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Biomarkers and prediction models for type 2 diabetes and diabetes related outcomes

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Document Version

Publisher's PDF, also known as Version of record

Publication date:

2013

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Abbasi, A. (2013). *Biomarkers and prediction models for type 2 diabetes and diabetes related outcomes*. s.n.

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Chapter 11

Peroxiredoxin 4, a novel circulating biomarker for oxidative stress and the risk of incident cardiovascular disease and all-cause mortality

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Abstract

Background Oxidative stress has been suggested to play a key role in the development of cardiovascular disease (CVD). The aim of our study was to investigate the associations of serum peroxiredoxin 4 (Prx4), a hydrogen peroxide degrading peroxidase, with incident CVD and all-cause mortality. We subsequently examined the incremental value of Prx4 for the risk prediction of CVD compared with the Framingham risk score (FRS).

Methods We performed Cox regression analyses in 8,141 participants without history of CVD (aged 28-75 years; women 52.6%) from the Prevention of Renal and Vascular End-stage Disease (PREVEND) study in Groningen, the Netherlands. Serum Prx4 was measured by an immunoluminometric assay in baseline samples. Main outcomes were: 1) incident CVD events or CVD mortality; and 2) all-cause mortality during a median follow-up of 10.5 years.

Results In total, 708 (7.8%) participants developed CVD events or CVD mortality, and 517 (6.3%) participants died. Baseline serum Prx4 levels were significantly higher in participants with incident CVD events or CVD mortality and in those who died than in participants who remained free of outcomes (both $P < 0.001$). In multivariable models with adjustment for Framingham risk factors, hazard ratios were 1.16 (95%CI, 1.06-1.27, $P < 0.001$) for incident CVD events or CVD mortality, and 1.17 (95%CI, 1.06-1.29, $P = 0.003$) for all-cause mortality per doubling of Prx4 levels. After addition of Prx4 to the FRS, the net reclassification improvement was 2.7% ($P = 0.01$) using 10-year risk categories of CVD.

Conclusions Elevated serum Prx4 levels are associated with significantly higher risk of incident CVD events or CVD mortality, and all-cause mortality after adjustment for clinical risk factors. Addition of Prx4 to the FRS marginally improved risk prediction of future CVD.

Introduction

Experimental and clinical studies suggest that oxidative stress plays a key role in the pathogenesis of cardiovascular disease (CVD) ¹⁻⁴. Oxidative stress status is usually defined as overproduction of reactive oxygen/nitrogen species in imbalance with endogenous antioxidant defences, which in turn result in increased oxidative damage ⁵. Several biomarkers, including target oxidation products and antioxidants have been proposed for assessment of the level of oxidative stress, but clinical data examining association between marker(s) and CVD independent of common risk factors are limited ⁵⁻⁷.

Recently, peroxiredoxin 4 (Prx4), which is a secretable and stable isoform of the Prx family of antioxidant peroxidases ⁸, has been found in the circulation of humans. Prx4 can be precisely measured by a validated immunoassay ⁹. Previous evidence showed an abundant cellular antioxidant activity of Prx4 and other Prx isoforms in all mammals protecting against oxidative stress ¹⁰⁻¹³. So far, a limited number of small-scale studies have evaluated association of the serum Prx4 with clinical data ^{9,14}. In these studies, serum levels of Prx4 were increased in septic patients when compared to that of healthy individuals and were positively associated with well-established inflammatory markers like procalcitonin, C-reactive protein (CRP) and interleukin 6 (IL-6) ^{9,14}. Recently, a study in patients presenting to emergency departments showed the incremental prognostic value of Prx4 to predict 30 day survival beyond usual risk predictors ¹⁵.

We aimed to investigate whether serum Prx4 is a predictor of CVD and all-cause mortality. For this study, we used data of a large scale, observational cohort of general population and examined the association of Prx4 with incident CVD events or CVD mortality, and all-cause mortality. Since a major clinical application of a biomarker lies within risk stratification and guided preventive strategies ¹⁶⁻¹⁹, we also evaluated the incremental predictive value of Prx4 above the Framingham risk score for the 10-year risk of CVD.

Methods

Study population and design

The study population was obtained from the Prevention of Renal and Vascular End-stage Disease (PREVEND) study, a Dutch cohort drawn from the general population (age ranged between 28 and 75 years) of the city of Groningen, the Netherlands between 1997 and 1998. We have reported details of the study design and recruitment of participants elsewhere ^{20, 21}. Briefly, 40,856 individuals (47.8%) completed a questionnaire on demographics, history of cardiovascular and metabolic outcomes, medication use and pregnancy prior to their first visit, collecting early morning urine sample in a vial to measure urinary albumin concentration. Those who were unable or unwilling to participate, individuals using insulin and pregnant women were

excluded. The baseline PREVEND were recruited from a total of 6,000 individuals with a urinary albumin concentration of 10 mg/1 or greater and a random control sample of individuals with a urinary albumin concentration of less than 10 mg/L (n=2,592).

In the baseline cohort, serum Prx4 assay was missing for 370 participants, leaving 8,222 for the baseline cross-sectional analyses. The PREVEND study was approved by the local medical ethics committee, University Medical Center Groningen, and conformed to the principles outlined in the Declaration of Helsinki. All participants gave written informed consent.

Clinical and biomarker measurements

In the baseline screening, study participants underwent two outpatient visits to assess demographics, anthropometric measurements, cardiovascular and metabolic risk factors, health behaviours, and medical family history and to collect two 24-hour urine samples on 2 consecutive days. Furthermore, information on medication use was substantiated with use of pharmacy-based data from all community pharmacies in the city of Groningen²². Smoking and alcohol use were based on self-reports.

Hypertension was defined based on self-report of diagnosis by a physician, measured hypertension ($\geq 140/90$ mmHg systolic/diastolic blood pressure) or the use of blood pressure-lowering agents. Prevalent cases of type 2 diabetes were ascertained if one or more of the following criteria were met: 1) a fasting plasma glucose of ≥ 7.0 mmol/l (126 mg/dl) or random sample plasma glucose of 11.1 mmol/l (200 mg/dl); or 2) self-report of diagnosis by a physician; or 3) use of glucose-lowering agents according to a central pharmacy registration²³. Prevalent CVD was defined based on self-reported physician diagnosis of cardiac, cerebral and peripheral events by a physician. Kidney disease was defined based on prior history of kidney disease requiring dialysis or estimated glomerular filtration rate (eGFR) below 60 mL/min/1.73m². We used the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation to calculate eGFR²⁴.

