u-CysC, low eGFR and high u-Alb and cardiovascular death may indicate that u-CysC adds information on cardiovascular mortality risk to the two most commonly used indices of kidney damage and dysfunction.

Renal dysfunction, indicated by increased concentrations of serum creatinine, cystatin C [6] or u-Alb, is now considered as an important fatal cardiovascular risk factor [22-24, 31]. The results of this study where mildly increased u-CystC clearly indicated a higher cardiovascular mortality risk is in line with previous studies suggesting that not only chronic renal glomerular dysfunction, but also chronic tubular dysfunction, may be important in the pathophysiology of nephropathies [32] and atherosclerosis. Increased urinary concentrations of other recently proposed biomarkers for tubular dysfunction such as urinary beta-2 microglobulin and urinary N-acetyl-beta-D-glucosaminidase (u-NAG) indicated a higher risk for cardiovascular disease in type 2 diabetic patients [33, 34]. Recent evidence supports the notion that the new tubular damage biomarkers urinary neutrophil gelatinase-associated lipocalin (u-NGAL) and urinary kidney injury molecule-1 (u-KIM-1) are associated with cardiovascular mortality and incident heart failure in the community [35, 36]. The findings in the present study and the findings of associations between u-NGAL and u-KIM-1 and adverse cardiovascular outcomes in the community further highlights that chronic tubular dysfunction is an important cardiovascular risk factor to consider in primary and secondary preventive care. Further studies are needed to evaluate the clinical utility of u-CystC.

This study may add mechanistic insight on the interplay between renal damage and fatal cardiovascular disease. Although based on analyses in small subgroups, a significant additive mortality risk effect was seen when all three biomarkers u-CysC, $eGFR_{CysC}$ and u-Alb were abnormal compared to only two or one of them, which may indicate that these three biomarkers reflect partly different pathological mechanisms. While increased serum cystatin C estimates glomerular dysfunction (expressed as decreased $eGFR_{CysC}$) it is still debated what underlying pathology albuminuria reflects. Pathological albuminuria may indicate glomerular dysfunction including defect podocytes or endothelial cell defects [37] as well as tubular dysfunction [38]. However, albuminuria has also been suggested to be a marker for impaired endothelial function in the whole vasculature [39, 40]. U-CystC primarily reflects proximal tubular cell defects [5] but the results of this study indicated that u-CysC was associated with cardiovascular mortality independent of u-Alb. Speculatively, u-CysC and u-Alb may mirror different aspects of tubular dysfunction. In other words that u-CysC is a biomarker of a pathological condition in the tubuli which is not fully detectable with the markers used in clinical practice today, i.e. $eGFR_{CysC}$ and u-Alb. Hyperglycemia is suggested to cause diabetic nephropathy through direct effects of elevated blood sugar and through oxidative stress [41, 42]. Decreased u-CysC reabsorption in the kidney may be an early marker of individuals with an initiated prediabetic nephropathy not accounted for by adjustments for diabetes at baseline. Finally, we cannot exclude the possibility that u-CystC is elevated in individuals who will suffer a more rapid decline in GFR. Rapid loss of kidney function has been shown to predict cardiovascular events independent of baseline GFR [43].

The clinical usefulness of measuring u-CysC for risk prediction purposes in patients and in the community needs to be further evaluated. However, considering economic aspects u-CysC may be an affordable and logistically preferable alternative for hospital laboratories compared

to other tubular dysfunction biomarkers emerging on the market (u-NGAL and u-KIM-1) as applications for serum and plasma CysC is available on many large analyzing platforms on the market which allows hospital laboratories to analyze CysC at a fairly low cost. The applications can easily be modified for measurement of CysC in urine.

U-Alb was initially reported as total amount excreted during 24 hours. Many laboratories also reported u-Alb excreted per minute or as a concentration. Presently, most u-Alb results are reported as a Albumin creatinine ratio (ACR) in an attempt to adjust for urinary dilution and ACR is often considered to be superior to u-Alb expressed in mg/L [44]. In analogy, it is not known if u-CysC should be reported as absolute values (in mg/L) or as a u-CysC creatinine ratio (in mg/mmol Cr). Our results indicate that u-CysC reported both in absolute values or as a creatinine ratio is indicative of an increased cardiovascular mortality risk. The hazard ratios were however generally higher for the creatinine ratio indicating that the adjustment for urinary creatinine might be beneficial for risk prediction purposes.

