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NIGRAM2+ consortium

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CLINICAL AND POPULATION STUDIES

Serum Calcification Propensity and the Risk of Cardiovascular and All-Cause Mortality in the General Population
The PREVEND Study

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OBJECTIVE: Vascular calcification contributes to the cause of cardiovascular disease. The calciprotein particle maturation time (T_{50}) in serum, a measure of calcification propensity, has been linked with adverse outcomes in patients with chronic kidney disease, but its role in the general population is unclear. We investigated whether serum T_{50} is associated with cardiovascular mortality in a large general population-based cohort.

APPROACH AND RESULTS: The relationship between serum T_{50} and cardiovascular mortality was studied in 6231 participants of the PREVEND (Prevention of Renal and Vascular End-Stage Disease) cohort. All-cause mortality was the secondary outcome. Mean (±SD) age was 53±12 years, 50% were male, and mean serum T_{50} was 329±58 minutes. A shorter serum T_{50} is indicative of a higher calcification propensity. Serum T_{50} was inversely associated with circulating phosphate, age, estimated glomerular filtration rate, and alcohol consumption, whereas plasma magnesium was positively associated with serum T_{50} ($P<0.001$, total multivariable model $R^2=0.281$). During median (interquartile range) follow-up for 8.3 (7.8–8.9) years, 364 patients died (5.8%), of whom 95 (26.1%) died from a cardiovascular cause. In multivariable Cox proportional hazard models, each 60 minutes decrease in serum T_{50} was independently associated with a higher risk of cardiovascular mortality (fully adjusted hazard ratio [95% CI], 1.22 [1.04–1.36], $P=0.021$). This association was modified by diabetes mellitus; stratified analysis indicated a more pronounced association in individuals with diabetes mellitus.

CONCLUSIONS: Serum T_{50} is independently associated with an increased risk of cardiovascular mortality in the general population and thus may be an early and potentially modifiable risk marker for cardiovascular mortality.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: association ■ calcification propensity (T_{50}) ■ calciprotein particles ■ cardiovascular diseases ■ diabetes mellitus ■ mortality ■ population

Coronary calcification is a risk factor for cardiovascular morbidity and mortality in the general population, independent of traditional cardiovascular risk factors.[1–4] Similarly, calcification of other vascular beds, including the thoracic[5,6] and abdominal aorta,[7] and the breast artery,[8] has been independently associated with cardiovascular morbidity and mortality. The susceptibility to vascular calcification and the strength of the relationship with outcomes may vary among vascular beds. In addition, different types of vascular calcification can

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Nonstandard Abbreviations and Acronyms

BMI	body mass index
CKD	chronic kidney disease
CPP	calciprotein particles
eGFR	estimated glomerular filtration rate
HR	hazard ratio
hsCRP	high-sensitivity C-reactive protein
ICD-9	International Classification of Diseases, Ninth Revision
PREVEND	Prevention of Renal and Vascular End-Stage Disease
serum T_{50}	measure of calcification propensity

Highlights

- The transformation time from primary to secondary calciprotein particles, known as the serum T_{50}, reflects the endogenous defense capacity against calcium-phosphate precipitation.
- Serum T_{50} is independently associated with an increased risk of cardiovascular mortality in the general population.
- Serum T_{50} may have a higher predictive value in subgroups that are at a higher risk of developing medial calcifications or cardiovascular disease, such as patients with diabetes mellitus.

(co-)occur, including intimal atherosclerotic calcification, arterial media calcification (which is common in patients with type 2 diabetes mellitus or chronic kidney disease [CKD]), and valvular calcification.[9] These calcification types seem to be driven by both local processes and systemic factors like inflammation and metabolic derangements,[9] which may concertedly result in a more general calcification propensity, predisposing to adverse clinical outcomes.[10] Currently used techniques to detect manifest vascular calcification, mostly based upon computed tomography, are incapable to quantify the intrinsic propensity to develop future calcifications.

An in vitro blood test has been developed that quantifies calcification propensity in serum.[11] Under physiological circumstances, the precipitation of supersaturated calcium and phosphate in serum is prevented by the formation of primary calciprotein particles (CPP), which may subsequently transform to more harmful, secondary CPP.[12,13] The transformation time from primary to secondary CPP, known as the serum T_{50}, reflects the endogenous defense capacity against calcium-phosphate precipitation. In patients prone to vascular calcification, such as patients with CKD, circulating CPP has been associated with aortic stiffness and vascular calcification.[14,15] Moreover, a shorter serum T_{50} (ie, accelerated precipitation time) has been associated with all-cause mortality in patients with CKD[16] and hemodialysis,[17] and with all-cause and cardiovascular mortality in renal transplant recipients,[18,19] independent of established cardiovascular risk factors. However, recently, it was shown in patients with CKD (stage 2–4), that the association of T_{50} with cardiovascular and all-cause mortality was not independent of renal function.[20]

To extend these findings, we broadened the research field of calcification propensity to the general population. We investigated the relationship between serum T_{50}, established cardiovascular risk factors and other relevant clinical and biochemical parameters, and whether serum T_{50} is independently associated with the risk of cardiovascular mortality and all-cause mortality in a large, general population-based cohort.

METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request. The article is written in compliance with the STROBE guidelines for observational studies.[21]

Study Population

The PREVEND study (Prevention of Renal and Vascular End-Stage Disease) is a prospective cohort that was designed to study the association of microalbuminuria with renal and cardiovascular disease in the general population. Details of the PREVEND study have been published previously.[22,23] In brief, between 1997 and 1998, inhabitants of the city of Groningen, The Netherlands, aged 28 to 75 years (n=85 421), received a questionnaire and a vial to collect an early morning urinary sample. Of these subjects, 40 856 responded (47.8%) and sent back their vial to a central laboratory where urinary albumin and creatinine concentrations were measured. Two subgroups were derived from this population: 9966 individuals with a urinary albumin concentration ≥10 mg/L and 30 890 subjects with urinary albumin concentration <10 mg/L. After the exclusion of subjects with insulin-dependent diabetes mellitus and pregnant women, 7768 subjects with a urinary albumin concentration ≥10 mg/L were invited to participate (n=6000 enrolled) and a randomly selected control group with a urinary albumin concentration <10 mg/L was invited (n=2592 enrolled). These 8592 individuals form the PREVEND cohort and were further investigated in an outpatient clinic. The PREVEND study has been approved by the medical ethics committee of the University Medical Center Groningen and was performed in accordance with the declaration of Helsinki. All participants provided written informed consent.

For the measurement of serum T_{50} in this cohort, serum samples were available from the second examination round, which took place between April 2001 and November 2003. This resulted in data from 6231 participants for the current analysis.

Measurements and Definitions

The procedures at each examination in the PREVEND study have been described in detail previously.[24,25] In brief, each examination included 2 visits to an outpatient clinic separated by 3 weeks. For the baseline survey, participants completed

a questionnaire obtaining information on demographics, race, cardiovascular and renal disease history, alcohol consumption, smoking status, and medication use. Self-reported medication use was complemented by information garnered from community pharmacies. Data on specific medications were based on pharmacy information only.

Furthermore, body weight, height, and waist and hip circumference were measured. During both visits of the second examination round, blood pressure was measured in a supine position on the right arm, every minute for 10 and 8 minutes, respectively, by an automatic Dinamap XL Model 9300 series device (Johnson-Johnson Medical, Tampa, FL). The mean of the last 2 recordings from each of the 2 visits was used. Hypertension was defined as systolic blood pressure >140 mm Hg, diastolic blood pressure >90 mm Hg, or use of antihypertensive medication.

Fasting blood samples were taken and stored at −80°C until further processing. Circulating albumin, hemoglobin, calcium, phosphate, magnesium, parathyroid hormone, creatinine, blood lipids, glucose, and hsCRP (high-sensitivity C-reactive protein) were determined using standard methods or as previously described.[26–28] Circulating calcium concentrations were corrected for albumin concentrations as follows: corrected calcium = calcium (mmol/L)+0.02×(40-albumin [g/L]). The estimated glomerular filtration rate (eGFR) was calculated by using the creatinine-cystatin C-based CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration) equation, taking into account age, sex, and race.[29] Diabetes mellitus was defined according to the guidelines of the American Diabetes Association as a fasting plasma glucose ≥7.0 mmol/L (126 mg/dL) or the use of blood glucose-lowering medication. Hypercholesterolemia was defined as total serum cholesterol >6.2 mmol/L (240 mg/dL) or the use of lipid-lowering medication. Albuminuria and urinary sodium excretion were measured in 24-hour urine samples, obtained after thorough oral and written instruction.[30]

Serum T_{50} measurements were performed as described previously.[18] In brief, samples were measured in a blinded manner in triplicates in 384-well plates at 37°C for 600 minutes in a Nephelostar nephelometer (BMG Labtech, Ortenberg, Germany). Stock calcium and phosphate solutions were used, with pH adjusted to 7.40 at 37°C in both solutions. For T_{50} measurements, 35 µL of calcium solution was mixed with 40 µL serum, and then 25 µL of the phosphate solution was added. Data analyses of nonlinear regression curves were performed using Microsoft Excel software to determine the half-maximal precipitation time (T_{50}). The analytical coefficients of variation of standards precipitating at 120, 260, and 390 minutes were 7.8%, 5.1%, and 5.9%, respectively.

Clinical End Points

The primary outcome of our longitudinal analyses was cardiovascular mortality, whereas the secondary outcome of our study was all-cause mortality. We studied incident fatal or nonfatal cardiovascular events as an exploratory outcome. Information on hospitalization for cardiovascular morbidity was obtained from PRISMANT, the Dutch national registry of hospital discharge diagnoses. Clinical event data were coded according to the *International Classification of Diseases, Ninth Revision* (*ICD-9*) and the classification of health interventions. Incident coronary artery disease was defined as fatal or nonfatal acute

myocardial infarction (*ICD-9* code 410), acute and subacute ischemic heart disease (code 411), coronary artery bypass grafting (code 414), or percutaneous transluminal coronary angioplasty. Stroke events were defined as subarachnoid hemorrhage (code 430), intracerebral hemorrhage (code 431), other intracranial hemorrhage (code 432), or occlusion or stenosis of the precerebral (code 433) or cerebral (code 434) arteries. Peripheral artery disease was defined as vascular interventions such as percutaneous transluminal angioplasty or bypass grafting of aorta and peripheral vessels. From the time of recruitment, the vital status of the participants was checked through the municipal register. The cause of death was obtained by linking the number of the death certificate to the primary cause of death as coded by a physician from the Central Bureau of Statistics in The Netherlands. The survival time was defined as the period from the date of serum collection of the participant until the date of first cardiovascular event, date of death, or end of follow-up.