In all participants, blood sample measurements for biomarkers were taken after an overnight fast. Serum Prx4 level was measured retrospectively in analogously stored baseline serum samples by a novel immunoluminometric assay, which was described previously⁹. The functional assay sensitivity (interassay coefficient of variation <20%) was 0.51 U/L. The intraassay coefficient of variation was <8% throughout range of Prx4 levels^{9,14}. Insulin was measured with an AxSym autoanalyzer (Abbott Diagnostics, Amstelveen, The Netherlands). Details on assays for total cholesterol, HDL-cholesterol, triglycerides, hs-CRP and procalcitonin have been described previously²⁵. These baseline assays were performed in EDTA-plasma aliquots that had been stored frozen at -80°C without previous thawing and refreezing. 24-hour urinary albumin excretion (UAE) - given as the mean of the two 24-hour urine excretions - was measured by nephelometry with a threshold of 2.3 mg/L and intra- and inter-assay coefficients of variation of less than 2.2% and less

than 2.6%, respectively (Dade Behring Diagnostic, Marburg, Germany). All technicians were blinded to the participants' characteristics.

Outcome definition

In prospective data, we ascertained the main outcomes as following: 1) incident CVD events, 2) incident CVD events or CVD mortality; and 3) all-cause mortality (up to January 1 2009). Information (on hospitalization) for incident CVD events was obtained from PRISMANT, the Dutch national registry of hospital discharge diagnoses. The validity of this database has been shown to be good, with 84% of primary diagnoses and 87% of secondary diagnoses matching the diagnoses recorded in patients' charts ²⁶. Data were coded according to the International Classification of Diseases (ICD), 9th revision and the classification of interventions. The incident CVD events were classified as acute myocardial infarction (ICD-code 410), acute and subacute ischemic heart disease (411), occlusion or stenosis of the precerebral (433) or cerebral arteries (434) and procedures including coronary artery bypass grafting or percutaneous transluminal coronary angioplasty, and other vascular interventions namely percutaneous transluminal angioplasty or bypass grafting of aorta and peripheral vessels.

Data on mortality were obtained through the municipal registration. Cause of mortality was ascertained by linking the number of the mortality certificate to the primary cause of mortality as coded by the Dutch Central Bureau of Statistics. Survival time was defined as the period from the baseline to the date of first incident CVD events, CVD mortality, date of death or 1 January 2009. In case a person had moved to an unknown destination, the date on which the person was removed from the municipal registry was used as censor date ²⁷.

Statistical Analyses

Data were shown as mean \pm standard deviation (SD) or median (quartiles 1 and 3 [Q1- Q3]) for continuous variables which were compared by one-way ANOVA or Kruskal- Wallis tests as appropriate. Frequency was used for categorical variables which were compared by χ^2 test across tertiles of Prx4. We calculated Spearman correlation coefficients of Prx4 with age, systolic blood pressure, body mass index (BMI), waist circumference, glucose, total Cholesterol, HDL cholesterol, triglyceride, hs-CRP, procalcitonin and 24-hour UAE. We used backward-elimination regression models to examine which of the clinical variables were independently associated with Prx4 as a dependent variable. The distribution of Prx4 was highly skewed. To normalise the distribution, we performed logarithmic transformation of the values of Prx4 prior to analyses. We used the logarithm base 2 (log2) to allow for interpretation of results per doubling of Prx4. Interpretation of results expressed per doubling of Prx4 seem more meaningful than interpretation per factor 10 change or per factor e change, which would have been the case respectively if transformation according to base 10 or transformation according to the natural logarithm would have been applied. We used

Cox proportional-hazards regression in crude and multivariable-adjusted models to examine the associations of Prx4 with incident CVD and all-cause mortality. We adjusted for age and sex in model 1. In model 2, we adjusted for the Framingham risk factors including age, sex, smoking, systolic blood pressure, use of antihypertensive therapy, diabetes at baseline, total cholesterol, and high-density lipoprotein (HDL)-cholesterol²⁸. We tested the assumptions of proportional-hazards for Cox regression models by Schoenfeld's global tests. Finally, in stepwise adjustments, we included alcohol use, triglycerides, high sensitivity (hs)-CRP and 24-hour urine albumin excretion (UAE) to model 2.

To assess incremental value of Prx4 for the risk prediction of CVD, we examined improvement of prediction of CVD as compared to the Framingham risk score. To do so we calculated 10-year general CVD risk based on the Framingham Risk Score²⁸ and based on a model with the Framingham Risk Score and \log_2 Prx4. Subsequently the models were compared in terms of the following measures, taking into account the time-to-event nature of the data^{18, 19, 29, 30}: 1) Harrell's C-statistic for the Cox proportional-hazards regression to quantify the discrimination performance of the models (ability to distinguish between individuals with and without outcome); 2) net reclassification improvement (NRI) to examine if individuals with and without outcome were correctly reclassified, (using the threshold values of <6%, 6 to 20% and $\geq 20\%$ for categories of low, medium and high risk, respectively^{28, 31}); and 3) integrated discrimination improvement (IDI), a continuous measure of reclassification.

Of the baseline sample of 8,592 participants, 451 had prior history of CVD. To do the prospective analyses, we first excluded these prevalent cases of CVD, leaving 8,141 participants. For most baseline variables, <1% was missing; however, this was up to 8% for self-reported variables. We performed a single imputation with predictive mean matching for missing data. This method can be used for skewed data with less than 10% missingness, because it produce less biased estimates for non-linear model and imputations are in the metric of the observed data^{32, 33}.

Moreover, a weighted method was performed to compensate the baseline enrichment for the PREVEND participants with urinary albumin concentration (UAC) ≥ 10 mg/L. Given the frequency of individuals with UAC ≥ 10 mg/L (24.4%) in our general population^{20, 21, 27}, we calculated the weight by sampling fractions. Those with UAC ≥ 10 mg/L had weight equal to 0.35 and those with UAC <10 mg/L weight equal to 2.51.