The community-based study design representing both u-CysC, serum CysC and detailed clinical and biochemical characterization of cardiovascular risk factors at baseline are strengths of this study. The follow-up time is extensive and due to the high quality of Swedish registry data [45], the loss to follow-up is very low. The study is obviously limited by the absence of women and other age and ethnic groups. The study population consisted of apparently healthy participants with generally low concentrations of u-CysC, and subsequently relatively small concentration differences between the quintiles. Risk estimates between nearby quintiles should for this reason, together with a limited total number of events in the quintiles, be interpreted with caution. The results may to some extent relate to reverse causation where high u-CysC is rather a consequence of prevalent disease with related low-

grade tubular damage at baseline. However, present or previous diagnosis of cardiovascular disease at baseline did not associate with u-CysC. Further, the results remained significant when we removed those who had a clinical diagnosis of cardiovascular disease at baseline; which indicates that reverse causation is not a substantial explanation to our findings. The subgroup analysis may be limited by the sometimes small number of participants in the subgroups leading to potentially less certain estimates.

5. Conclusion

This is the first study to explore u-CysC as a biomarker of chronic low-grade tubular damage and the results suggest that even mildly increased concentrations of u-CysC indicate higher risk of cardiovascular death independent of other cardiovascular risk factors, glomerular filtration and u-Alb. However, the clinical usefulness of measuring u-CysC for risk prediction purposes in different age groups and in the community remains to be evaluated.

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Tables

Table 1. Baseline characteristics of the study population (n = 604) in quintiles of urinary Cystatin C (mg/mmol Cr)

	All	**Quintiles**					**P**
	Mean (SD)	**Mean (SD)**					
Variable		**1 (lowest)**	**2**	**3**	**4**	**5 (highest)**	
Urinary Cystatin C/creatinine ratio (mg/mmol Cr)	0.018 (0.035)	0.00059 (0.00017)	0.0030 (0.0018)	0.013 (0.0038)	0.024 (0.0028)	0.051 (0.067)	-
Urinary Cystatin C (mg/L)	0.14 (0.21)	0.0059 (0.0019)	0.028 (0.020)	0.11 (0.053)	0.21 (0.090)	0.38 (0.35)	<0.001
Urinary creatinine (mmol/L)	9.0 (3.3)	10 (3.0)	8.8 (3.0)	8.7 (3.2)	8.8 (3.5)	8.1 (3.3)	<0.001
Age (years)	78 (0.8)	78 (0.8)	78 (0.8)	78 (0.8)	78 (0.7)	78 (0.7)	0.96
BMI (kg/m^2)	26 (3.4)	26 (3.4)	26 (3.4)	26 (3.4)	26 (3.2)	26 (3.8)	0.79
Systolic blood pressure (mmHg)	151 (21)	150 (21)	149 (19)	150 (21)	151 (21)	153 (21)	0.53
$eGFR_{CysC}$ ($mL/min/1.73\ m^2$)	74 (17)	76 (15)	70 (16)	73 (17)	76 (20)	74 (17)	0.06

Total cholesterol (mmol/L)	5.4 (1.0)	5.4 (0.9)	5.5 (1.0)	5.3 (1.0)	5.3 (0.9)	5.4 (1.1)	0.40
HDL (mmol/L)	1.3 (0.33)	1.3 (0.35)	1.3 (0.32)	1.3 (0.30)	1.3 (0.33)	1.3 (0.34)	0.86
	N (%)						**P**
Previous clinical cardiovascular disease	162 (27)	30 (25)	35 (29)	34 (28)	35 (29)	28 (23)	0.80
Smoking	41 (6.8)	5 (4.1)	7 (5.7)	10 (8.2)	7 (5.7)	12 (10)	0.39
Diabetes	82 (14)	11 (9.0)	17 (14)	20 (17)	18 (15)	16 (13)	0.53
Antihypertensive medication	291 (48)	60 (50)	63 (52)	56 (46)	64 (53)	48 (40)	0.26
Lipid lowering medication	111 (18)	19 (16)	22 (18)	22 (18)	31 (26)	17 (14)	0.18

Cr, urinary creatinine, $eGFR_{CysC}$, estimated glomerular filtration rate by serum cystatin C

Table 2. Associations between urinary cystatin C/creatinine ratio (mg/mmol Cr) and urinary cystatin C (mg/L) and cardiovascular mortality risk in Cox regression models