Statistical Analyses

Continuous variables with a normal distribution are expressed as mean with SD. Variables with a skewed distribution are given as median (interquartile range) and were normalized using natural logarithmic transformation before use in parametric tests. *P* trend over the quintiles of serum T_{50} (based on the median T_{50} values for each quintile) was calculated by linear regression analysis for continuous variables or χ^2 linear-by-linear association for categorical data.

For the screening of the PREVEND study, subjects with an elevated albuminuria were overselected to acquire sufficient subjects with microalbuminuria. To overcome the effect of oversampling of subjects with elevated albuminuria, all models took the sampling design into account by specifying stratum-specific baseline hazard functions. Owing to this statistical weighing method, our conclusions may also be generalized to subjects with normal levels of albuminuria.

Our study cohort was used to examine the association of T_{50} with traditional cardiovascular risk factors, renal function, and other relevant clinical and biochemical parameters. Main determinants of serum T_{50} were evaluated using a backwards linear regression model in which variables were included that were significantly associated with T_{50} upon univariable linear regression. In longitudinal primary analyses, the association of T_{50} with cardiovascular mortality was investigated using Cox proportional hazard models. Model 1 is a basic model adjusted for age and sex. In model 2, we further adjusted for cardiovascular risk factors (smoking, systolic blood pressure, use of antihypertensive medication, plasma glucose, use of glucose-lowering medication, total cholesterol, use of lipid-lowering medication, history of cardiovascular events, body mass index [BMI], and hsCRP). In model 3, we additionally adjusted for alcohol consumption and eGFR, representing the determinants of serum T_{50} (derived from the backwards linear regression model) without a known direct (in vitro) accelerating (eg, phosphate) or delaying (eg, magnesium) effect on T_{50}. Using the same Cox regression models, we conducted secondary analysis to investigate the association of T_{50} with all-cause mortality.

To investigate the value of serum T_{50} over plasma phosphate as a risk marker for (cardiovascular) mortality, the same models were used replacing T_{50} with phosphate. In additional exploratory

analyses, we studied the association of decreasing serum T_{50} with incident fatal or nonfatal cardiovascular events using the Cox regression models described above (except for adjustment for history of cardiovascular events). For these analyses, subjects with a history of the studied cardiovascular event outcome (eg, incident cardiovascular events, coronary artery disease, stroke, heart failure, and peripheral artery disease) were excluded beforehand.

To retain the number of events in adjusted Cox models, missing data in covariables (for details see Table I in the Data Supplement) were handled by multiple (n=5) imputations[31,32] using the linear regression method in SPSS.

The ability of the models to distinguish those with an event from those without, was evaluated with Harrell C index. Harrell C index is analogous to the area under the receiver operating characteristic curve, for which larger values indicate better discrimination. We examined potential nonlinear relationships by fitting restricted cubic spline transformations with 3 knots on a Cox model adjusted for age and sex and comparing them with linear splines. We explored possible effect modification by age, sex, BMI, eGFR, diabetes mellitus, plasma magnesium, and plasma phosphate for the association between T_{50} and cardiovascular and all-cause mortality by using multiplicative interaction terms (where applicable with continuous data), followed by stratified Cox regression analysis based on median values.

Statistical analyses were performed using SPSS version 23.0 for Windows (IBM Corporation, Chicago, IL) and STATA Statistical Software: Release 14 (StataCorp, College Station, TX). Figures were made using GraphPad Prism 7.02 (GraphPad Software, San Diego, CA) and R version 3.5.1 (Vienna, Austria; http://cran.r-project.org/).

RESULTS

Subjects

The mean age of participants was 53.5±12.0 years, 49.9% were male, and mean serum T_{50} was 329±58 minutes. The prevalence of diabetes mellitus was 2.7%, 33.2% had hypertension, 18.5% were obese (BMI ≥30 kg/m^2), 30.3% had hypercholesterolemia, and 28.2% were current smokers. Mean eGFR was 92.2±17.1 mL/min per 1.73 m^2, and 4.3% of the participants had an eGFR <60 mL/min per 1.73 m^2. Baseline characteristics of the 6231 participants, according to quintiles of T_{50}, are presented in Table 1. Subjects with a shorter T_{50} (ie, higher calcification propensity) were more likely to be female, to consume more alcohol and to smoke (all $P<0.001$). The mean difference in serum T_{50} between men and women was ≈20 minutes (339±58 and 320±56 minutes, respectively).

During follow-up for a median of 8.3 (7.8–8.9) years, 364 patients died (5.8%), of whom 95 (26.1%) from a cardiovascular cause.