Subsequently, we performed secondary analyses to take into account residual confounding. To do this, we incorporated other covariates which might be confounding of the association between Prx4 and the risk of incident CVD in combination with the Framingham risk factors (model 2). First, we further adjusted for BMI or waist circumference in separate models. Second, we adjusted for family history of CVD and examined the effects of kidney disease on this association. Third, we calculated the metabolic syndrome which was defined according to the National Cholesterol Education Program's Adult Treatment Panel III report criteria²⁵. And then we adjusted for the metabolic syndrome or insulin in combination with variables

in model 2. Next, we examined the association of Prx4 with each component of CVD events or CVD mortality including myocardial infarction, cerebrovascular disease and cardiovascular mortality. In addition, we performed another analysis including those who had prior history of CVD. In total population, we further adjusted history of CVD in combination with variables in model 2. And then, we performed a similar analysis in participants with prior history of prior CVD. We used Cox proportional-hazards regression with fractional polynomials to search for the best fitting functional form of Prx4 in the model for incident CVD (model 2).

Given the number of each event, we had 80% power at a 0.05 significance level to detect a HR equal to 1.29 for myocardial infarction, 1.25 for cerebrovascular disease and 1.23 for CVD mortality. All the statistical analyses were carried out using IBM SPSS 19.0 and R version 2.10.1 (Vienna, Austria) (<http://75.cran.r-project.org/>).

Results

Baseline characteristics

Baseline clinical characteristics of total population and corresponding tertiles of serum Prx4 are summarized in Table 1. Median (Q1-Q3) Prx4 levels were 0.71 (0.45-1.16) U/L in men and 0.66 (0.42-1.08) U/L in women ($P<0.001$). Across tertiles of Prx4, those who had higher Prx4 levels were older, more obese, less frequent alcohol drinker and more likely to have a history of CVD, hypertension and prevalent diabetes.

Prx4 levels were positively correlated with age, BMI, waist circumference, systolic blood pressure, glucose, triglyceride, insulin, 24-hour UAE and inflammatory biomarkers of hs-CRP and procalcitonin, and inversely correlated with HDL-cholesterol ($p<0.001$ for all correlations, Table 2). In backward-elimination regression model, \log_2 Prx4 were positively associated with age ($\beta=0.006$, $P<0.001$), prior history of CVD ($\beta=0.203$, $P=0.001$), triglyceride ($\beta=0.048$, $P<0.001$), \log_2 hs-CRP ($\beta=0.102$, $P<0.001$), \log_2 UAE ($\beta=0.050$, $P<0.001$) and \log_2 insulin ($\beta=0.102$, $P<0.001$) and inversely associated with female sex ($\beta= -0.059$, $P=0.03$), alcohol use ($\beta= -0.083$, $P<0.001$) and total cholesterol ($\beta= -0.060$, $P<0.001$) (Table 3).

Incident CVD and all-cause mortality

During median (Q1-Q3) follow-up of 10.5 (9.9-10.8) years, 608 participants (7.5%) developed incident CVD events, 708 participants (8.7%) developed incident CVD events or CVD mortality, and 517 (6.3%) participants died (of which 135 from cardiovascular causes). Median (Q1-Q3) Prx4 levels were 0.88 (0.54-1.45) U/L and 0.85 (0.53-1.46) U/L in participants who developed incident CVD events or CVD mortality and all-cause mortality, respectively. In Figure 1, the cumulative survival to incident CVD events and to incident CVD events or CVD mortality is shown based on tertile groups. The crude cumulative incidence rates (per 1,000 person-year), and hazard ratios (HR) (95% confidence interval [CI]) in crude and multivariable-adjusted

Table 1. Baseline characteristics of study participants for total population's corresponding tertiles of serum peroxiredoxin 4 *

Characteristic	Tertiles†			
	Total	2,730	2,671	2,821
Peroxiredoxin 4 — (U/l)	8,222	0.37 (0.37-0.44)	0.68 (0.59-0.78)	1.38 (1.10-1.97)
Age — yr	49.2±12.7	47.1±11.9	48.7±12.4	51.8±13.2
Male sex — no.(%)	4107 (50.0)	1276 (46.7)	1325 (49.6)	1506 (53.4)
Prior history of CVD — no.(%)	431 (5.4)	80 (3.0)	116 (4.5)	235 (8.7)
Family history of CVD — no.(%)	3,817 (50.2)	1,292 (50.7)	1,249 (50.3)	1,276 (49.7)
Current smoker— no.(%)	2787 (34.0)	1071 (39.4)	897 (33.7)	819 (29.1)
Ex-smoker— no.(%)	2984 (36.4)	886 (32.6)	954 (35.9)	1144 (40.7)
Alcohol drinker— no.(%)	6110 (74.7)	2121 (78.0)	2021 (75.9)	1968 (70.2)
BMI—kg/m ²	26.1±4.2	25.3±3.8	26.1±4.2	26.8±4.5
Waist circumference—cm	88.5±13.0	86.0±12.1	88.3±12.8	91.2±13.8
Systolic blood pressure— mmHg	124.5±19.6	121.0±17.5	124.0±19.3	128.3±21.1
Diastolic blood pressure—mmHg	71.8±9.7	70.3±9.0	71.8±9.8	73.3±10.2
Antihypertensive therapy— no.(%)	1282 (15.6)	294 (10.8)	396 (14.8)	592 (21.0)
Prevalent diabetes— no.(%)	315 (4.0)	49 (1.9)	96 (3.7)	170 (6.2)
Fasting glucose— mmol/l	4.9±1.2	4.7±0.9	4.9±1.2	5.0±1.4
Total cholesterol— mmol/l	5.64±1.13	5.63±1.09	5.60±1.12	5.70±1.16
HDL cholesterol— mmol/l	1.32±0.40	1.37±0.40	1.32±0.39	1.27±0.40
Triglyceride— mmol/l	1.16 (0.85-1.68)	1.11 (0.81-1.56)	1.13 (0.83-0.164)	1.26 (0.90-1.88)
Metabolic syndrome— no.(%)	1,378 (18.2)	314 (12.3)	444 (17.9)	629 (24.5)
Fasting insulin— mU/l	8.0 (5.6-12.1)	7.2 (5.2-10.2)	8.0 (5.5-11.9)	9.1 (6.2-14.3)
hs-CRP— mg/l	1.27 (0.55-2.96)	0.93 (0.42-2.08)	1.22 (0.55-2.82)	1.85 (0.79-4.27)
Procalcitonin—ng/ml	0.016 (0.013-0.20)	0.015 (0.013-0.018)	0.016 (0.013-0.019)	0.017 (0.014-0.021)
UAE—mg/24h	9.45 (17.8-6.33)	8.76 (6.16-14.36)	9.11 (6.21-16.23)	10.87 (6.64-24.99)