	Continuous	**Quintiles**				
	1 SD-increase	1 (lowest)	2	3	4	5 (highest)
	HR (95 % CI)	HR (95 % CI)	HR (95 % CI)	HR (95 % CI)	HR (95 % CI)	HR (95 % CI)
Urinary Cystatin C/creatinine ratio (mg/mmol Cr)						
Number of events/number at risk	90/604	10/121	20/121	20/121	14/121	26/120
1. Crude model	1.2*** (1.1-1.4)	referent	2.1 (0.98-4.5)	2.1 (0.99-4.5)	1.5 (0.68-3.5)	2.8** (1.3-5.8)
2. Model A	1.2** (1.1-1.3)	referent	2.0 (0.91-4.2)	2.0 (0.93-4.3)	1.4 (0.63-3.2)	2.6* (1.3-5.5)
3. Model B	1.1 (1.0-1.2)	referent	1.7 (0.77-3.6)	1.9 (0.88-4.0)	1.3 (0.58-3.0)	2.5* (1.2-5.3)
4. Model C	1.1 (0.97-1.2)	referent	1.7 (0.91-4.2)	1.9 (0.93-4.3)	1.3 (0.63-4.2)	2.5* (1.2-5.2)
Urinary Cystatin C (mg/L)						
Number of events/number at risk	90/604	16/165	15/80	26/132	9/108	24/119

1. Crude model	1.2** (1.1-1.4)	referent	2.0* (1.0-4.1)	2.2* (1.2-4.1)	0.96 (0.42-2.2)	2.1* (1.1-4.0)
2. Model A	1.2** (1.1-1.4)	referent	1.6 (0.75-3.2)	1.9 (1.0-3.6)	0.83 (0.37-1.9)	2.2* (1.2-4.2)
3. Model B	1.1* (1.0-1.3)	referent	1.5 (0.71-3.0)	1.9* (1.0-3.5)	0.76 (0.33-1.7)	2.3* (1.2-4.4)
4. Model C	1.1 (0.96-1.3)	referent	1.5 (0.71-3.0)	1.9 (1.0-3.5)	0.73 (0.31-1.7)	2.2* (1.2-4.2)

HR, Hazard Ratio; ***P<0.001, **P<0.01,*P<0.05

Model A. Adjusted for systolic blood pressure, body mass index, smoking status, diabetes and prevalent cardiovascular disease

Model B. Adjusted for the covariates in model A and $eGFR_{CysC}$ (glomerular filtration rate estimated by serum cystatin C)

Model C. Adjusted for the covariates in model B and urinary albumin

Figure legends

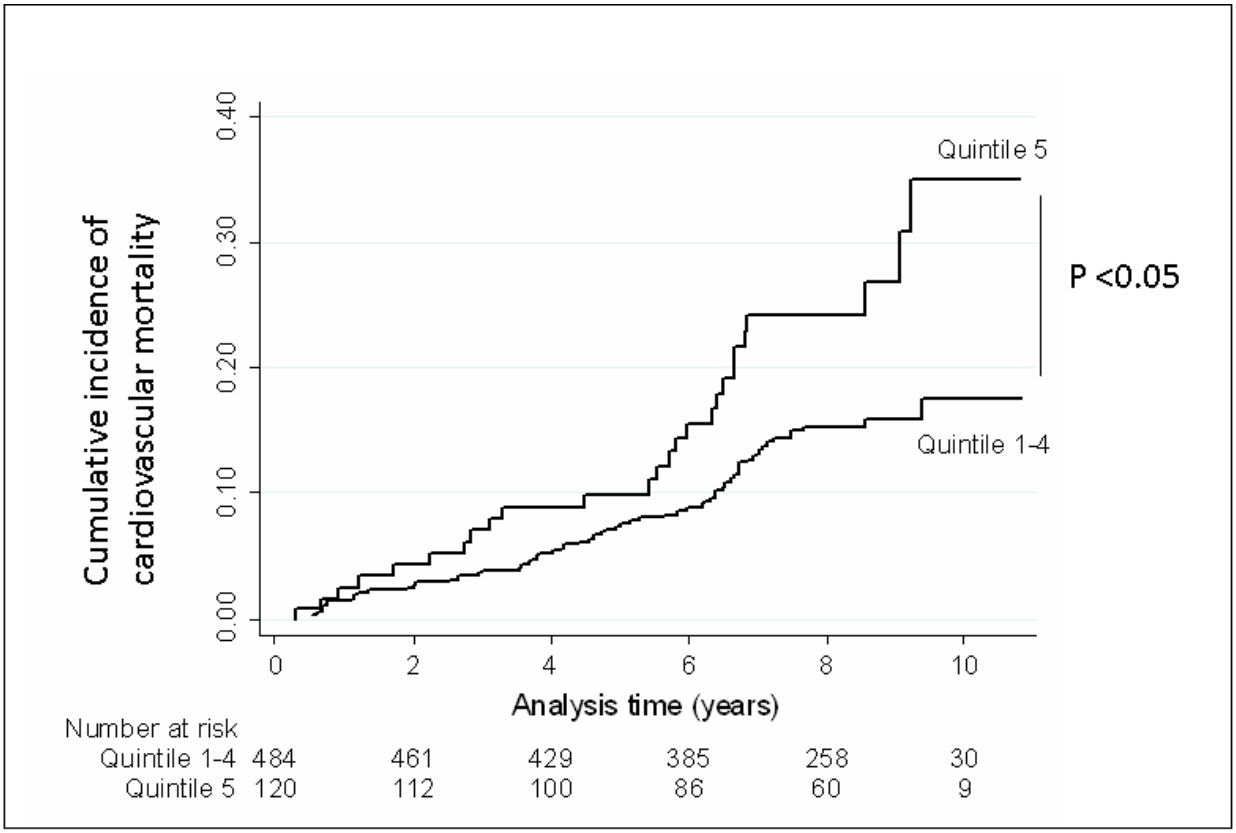
Figure 1. Nelson-Aalen plot of cumulative incidence of cardiovascular mortality by participants divided in quintiles according to concentrations of urinary cystatin C. The quintiles 1-4 corresponds to urinary Cystatin C concentrations of 0.0005-0.029 mg/mmol Cr and quintile 5 to >0.029 mg/mmol Cr.