Determinants of Serum T_{50}

Multivariable linear regression analysis (Table 2) showed that plasma phosphate, age, and plasma magnesium were the strongest determinants of serum T_{50}. Higher phosphate concentrations and older age were associated with a shorter T_{50}, whereas higher magnesium concentrations were associated with a longer T_{50} (ie, lower calcification propensity). In addition, eGFR and alcohol intake were inversely associated with T_{50} (ie, increased calcification propensity with higher eGFR and more alcohol consumption). Other determinants of serum T_{50} in this population were albumin, smoking, calcium, cholesterol, glucose, parathyroid hormone, and systolic blood pressure (see Table 2 for directions of effects). The total multivariable model had an overall R^2 of 0.281.

T_{50} and Cardiovascular Mortality

In a basic Cox regression model adjusted for age and sex, a shorter serum T_{50} was associated with an increased risk of cardiovascular mortality (model 1, hazard ratio [HR; 95% CI], 1.24 [1.07–1.38], $P=0.007$; Table 3). This relationship is depicted as a linear spline curve in Figure 1A. Serum T_{50} was associated with cardiovascular mortality independent of other cardiovascular risk factors (model 2), and independent of other possible confounders (model 3, fully adjusted HR, 1.22 [1.04–1.36], $P=0.021$).

For BMI, plasma magnesium, and diabetes mellitus significant effect modification was found in the association between T_{50} and cardiovascular mortality ($P_{interaction}$ <0.1). Stratified analyses indicated a more pronounced relationship between serum T_{50} and the risk of cardiovascular mortality in subjects with diabetes mellitus (HR, 1.54 [1.15–1.76], $P=0.013$). In addition, the associations were mainly present for subjects with a higher BMI (>26.1 kg/m^2) or lower plasma magnesium (<0.82 mmol/L; Figure 2).

T_{50} and All-Cause Mortality

Multivariable Cox regression analysis also revealed that serum T_{50} was associated with all-cause mortality in a basic model adjusted for age and sex (model 1, HR, 1.12 [1.03–1.21], $P=0.014$), depicted as a spline curve in Figure 1B. The association did not remain significant after adjustment for several cardiovascular risk factors and other potential confounders (fully adjusted HR [model 3], 1.10 [0.99–1.19], $P=0.064$).

Significant effect modification by diabetes mellitus was observed in the association between T_{50} and all-cause mortality ($P_{interaction}$ <0.1). Stratified analyses indicated that the increased risk for all-cause mortality per 60 minutes decrease in T_{50} was most pronounced in participants with diabetes mellitus (HR, 1.43 [1.14–1.62], $P=0.007$; Figure I in the Data Supplement).

Exploratory Analyses—Phosphate and (Cardiovascular) Mortality

The value of serum T_{50} over plasma phosphate as a risk marker for (cardiovascular) mortality was investigated