* Data are mean (±SD) and median (quartiles 1 and 3) for continuous variables and percentage for categorical variables in complete baseline data set. For clinical variables, up to 1.2% was missing. For self-reported data, 0.4-7.8% was missing. For biomarkers, 0.2-6.4% was missing. BMI denotes body mass index which is the weight in kilogram divided by the square of the height in meters. CVD denotes cardiovascular disease, BMI body mass index which is the weight in kilogram divided by the square of the height in meters, HDL high-density lipoprotein, UAE urine albumin excretion and hs-CRP high sensitivity C-reactive protein. The metabolic syndrome was defined according to the National Cholesterol Education Program's Adult Treatment Panel III report criteria.

† P <0.001 for the comparison among all peroxiredoxin 4 tertile group, except for total cholesterol (P=0.005) and family history of CVD (P=0.77).

models for the risk of developing incident CVD events, incident CVD events or CVD mortality, and all-cause mortality are shown in Table 4. Age-and sex-adjusted HR (95% CI) were ranging from 1.32 (1.07-1.63) for incident CVD events to 1.40 (1.08-1.79) for all-cause mortality when compared the highest tertile to the first tertile of Prx4 (p for trend <0.001). In a model adjusted for the Framingham risk factors, Prx4 was significantly associated with the increased risk of incident CVD and all-cause mortality. The proportional-hazards assumptions were met for all models.

Table 2. Spearman correlation coefficients of serum peroxiredoxin 4 with baseline variables*

Variables	correlation coefficients (95%CI)
Age	0.154 (0.131 to 0.177)
Systolic blood pressure	0.148 (0.117 to 0.164)
Body mass index	0.150 (0.128 to 0.170)
Waist circumference	0.167 (0.146 to 0.187)
Glucose	0.114 (0.088 to 0.135)
Total cholesterol	0.020(0.000 to 0.044)
HDL-cholesterol	-0.118 (-0.141 to -0.098)
Triglyceride	0.105 (0.080 to 0.133)
Insulin	0.181 (0.160-0.201)
hs-CRP	0.229 (0.205 to 0.249)
Procalcitonin	0.129 (0.107 to 0.153)
UAE	0.126 (0.104 to 0.150)

BMI denotes body mass index which is the weight in kilogram divided by the square of the height in meters, HDL high-density lipoprotein, hs-CRP high sensitivity C-reactive protein and UAE urine albumin excretion.

* Data were available for 7,638 to 8,222 participants. We used bootstrapping method to calculate 95% confidence interval (CI). P values were <0.001 for all correlations, except for total cholesterol (p=0.096).

In further models, stepwise adjustment for alcohol use, triglyceride, hs-CRP and 24-hour UAE minimally attenuated the associations of Prx4 with incident CVD events or CVD mortality. This was comparable to calculation of HR per doubling Prx4 levels for each outcome. We observed a 15% increased risk of incident CVD events independent of the Framingham risk factors per doubling Prx4 levels (HR, 1.15; 95% CI, 1.05 to 1.26). This was a 16% and a 17% increase for incident CVD events or CVD mortality (HR, 1.16; 95% CI, 1.06 to 1.27), and all-cause mortality (HR, 1.17; 95% CI, 1.06 to 1.29), respectively (Table 4). In subsequent analyses, the associations of Prx4 with the risk of either incident CVD or all-cause mortality were similar for men and women (data not shown).

To assess the incremental predictive value of Prx4 for the risk of CVD, we added Prx4 to the Framingham Risk Score as continuous variable²⁸. In our data set, the Framingham risk score had a C-statistic (95% CI) of 0.80 (0.78-0.82) for the 10-year risk of CVD. Addition of Prx4 improved modestly C-statistic to 0.81 (0.79-0.82) (P=0.02), and led to IDI of 0.003 (P<0.001) and NRI of 2.7% (95%CI, 0.7% to 4.7%; P=0.01) (Table 5). In patients without incident CVD events or CVD mortality, use of Prx4 reclassified 1% and 4% of participants in lower and higher risk categories (<6%

and above 20%), respectively. In patients with incident CVD events or CVD mortality, use of Prx4 reclassified 2% and 4% of participants in lower and higher risk categories, respectively.