Figure 2. Regression spline curve for the relation between u-Cystatin C and the risk for cardiovascular mortality. The black line shows estimated hazard ratios and the dotted line indicates 95 % confidence intervals. The knots were entered at the 10th, 50th, 90th percentile corresponding to urinary cystatin C concentrations of 0.0006, 0.012 and 0.039 mg/mmol Cr.

Figure 3. Cardiovascular mortality risk in participants with urinary Cystatin C above and below 0.029 mg/mmol Cr (highest quintile) and a) Urinary Albumin above and below 3 mg/mmol Cr and b) eGFR below and above 60 ml/min estimated by Cox regression models. The hazard ratios are adjusted for systolic blood pressure, body mass index, smoking status, diabetes and prevalent cardiovascular disease. *** P<0.001,* P<0.05

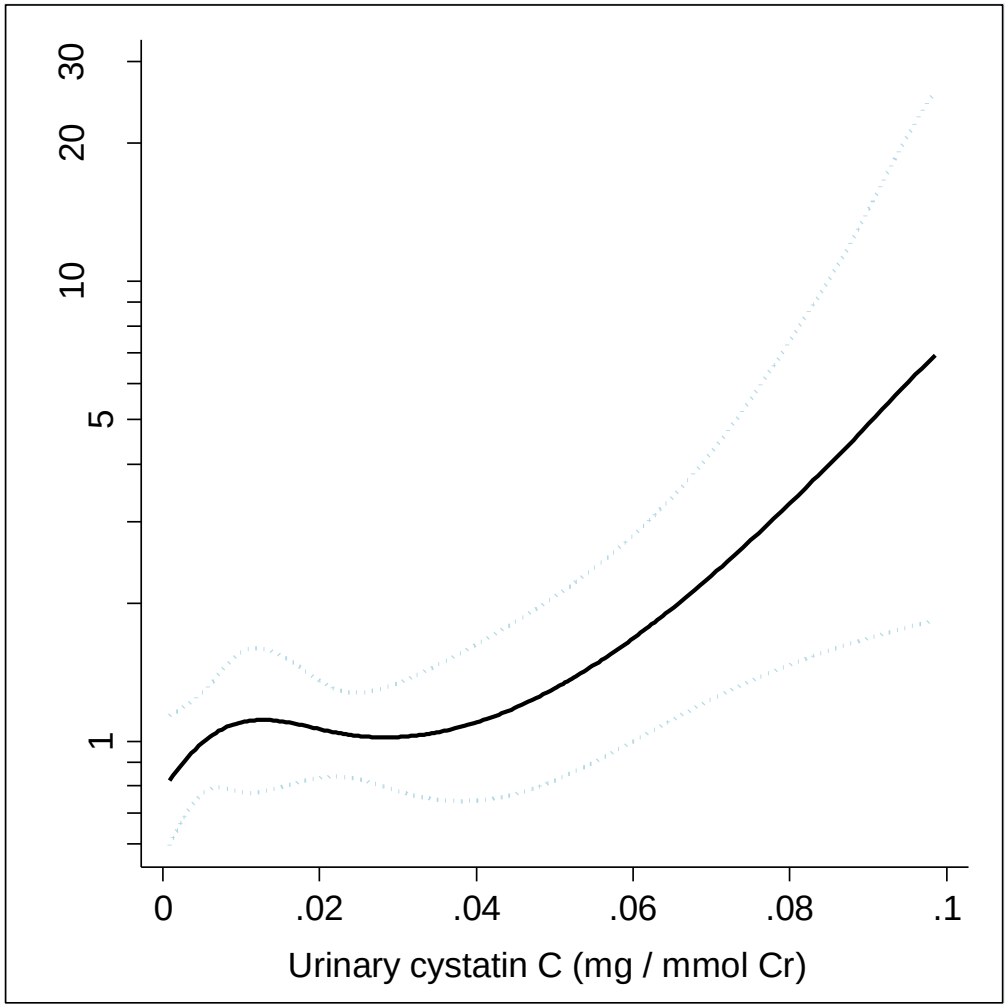
Figures

Figure 1. Nelson-Aalen plot of cumulative incidence of cardiovascular mortality by participants divided in quintiles according to concentrations of urinary cystatin C



The quintiles 1-4 corresponds to urinary Cystatin C concentrations of 0.0005-0.029 mg/mmol Cr and quintile 5 to >0.029 mg/mmol Cr.

Figure 2. Regression spline curve for the relation between u-Cystatin C and cardiovascular mortality



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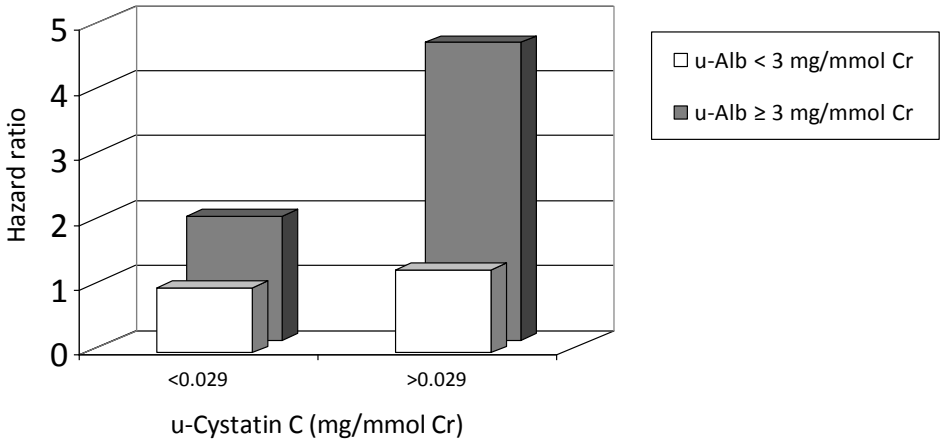
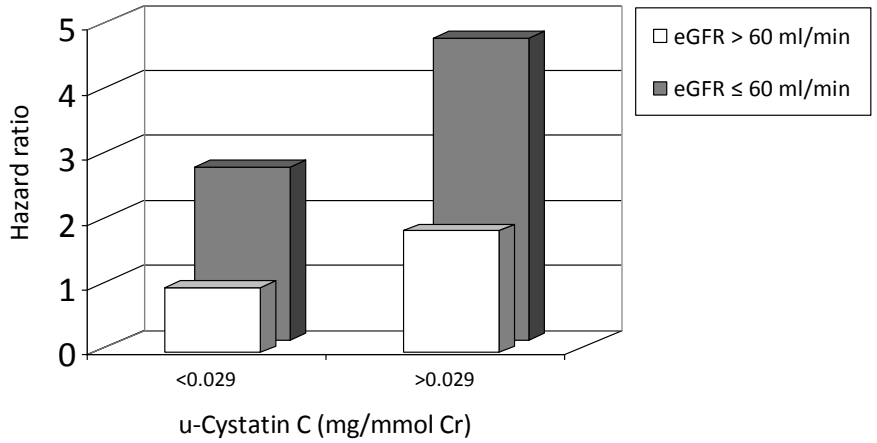
a)



b)



The hazard ratios are adjusted for systolic blood pressure, body mass index, smoking status, diabetes and prevalent cardiovascular disease. *** P<0.001,* P<0.05