Table 1. Baseline Characteristics of the Cohort Per Quintiles of Serum T$_{50}$*

T$_{50}$ (min)	Quintile 1 N=1245; <284	Quintile 2 N=1243; 284–315	Quintile 3 N=1244; 315–344	Quintile 4 N=1249; 344–378	Quintile 5 N=1250; >378	P for Trend
Demographics						
Male sex, n (%)	476 (38.2)	574 (46.2)	601 (48.3)	667 (53.4)	793 (63.4)	<0.001
Age, y	54.0±11.7	53.5±11.7	54.2±12.0	52.7±12.0	53.1±12.7	0.024
Current smoking	459 (37.3)	360 (29.1)	322 (26.2)	314 (25.5)	283 (23.0)	<0.001
Alcohol consumption						<0.001
No, almost never	265 (21.5)	302 (24.5)	308 (25.0)	332 (26.8)	334 (27.0)	
1–4 drinks/mo	191 (15.5)	198 (16.0)	223 (18.1)	214 (17.3)	239 (19.3)	
2–7 drinks/wk	351 (28.4)	391 (31.7)	399 (32.3)	391 (31.6)	418 (33.8)	
1–3 drinks/d	347 (28.1)	295 (23.9)	255 (20.7)	249 (20.1)	214 (17.3)	
>3 drinks/d	80 (6.5)	49 (4.0)	49 (4.0)	51 (4.1)	33 (2.7)	
History of CV events	75 (6.0)	86 (6.9)	97 (7.8)	74 (5.9)	78 (6.2)	0.861
Body composition						
BMI, kg/m^2	26.0±4.2	26.5±4.4	27.1±4.4	26.8±4.2	26.8±4.2	<0.001
Waist/hip, ratio	0.88±0.09	0.90±0.09	0.90±0.08	0.90±0.08	0.92±0.08	<0.001
Blood pressure						
Systolic BP, mm Hg	123.2±18.9	125.2±18.8	126.2±18.3	126.9±18.6	129.1±18.9	<0.001
Diastolic BP, mm Hg	72.4±9.3	73.1±8.9	73.2±8.9	73.7±9.2	74.5±9.3	<0.001
Renal function						
eGFR, mL/min per 1.73 m^2	93.3±17.3	92.8±16.8	91.2±17.1	92.7±16.7	90.9±17.5	0.002
Albuminuria, mg/24 h	8.7 (6.0–15.4)	9.0 (6.2–15.6)	8.4 (6.0–15.6)	8.7 (6.1–16.5)	9.0 (6.2–16.5)	0.186
ACR, mg/mmol	0.79 (0.54–1.36)	0.77 (0.54–1.37)	0.72 (0.50–1.37)	0.71 (0.50–1.36)	0.72 (0.48–1.37)	0.064
Other urinary parameter						
Sodium excretion, mmol/24 h	139±52	144±54	147±54	146±56	148±57	<0.001
Glucose						
Type 2 diabetes mellitus, n (%)	29 (2.3)	34 (2.7)	39 (3.1)	31 (2.5)	34 (2.7)	0.681
Glucose, mmol/L	4.7 (4.4–5.2)	4.7 (4.4–5.3)	4.8 (4.4–5.3)	4.8 (4.4–5.3)	4.8 (4.5–5.3)	<0.001
Lipids						
Total cholesterol, mmol/L	5.41±1.05	5.40±1.06	5.44±1.01	5.44±1.06	5.49±1.06	0.024
HDL cholesterol, mmol/L	1.32±0.34	1.27±0.30	1.24±0.31	1.23±0.30	1.23±0.31	<0.001
Triglycerides, mmol/L	0.99 (0.73–1.40)	1.07 (0.77–1.53)	1.13 (0.83–1.65)	1.15 (0.83–1.65)	1.24 (0.88–1.76)	<0.001
Other plasma parameters						
Hemoglobin, mmol/L	8.3±0.8	8.4±0.7	8.5±0.7	8.6±0.7	8.7±0.8	<0.001
Calcium (corrected), mmol/L	2.23±0.08	2.23±0.07	2.23±0.07	2.23±0.08	2.24±0.07	0.021
Phosphate, mmol/L	1.11±0.15	1.04±0.15	0.99±0.15	0.95±0.14	0.90±0.14	<0.001
Magnesium, mmol/L	0.81±0.06	0.82±0.05	0.82±0.05	0.83±0.05	0.84±0.05	<0.001
PTH, pmol/L	5.01±1.85	5.03±1.45	5.10±1.73	5.17±1.62	5.30±1.71	<0.001
Albumin, g/L	43.4±2.5	43.5±2.6	43.5±2.5	43.9±2.7	44.3±2.9	<0.001
hsCRP, mg/L	1.18 (0.56–2.73)	1.37 (0.61–3.07)	1.35 (0.63–3.19)	1.40 (0.63–3.09)	1.40 (0.65–2.96)	0.069
Medication use						
Aspirin	28 (2.3)	31 (2.6)	37 (3.0)	36 (2.9)	33 (2.7)	0.415
Vitamin K antagonist	35 (2.9)	22 (1.8)	24 (2.0)	16 (1.3)	19 (1.6)	0.011
Antihypertensive drug use	238 (19.1)	260 (20.9)	276 (22.2)	259 (20.7)	279 (22.3)	0.112
Diuretics	63 (5.3)	70 (5.9)	71 (6.0)	71 (6.0)	97 (8.2)	0.006
β-blocker	112 (9.4)	126 (10.6)	131 (11.1)	116 (9.7)	124 (10.5)	0.555
ACE inhibitors/ARBs	108 (8.9)	98 (8.1)	114 (9.4)	100 (8.2)	106 (8.8)	0.753
Lipid-lowering drug use	105 (8.4)	113 (9.1)	137 (11.0)	114 (9.1)	113 (9.0)	0.626

(*Continued*)

Table 1. Continued

T_{50} (min)	Quintiles of Serum T_{50}					*P* for Trend
	Quintile 1 N=1245; <284	Quintile 2 N=1243; 284–315	Quintile 3 N=1244; 315–344	Quintile 4 N=1249; 344–378	Quintile 5 N=1250; >378	
Statines	83 (7.0)	94 (7.9)	114 (9.6)	91 (7.6)	88 (7.5)	0.733
Glucose-lowering drug use	20 (1.6)	26 (2.1)	25 (2.0)	19 (1.5)	13 (1.0)	0.151

ACE indicates angiotensin-converting enzyme; ACR, albumin creatinine ratio; ARBs, angiotensin receptor blockers; BMI, body mass index; BP, blood pressure; CV, cardiovascular; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; hsCRP, high-sensitivity C-reactive protein; IQR, interquartile range; and PTH, parathyroid hormone.

*Values are mean±SD if normally distributed, median (IQR) if non-normally distributed, or number (%) for categorical variables. *P* value was calculated by linear regression analysis (after natural logarithmic transformation where applicable) for continuous variables or χ^2 linear-by-linear association for categorical data.

with the same Cox regression models. Circulating phosphate was not associated with cardiovascular mortality nor with all-cause mortality (Table II in the Data Supplement).

Exploratory Analyses—T_{50} and Cardiovascular Events

Cox regression models exploring the possible association of serum T_{50} with incident cardiovascular events, coronary artery disease, stroke, heart failure, or peripheral artery disease are shown in Table III in the Data Supplement. No significant associations were observed with the incidence of specific cardiovascular events.

DISCUSSION

In this prospective cohort study, we addressed the association between serum T_{50} and the risk of cardiovascular mortality and all-cause mortality in the general population.