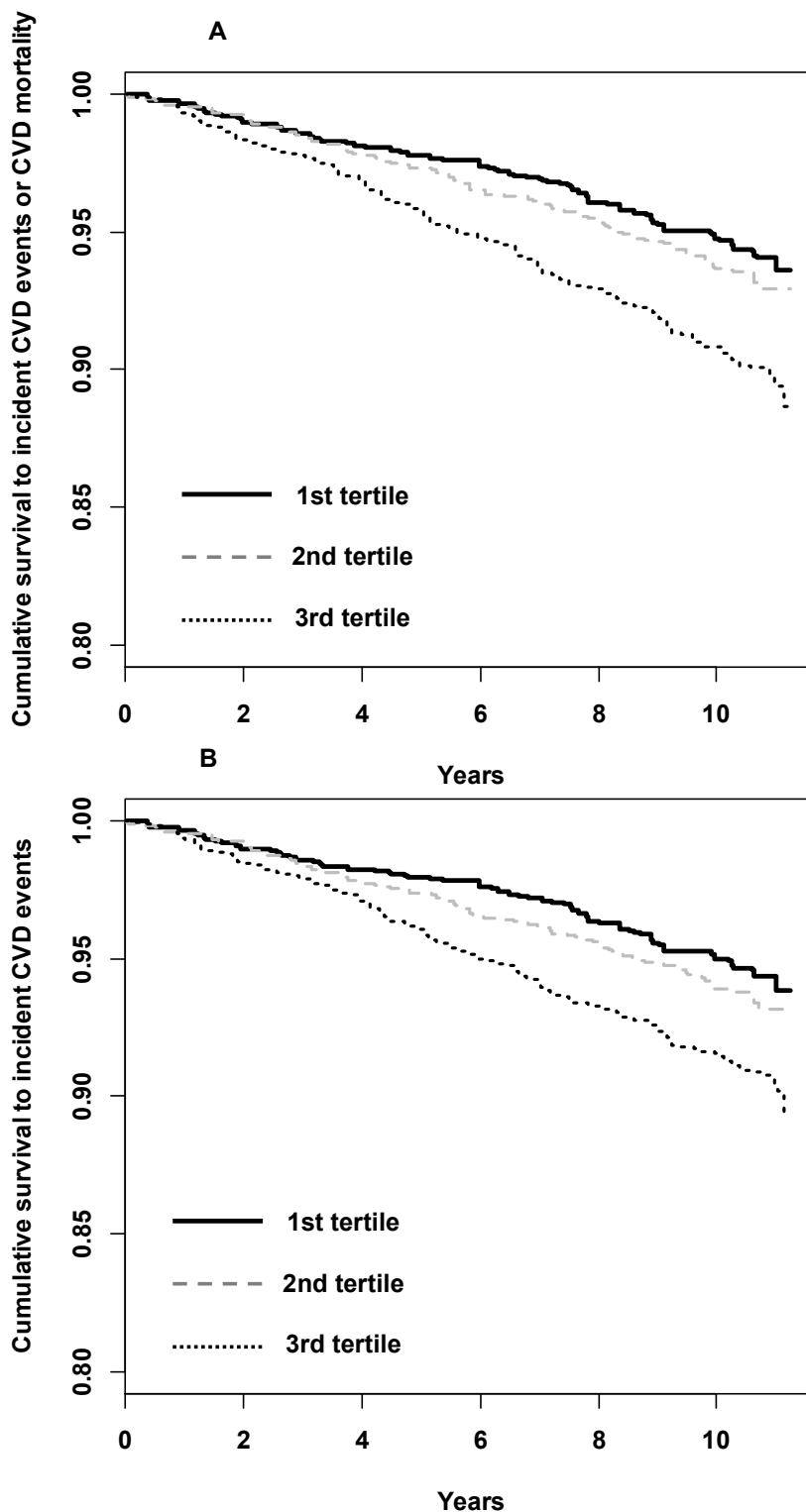


Figure 1. The cumulative probability of incident CVD events (Panel A) and incident CVD events or CVD mortality (Panel B) is shown by tertiles of Prx4. In overall, the log-Rank tests were significant for all outcomes according to the tertiles of serum peroxiredoxin 4 ($P < 0.001$).

Table 3. Association of baseline variables with serum peroxiredoxin 4 as dependent variable*

	Unadjusted		Age- and sex-adjusted		Multivariable-adjusted†	
	β coefficients (SE)	P value	β coefficients (SE)	P value	β coefficients (SE)	P value
Age, per increase of 1 year	0.011 (0.001)	<0.001	0.011 (0.001)	<0.001	0.006 (0.001)	<0.001
Sex, female vs. male	-0.081 (0.022)	<0.001	-0.061 (0.022)	0.005	-0.059 (0.027)	0.03
Prior history of CVD, yes vs. no	0.406 (0.049)	<0.001	0.261 (0.050)	<0.001	0.203 (0.052)	0.001
Smoking, yes vs. no	0.032 (0.013)	0.02	0.006 (0.013)	0.67
Alcohol use, yes vs. no	-0.172 (0.025)	<0.001	-0.158 (0.025)	<0.001	-0.083 (0.026)	0.001
BMI, per increase of 1 kg/m ²	0.029 (0.003)	<0.001	0.021 (0.003)	<0.001
Waist circumference, per increase of 1 cm	0.010 (0.001)	<0.001	0.008 (0.001)	<0.001	-0.002 (0.001)	0.071
Systolic blood pressure, per increase of 1 mmHg	0.007 (0.001)	<0.001	0.004 (0.001)	<0.001	0.001 (0.001)	0.074
Antihypertensive therapy, yes vs. no	0.309 (0.030)	<0.001	0.194 (0.023)	<0.001	0.059 (0.035)	0.10
Prevalent diabetes, yes vs. no	0.422 (0.057)	<0.001	0.291 (0.058)	<0.001
Total cholesterol, per increase of 1 mmol/l	0.027 (0.035)	0.006	-0.015 (0.010)	0.13	-0.060 (0.011)	<0.001
HDL cholesterol, per increase of 1 mmol/l	-0.227 (0.027)	<0.001	-0.204 (0.029)	<0.001
Triglyceride, per increase of 1 mmol/l	0.104 (0.011)	<0.001	0.081 (0.011)	<0.001	0.048 (0.013)	<0.001
Insulin, per increase of log ₂ -unit	0.198 (0.013)	<0.001	0.171 (0.013)	<0.001	0.102 (0.017)	<0.001
hs-CRP, per increase of log ₂ -unit	0.220 (0.023)	<0.001	0.114 (0.007)	<0.001	0.102 (0.008)	<0.001
Procalcitonin, per increase of log ₂ -unit	0.127 (0.007)	<0.001	0.152 (0.024)	<0.001	0.049 (0.026)	0.10
24-hour UAE, per increase of log ₂ -unit	0.101 (0.009)	<0.001	0.075 (0.009)	<0.001	0.050 (0.010)	<0.001

BMI denotes body mass index which is the weight in kilogram divided by the square of the height in meters, SE standard error, HDL high-density lipoprotein, hs-CRP high sensitivity C-reactive protein and UAE urine albumin excretion.

* Base-two logarithmically-transformed serum level of peroxiredoxin 4 was considered as dependent variable.

† Backward selection was used when dropped non-significant variables (probability for removal was 0.10 by F test).