Table 2. Multivariable Linear Regression Model for Serum T_{50}*

Variable	St Beta	95% CI		*P* Value
		Lower	Upper	
Plasma phosphate	−0.456	−0.477	−0.429	<0.001
Age	−0.171	−0.205	−0.137	<0.001
Plasma magnesium	0.157	0.133	0.181	<0.001
eGFR (creat-cysC)	−0.114	−0.146	−0.083	<0.001
Alcohol intake	−0.113	−0.137	−0.090	<0.001
Plasma albumin	0.105	0.079	0.130	<0.001
Current smoking	−0.068	−0.092	−0.045	<0.001
Total cholesterol	0.056	0.033	0.080	<0.001
Plasma calcium (corrected)	0.051	0.027	0.076	<0.001
Plasma PTH	−0.040	−0.064	−0.017	0.001
Plasma glucose	0.037	0.014	0.064	0.002
Systolic blood pressure	0.030	0.003	0.058	0.027
Plasma hemoglobin	0.025	−0.002	0.051	0.069

BMI indicates body mass index; eGFR, estimated glomerular filtration rate; and PTH, parathyroid hormone.

*Multivariable linear regression model obtained using backward selection of covariables (using Z scores) associated with serum T_{50} at baseline. Explained variance R^2=0.281. Variables not independently associated with serum T_{50} included hemoglobin, body mass index, urinary sodium excretion, and sex.

The primary finding is that a shorter serum T_{50}, reflecting higher calcification propensity, is associated with a higher risk of cardiovascular mortality. This association was independent of established cardiovascular risk factors, and more pronounced in certain subgroups, such as participants with diabetes mellitus, overweight or low plasma magnesium. There was no association with all-cause mortality after multivariable adjustment.

The determination of calcification propensity, reflected by the serum T_{50}, is an increasingly used risk prediction tool in medicine,[11] especially in the field of nephrology. When serum of individual subjects is challenged with supersaturated calcium and phosphate solutions, spontaneous formation of (additional) primary CPP, which contain amorphous calcium phosphate, is triggered. These nano-sized particles then undergo, at time point T_{50}, which is specific for individual serum samples, spontaneous conversion to more harmful crystalline calcium phosphate-containing secondary CPP.[12] Thereby, calcification propensity, as measured by the T_{50} test, reflects the functional integrity of a relevant protective physiological system, which so far has clinically not been

Table 3. Hazard Ratios of Cardiovascular Mortality and All-Cause Mortality Per 60 Minutes Decrease in T_{50}*

	Per 60 Minutes Decrease in T_{50}		
	HR (95% CI)	*P* Value	Harrell C
Cardiovascular mortality (95 events)			
Model 1	1.24 (1.07–1.38)	0.007	0.873
Model 2	1.20 (1.02–1.35)	0.032	0.908
Model 3	1.22 (1.04–1.36)	0.021	0.908
All-cause mortality (364 events)			
Model 1	1.12 (1.03–1.21)	0.014	0.820
Model 2	1.09 (0.99–1.18)	0.079	0.840
Model 3	1.10 (0.99–1.19)	0.064	0.839

Model 1: adjusted for age and sex; Model 2: model 1 + adjusted for smoking, systolic blood pressure, use of antihypertensive medication, plasma glucose, use of glucose-lowering medication, total cholesterol, use of lipid-lowering medication, history of CV events, BMI, and hsCRP (CV risk factors); and Model 3: model 2 + adjusted for alcohol consumption and eGFR (determinants of serum T_{50} without a known direct (in vitro) accelerating or delaying effect on T_{50}). BMI indicates body mass index; CV, cardiovascular; eGFR, estimated glomerular filtration rate; HR, hazards ratio; and hsCRP, high-sensitivity C-reactive protein.

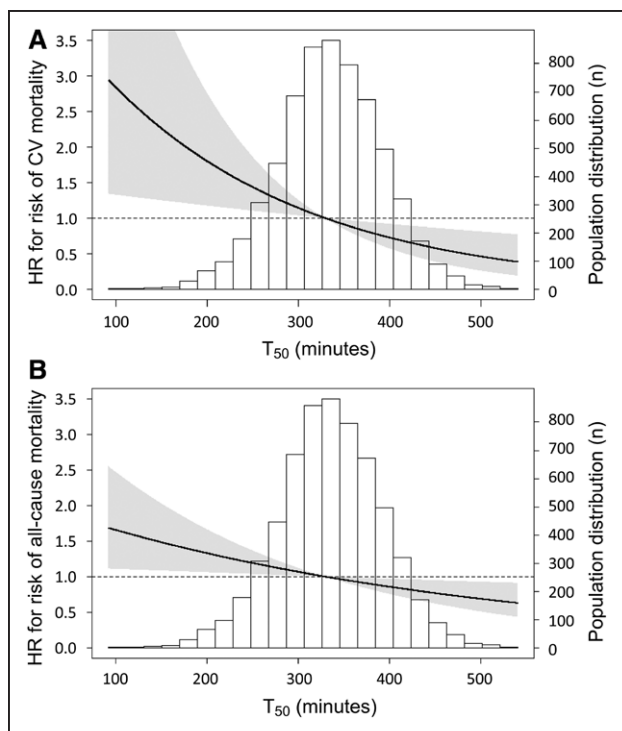*Hazard ratios and 95% CI were derived from Cox proportional hazards regression models. N=6231.