Table 4. Association of serum peroxiredoxin 4 with incident CVD events, incident CVD events or CVD mortality, and all-cause mortality (n=8,141)*

	HR (95% CI) or No. of Cases (Incidence Rate) According to Tertiles of Prx4			HR (95% CI) Per Log2 Unit Increase*	P
	1	2	3		
Incident CVD events					
No. of cases, per 1000 person-years	151 (5.8)	204 (7.8)	308 (12.3)		
Unadjusted analysis	1.00	1.19 (0.95 to 1.48)	1.73 (1.40 to 2.13)	1.32 (1.21 to 1.44)	<0.00
Model 1	1.00	1.04 (0.84 to 1.30)	1.32 (1.07 to 1.63)	1.21 (1.10 to 1.33)	<0.00
Model 2	1.00	1.06 (0.84 to 1.32)	1.26 (1.01 to 1.57)	1.15 (1.05 to 1.26)	0.003
Model 2+alcohol use	1.00	1.06 (0.84 to 1.32)	1.25 (1.01 to 1.55)	1.14 (1.04 to 1.25)	0.004
Model 2+TG	1.00	1.06 (0.85 to 1.33)	1.28 (1.03 to 1.59)	1.16 (1.06 to 1.27)	0.001
Model 2+CRP	1.00	1.05 (0.84 to 1.31)	1.22 (0.98 to 1.52)	1.11 (1.01 to 1.22)	0.02
Model 2+UAE	1.00	1.05 (0.84 to 1.32)	1.25 (1.01 to 1.55)	1.15 (1.05 to 1.25)	0.003
Model 2+alcohol use, TG, CRP, and UAE	1.00	1.06 (0.84 to 1.32)	1.23 (1.00 to 1.53)	1.12 (1.02 to 1.23)	0.02
Incident CVD events or CVD mortality					
No. of cases, per 1000 person-years	160 (6.1)	215 (8.2)	333 (13.3)		
Unadjusted analysis	1.00	1.17 (0.94 to 1.45)	1.78 (1.46 to 2.19)	1.33 (1.23 to 1.45)	<0.00
Model 1	1.00	1.02 (0.82 to 1.27)	1.35 (1.10 to 1.66)	1.22 (1.12 to 1.33)	<0.00
Model 2	1.00	1.03 (0.83 to 1.29)	1.29 (1.05 to 1.59)	1.16 (1.06 to 1.27)	<0.00
Model 2+alcohol use	1.00	1.03 (0.83 to 1.29)	1.29 (1.04 to 1.58)	1.16 (1.06 to 1.26)	0.001
Model 2+TG	1.00	1.04 (0.84 to 1.30)	1.32 (1.07 to 1.62)	1.18 (1.08 to 1.28)	<0.00
Model 2+CRP	1.00	1.03 (0.82 to 1.28)	1.26 (1.02 to 1.56)	1.13 (1.03 to 1.23)	0.01
Model 2+UAE	1.00	1.03 (0.83 to 1.29)	1.29 (1.05 to 1.59)	1.16 (1.06 to 1.27)	0.001
Model 2+alcohol use, TG, CRP, and UAE	1.00	1.03 (0.83 to 1.29)	1.27 (1.03 to 1.57)	1.13 (1.03 to 1.24)	0.008
Incident all-cause mortality					
No. of cases, per 1000 person-years	117 (4.4)	160 (5.9)	240 (9.1)		
Unadjusted analysis	1.00	1.43 (1.10 to 1.86)	2.00 (1.56 to 2.57)	1.36 (1.23 to 1.50)	<0.00
Model 1	1.00	1.25 (0.96 to 1.62)	1.40 (1.08 to 1.79)	1.20 (1.08 to 1.33)	<0.00

Model 2	1.00	1.27 (0.98 to 1.66)	1.41 (1.09 to 1.82)	1.17 (1.06 to 1.29)	0.003
Model 2+alcohol use	1.00	1.27 (0.98 to 1.66)	1.41 (1.09 to 1.82)	1.17 (1.06 to 1.30)	0.002
Model 2+TG	1.00	1.26 (0.97 to 1.65)	1.39 (1.07 to 1.79)	1.16 (1.05 to 1.29)	0.004
Model 2+CRP	1.00	1.24 (0.95 to 1.62)	1.26 (0.97 to 1.64)	1.09 (0.98 to 1.21)	0.11
Model 2+UAE	1.00	1.27 (0.98 to 1.66)	1.40 (1.08 to 1.81)	1.15 (1.04 to 1.28)	0.006
Model 2+alcohol use, TG, CRP, and UAE	1.00	1.23 (0.94 to 1.60)	1.24 (0.96 to 1.61)	1.08 (0.97 to 1.20)	0.18

Cardiovascular disease (CVD) events were defined as a composite of incident cardiac, cerebral and peripheral vascular events. Participants with history of CVD were excluded. These associations did not differ by sex. Hazard ratios (HR) (95% confidence interval [CI]) have been adjusted for:

Model 1: age and sex

Model 2: the Framingham risk factors including age, sex, smoking, systolic blood pressure, use of anti-hypertensive therapy, diabetes at baseline, total cholesterol, HDL-cholesterol.

* Base-two logarithmically-transformed Ptx4, CRP, and 24-hour UAE were analyzed as continuous variable. Individuals with prevalent CVD were excluded.

Table 5. Reclassification of participants for the 10-year risk prediction of cardiovascular disease corresponding to the Framingham risk score and after adding serum peroxiredoxin 4*

Framingham risk category	Framingham risk score with Prx4			Reclassification (%)
	Low risk	Intermediate risk	High risk	
In participants without outcome				
Low risk	4647	38	0	1.0
Intermediate risk	207	1984	81	13.0
High risk	0	23	494	4.0
In participants with outcome				
Low risk	104	2	0	2.0
Intermediate risk	9	299	24	10.0
High risk	0	9	213	4.0
Total sample				
Low risk	4751	40	0	1.0
Intermediate risk	216	2283	105	12.0
High risk	0	32	707	4.0

* Corresponding the Framingham risk score and after adding serum peroxiredoxin 4, low risk denotes less than 6%, intermediate risk 6 to 20% and high risk more than 20% for the 10-year of cardiovascular disease. Net reclassification index (95%CI) was 2.7 (0.7 to 4.7); P= 0.01, and integrated discrimination improvement (95% CI) of 0.0032 (0.0014 to 0.0049); P value< 0.001

Secondary analyses

Tables 6 and 7 show the results of secondary analyses. Separately, adjustment for BMI or waist circumference in combination with the Framingham risk factors (i.e., model 2) did not materially change the association of Prx4 with risk of incident CVD events or CVD mortality. Moreover, our results adjusted for both kidney disease and family history of CVD were similar to that of model 2. Additionally, further adjustment for the metabolic syndrome or insulin in model 2 did not affect on the association. We also investigated whether Prx4 was associated with the components of incident CVD events or CVD mortality including MI, cerebrovascular disease and CVD mortality. After adjustment for the variables in model 2, the HRs (95%CI) in the highest tertile compared to the first tertile of Prx4 were 1.03 (0.71-1.50), 1.28 (0.85-1.93) and 1.22 (0.71-2.12) for myocardial infarction, cerebrovascular disease and CVD mortality, respectively.

Table 6. Association of serum peroxiredoxin 4 with incident cardiovascular events or mortality (n=8,141)

Model	HR (95%CI) by tertiles of peroxiredoxin 4 (U/L)		
	1	2	3
Adjusted for model 2	1.00	1.03 (0.83-1.29)	1.29 (1.05-1.59)
Adjusted for model 2 + BMI	1.00	1.03 (0.83-1.28)	1.29 (1.05-1.59)
Adjusted for model 2 + waist circumference	1.00	1.03 (0.83-1.28)	1.31 (1.06-1.59)
Adjusted for model 2 + family history of CVD	1.00	1.04 (0.83-1.29)	1.30 (1.06-1.60)
Adjusted for sex, age, smoking + metabolic syndrome	1.00	1.03 (0.82-1.28)	1.38 (1.12-1.76)
Adjusted for model 2 + insulin	1.00	1.04 (0.83-1.30)	1.31 (1.06-1.61)
Adjusted for model 2 + kidney disease	1.00	1.03 (0.83-1.29)	1.30 (1.05-1.60)

Hazard ratios (HR) (95% confidence interval [CI]) have been adjusted for model 2, in which the Framingham risk factors age, sex, smoking, systolic blood pressure, use of anti-hypertensive therapy, diabetes at baseline, total cholesterol, HDL-cholesterol were included. Kidney disease was defined based on prior history of kidney disease requiring dialysis or estimated glomerular filtration rate (eGFR) below 60 mL/min/1.73m². We used the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation to calculate eGFR.

In another analysis, we examined the association of Prx4 with risk of incident CVD after adjustment for the variables in model 2 and prior history of CVD in total population. The adjusted HR (95%CI) in the highest tertile compared to the first tertile of Prx4 was 1.32 (1.11-1.59) for incident CVD events or CVD mortality. In participants with prior history of CVD, the adjusted HR (95%CI), for the variables in model 2, in the highest tertile compared to the first tertile of Prx4 was 1.07 (0.73-1.56) for incident CVD events or CVD mortality (n=181).

Figure 2 depicts the relationship of continuous Prx4 with incident CVD events or CVD mortality. We plotted for Framingham risk factors adjusted (model 2) HRs and their 95%CIs as a function of Prx4. The optimal transformation was one in which the terms $(Prx4)^{1/2}$ and $(Prx4)^{1/2} \times \ln(Prx4)$ were incorporated. The solid line demonstrates that after a slight decrease in risk with levels slightly higher than the

lowest ones that can be detected, the risk associated with increasing levels of Prx4 steeply increases, until a plateau is reached with high levels of Prx4. Incremental value for risk prediction with addition of $(Prx4)^{1/2}$ and $(Prx4)^{1/2} \times \ln(Prx4)$ to the model rather than log2-linear transformed Prx4 (C-statistic=0.81, 95%CI, 0.79-0.82; IDI=0.004, P<0.001; NRI=3.6% 95%CI, 1.5% to 5.7%, P<0.001) was slightly higher, but very similar to that with log2-linear transformed Prx4.

Table 7. Association of serum peroxiredoxin 4 with incident myocardial infarction, cerebrovascular events and cardiovascular mortality (n=8,141)

	Tertiles of peroxiredoxin 4 (U/L)		
	1	2	3
Incident myocardial infarction			
No. of Cases (%)	51 (5.8)	63 (7.8)	94 (10.1)
Unadjusted HR (95%CI)	1.00	1.04 (0.71-1.52)	1.43 (1.00-2.06)
Multivariate-adjusted HR (95%CI)*	1.00	0.98 (0.67-1.43)	1.06 (0.74-1.53)
Incident cerebrovascular disease			
No. of Cases (%)	39 (6.1)	58 (8.2)	88 (13.3)
Unadjusted HR (95%CI)	1.00	1.00 (0.64-1.55)	1.73 (1.16-2.58)
Multivariate-adjusted HR (95%CI)*	1.00	0.93 (0.60-1.45)	1.28 (0.85-1.91)
Incident cardiovascular mortality			
No. of Cases (%)	28 (1.0)	44(5.9)	63 (9.1)
Unadjusted HR (95%CI)	1.00	1.48 (0.84-2.59)	2.10 (1.23-3.58)
Multivariate-adjusted HR (95%CI)*	1.00	1.35 (0.77-2.36)	1.38 (0.80-2.36)

* Hazard ratios (HR) (95% confidence interval [CI]) have been adjusted for model 2 in which the Framingham risk factors included, age, sex, smoking, systolic blood pressure, use of anti-hypertensive therapy, diabetes at baseline, total cholesterol, HDL-cholesterol.

Discussion

In this study, we demonstrated that serum Prx4, a circulating biomarker with antioxidant properties, was associated with the most common risk factors of CVD in a general population cohort enriched with individuals with microalbuminuria. We found it to have an statistically significant positive association with age, history of CVD, systolic blood pressure, antihypertensive therapy, triglycerides, hs-CRP, UAE and procalcitonin, while there was an inverse association with alcohol use and total cholesterol. Moreover, higher serum Prx4 levels were associated with significantly higher risk of incident CVD and all-cause mortality. For potential clinical application, we examined the incremental predictive value of Prx4 compared with the Framingham risk score. Particularly, Prx4 modestly improved prediction for the 10-year CVD risk when added to the Framingham risk score in terms of discriminative ability and net reclassification.