Figure 1. Spline Cox proportional hazards regression models for the association of T_{50} with mortality.
Linear spline curve for cardiovascular (CV) mortality (**A**), and all-cause mortality (**B**). Models are adjusted for age and sex (model 1).

taken into consideration. This system is thought to consist of calcification promotors and inhibitors, where an imbalance (eg, due to disease conditions such as kidney failure) of this dynamic system would lead to increased calcifications.[14,15]

From the multivariable regression analysis, phosphate appeared as the strongest determinant of T_{50}, showing an inverse association with serum T_{50}, as expected due to its prominent role in CPP formation. In a study in people with moderate CKD, higher serum phosphate concentrations, although still within the normal range, were previously associated with a greater prevalence of vascular calcification.[33] Magnesium was also a strong determinant of serum T_{50}, with higher plasma magnesium concentrations being associated with a longer T_{50} (eg, decreased calcification propensity), which is in line with results from the Framingham Heart Study, where magnesium intake was inversely associated with coronary artery calcification.[34] This could imply that magnesium acts as an inhibitor of calcification, as observed in in vitro studies, where phosphate-induced calcification of vascular smooth muscle cells is prevented by magnesium, by interfering with secondary CPP crystal formation.[35,36] Confirmatory ex vivo experiments in human serum demonstrated that the addition of 0.2 mmol/L Mg^{2+} increased T_{50} from both healthy controls and patients with CKD by ≈40 to 50 minutes.[36] In addition, similar improvements of calcification propensity by magnesium was shown in randomized

controlled intervention studies in CKD and dialysis patients.[37,38] Multivariable regression analysis further indicated that age and alcohol use were independently associated with serum T_{50}. This latter observation can at least partly be reconciled with the established risk of cardiovascular events related to alcohol intake,[39] although alcohol intake has not been consistently linked with vascular calcification.[40,41] Together, these findings suggest that serum T_{50} and the accompanying increased risks would be modifiable, for instance by changes in diet, but also by targeted therapeutic interventions aimed at, for example, magnesium and phosphate. It should, however, be noted that the variables remaining in the model (Table 2) only explain ≈28% of the variation in T_{50}.

The association between serum T_{50} and the increased risk for cardiovascular mortality in the current population is in agreement with previous studies in renal transplant recipients[18,19] but was so far not shown in the general population. If such an association would be confirmed in other general population-based cohorts, serum T_{50} may prove useful as an independent cardiovascular risk marker in the general population. Nevertheless, serum T_{50} may have a higher predictive value in subgroups that are at a higher risk of developing medial calcifications or cardiovascular disease. In line with this, we observed more pronounced associations between serum T_{50} and cardiovascular mortality risk in subjects with diabetes mellitus, despite the small number of patients with diabetes mellitus in this cohort. Similarly, there was an interaction with BMI and plasma magnesium, showing more pronounced associations in subjects with a higher BMI and lower plasma magnesium, both characteristics that are also present in subjects with type 2 diabetes mellitus.[42] Recently, in a study with type 1 diabetes mellitus patients serum T_{50} was associated with indices of increased mineral stress, but not with the development of long-term macrovascular complications, possibly due to the small sample size.[43] Future studies may clarify the role of T_{50} as a marker of cardiovascular risk in persons with diabetes mellitus.

The association between serum T_{50} and all-cause mortality was weaker and lost significance after multivariable adjustment. All-cause mortality includes, next to cardiovascular-related deaths, a substantial number of malignancy-related deaths (n=185), showing (as expected) no relation to serum T_{50} (data not shown). In addition, given the associations with cardiovascular mortality, we expected to find relationships between serum T_{50} and incident cardiovascular events in exploratory analyses, but these were not found in the present cohort. This may suggest that T_{50} is linked with more severe cardiovascular events, leading to mortality.

It may be questioned whether the association between T_{50} and cardiovascular mortality is not mainly driven by phosphate concentrations. Besides studies with CKD patients, another study in the general
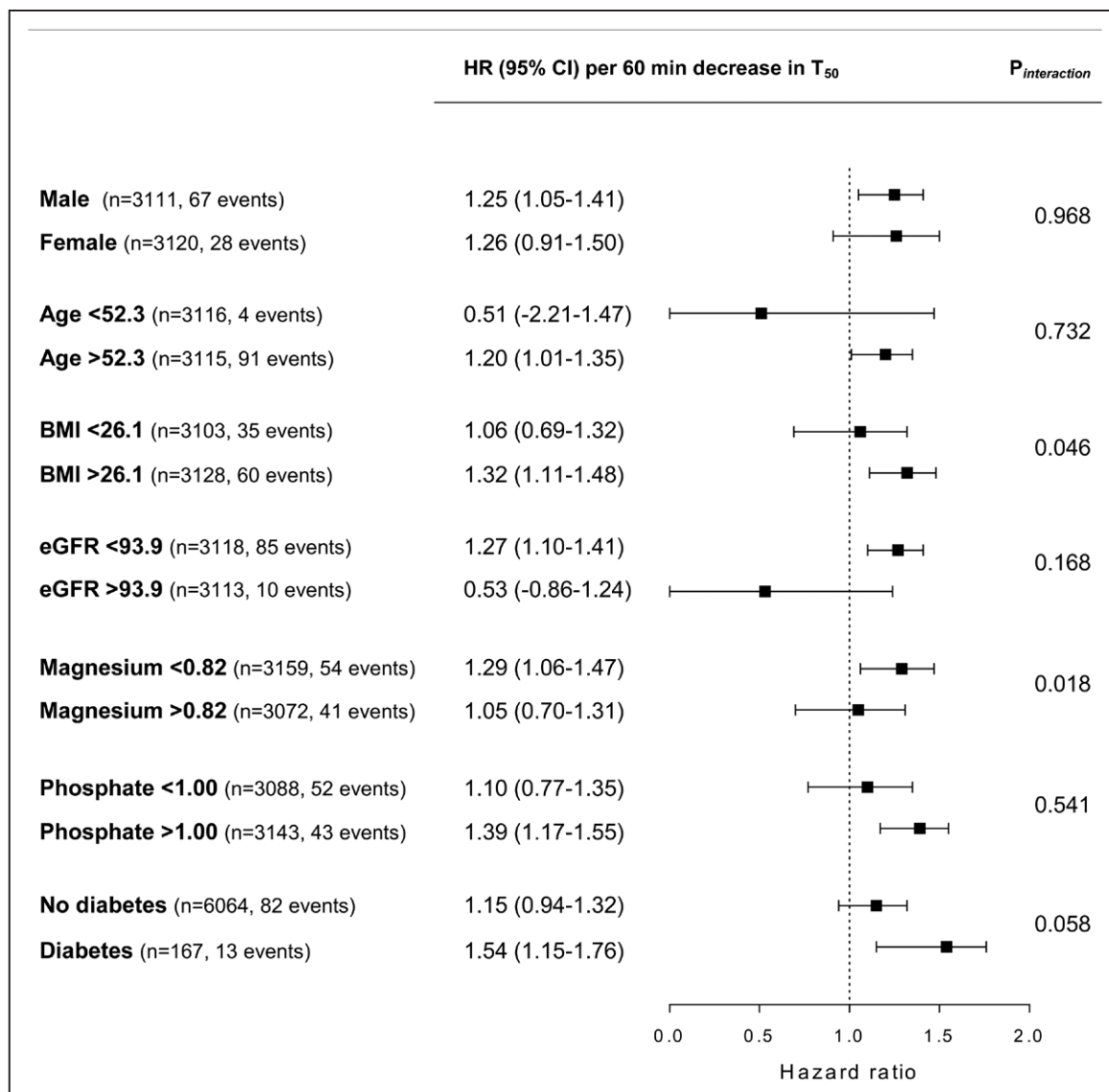
Figure 2. Forest plot of subanalyses for cardiovascular mortality.
Stratification was based on median values for age (< or >52.3 y), body mass index (BMI; < or >26.1 kg/m²), estimated glomerular filtration rate (eGFR; < or > 93.9 mL/min per 1.73 m2), plasma magnesium (< or >0.82 mmol/L), and plasma phosphate (< or >1.00 mmol/L).

population found that fasting serum phosphate is associated with mortality,[44] and phosphate is a strong determinant of T_{50}. In an additional exploratory analysis, we, therefore, assessed whether phosphate is also associated with cardiovascular mortality and all-cause mortality, but these associations were absent in our cohort. This emphasizes that the T_{50} test gives additional, clinically relevant information over the measurement of phosphate concentrations.

In contrast to primary CPP, secondary CPP can induce calcification of vascular smooth muscle cells in vitro.[45] Furthermore, exposure of macrophages or vascular smooth muscle cells to secondary CPP induces a strong proinflammatory response and oxidative stress.[45,46] This inflammatory response was found to further enhance the calcification process.[45] Therefore,

secondary CPP may contribute to the formation of calcium-phosphate precipitation and inflammation of soft tissue, including the arterial wall. If substantiated in future studies, these proposed mechanisms may causally link the result of the T_{50} test with cardiovascular mortality in the general population.

To our knowledge, this is the first study to investigate the relationship between serum calcification propensity and outcomes in the general population. Other strengths of our study include the large sample size, the long duration of follow-up, the well-phenotyped cohort, allowing adjustment for relevant potential confounders, and the validated way in which causes of death were determined. A limitation is that serum T_{50} was measured only at a single visit; therefore, we could not take changes in calcification propensity over time

into account. However, the availability of a single measurement may lead to underestimation, rather than overestimation, of the true association between T_{50} and outcomes. Finally, as with any observational study, residual confounding could, in part, explain the association between serum T_{50} and the risk of cardiovascular mortality, despite the multivariable adjustment.

In conclusion, we found that a shorter serum T_{50}, reflecting an increased calcification propensity, is associated with a higher risk of cardiovascular mortality in a large prospective general population-based cohort. Serum T_{50} may be considered a novel independent, and potentially modifiable, risk marker for cardiovascular mortality.

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