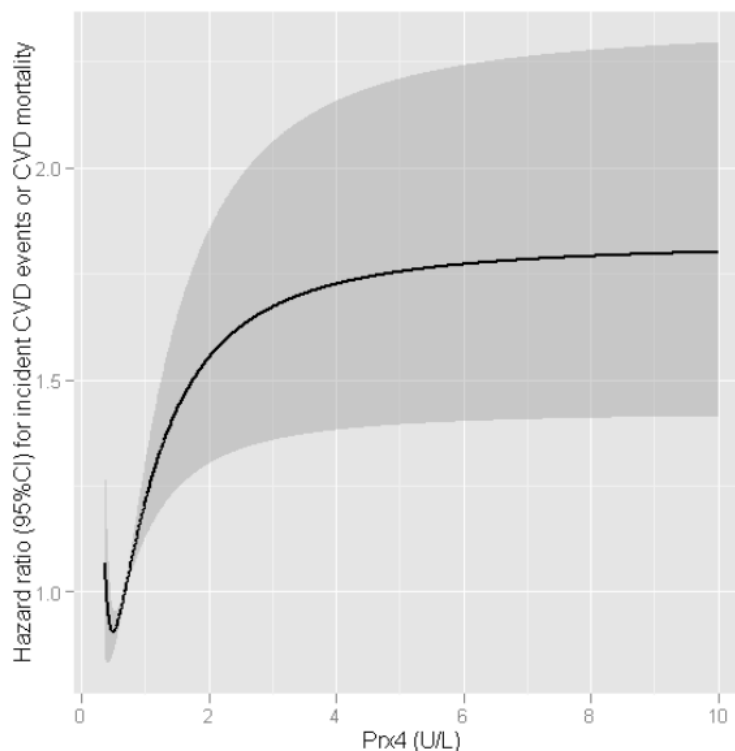


Figure 2. The relationship of peroxiredoxin 4 with incident CVD events or CVD mortality. Data are shown for 8,141 participants without CVD at baseline. The plotted hazard ratio (95%CI) was adjusted for the Framingham risk factors and centered on the Prx4 median value. The optimal transformation of Prx4 consisted of one in which the terms $(Prx4)^{1/2}$ and $(Prx4)^{1/2} \times \ln(Prx4)$ were incorporated.

Our findings showing the associations of serum Prx4 levels with cardiovascular risk factors and events support previous clinical studies suggesting a role of oxidative stress in the pathogenesis of CVD ^{1, 2, 4, 34}. In an earlier study, a higher urinary excretion of oxidative stress indices was reported in individuals with renovascular hypertension which was correlated with endothelium-dependent vasodilatation ⁴. Another study has shown the independent association of oxidized low-density lipoprotein with the incidence of metabolic syndrome ². In line with this, genotypes and serum activity of paraoxonase 1, an HDL-related antioxidant, has been shown to be associated with systemic oxidative stress and cardiovascular outcomes in humans ¹. We now extend accumulating information obtained from animal and human studies on Prx4 of the family of thiol-dependent antioxidants in this era. An animal model of type 1 diabetes has indicated that Prx4 may have a pivotal role in the suppression of apoptosis and the proliferation of progenitor cells to protect against oxidative stress-induced β -cell dysfunction ¹². In line with this, a higher gene expression of Prx4 has been found in the islets of high-fat diet model of β -cell dysfunction ³⁵ and down-regulated expression of Prx4 in islet in diabetic mice with chronic hyperglycaemia ³⁶. Moreover, recent studies suggested that Prx4 might be involved in the protection against celiac disease and cancer in pancreas and lung with an increased expression and production of Prx4 in the related human tissues. ^{12, 37, 38}.

Consistently, Prx1, another member of the Prx family, has also shown to be involved in a broad range of oxidative stress-related outcomes in the cardiovascular system ^{39, 40}.

Importantly, Prx4 is the only secretable member of the family in the animals and humans ^{8, 11}. Recently, clinical data have shown that Prx4 levels were increased in serum of septic patients compared to healthy individuals ^{14, 15}. Consistent with this, we showed the relation between Prx4 and inflammatory markers, and also explored its relation with measures of adiposity, blood pressure, glycaemia index, lipids, and albuminuria. All these markers are underlying in central biological pathways of metabolic traits and CVD ^{27, 41}. Other recent data from patients who presented to emergency departments showed that serum Prx4 levels were increased in non-survivors and improved prognostic information for survival at 30 days when added to a clinical risk score ¹⁵. One main explanation for these findings may be that concomitant production and secretion of Prx4 can be augmented to protect against the increased rate of oxidative stress in relation with adverse cardiovascular and metabolic conditions ⁴². Interestingly, in secondary analyses with fractional polynomials, we found indication for the notion that very low levels of Prx4 as associated with slightly increased risk as compared to somewhat higher levels. Possibly this makes up a subgroup of subjects with constitutively low levels of Prx4, which predisposes to CVD. The high risk with more elevated levels of Prx4 is consistent with compensatory increase of Prx4 in response to existing oxidative stress. At cellular level, Prx4 may promote antioxidant activity via several pathways, such as the nuclear factor kB ⁴³, p53 ⁴⁴, thromboxane A2 receptor ⁴⁵ and NF-E2-related factor 2 (Nrf2) ⁴⁶. Despite equivocal findings of supplementing antioxidant vitamins to prevent cardiovascular and metabolic diseases ^{1, 47}, a recent trial ⁴⁸ showed an effective intervention of a specific antioxidant, that is Nrf2 antagonist which is related to Prx4 pathway, against decline of renal function in patients with chronic kidney disease and type 2 diabetes. Therefore, this might be an opening line for novel therapeutic targets and monitoring tools for metabolic traits and CVD.

Next, we assessed the clinical application of Prx4 for the risk prediction of CVD. To do this, we used information from general 10-year risk of CVD based on the Framingham risk score ²⁸, and examined whether Prx4 might have incremental predictive value above this risk score. We did not develop new models, but chose an appropriate approach in prediction research by using the information retained in the existing prediction model ^{19, 30, 31, 49}. Of note, there are usually missing values for baseline data in an observational study. We had less than 10% missing data and we used a single imputation and predictive mean matching. Imputation for missing values will increase the power and precision of study and minimize the risk of biased findings ⁵⁰. Finally, we used reclassification measures, that are more sensitive and more clinically relevant than C-statistic alone ^{18, 19, 49}. For CVD, addition of Prx4 to the Framingham risk score marginally improved prediction in terms of discrimination and reclassification. Overall, addition of Prx4 correctly reclassified 2.7% of participants for the risk of CVD obtained by the Framingham risk score. Further

studies are warranted to confirm our findings for this oxidative stress biomarker in different setting ¹⁷ and among individuals with other co-morbidities such as diabetes.

There are some limitations to this study that should be addressed. Our cohort is predominantly comprised of white adults, and it is therefore unknown whether our findings can be generalized for non-whites. The PREVEND cohort was enriched for microalbuminuria at baseline. However, a weighted method was performed to compensate this, and this did not affect results. Moreover, we investigated the association of only baseline and of not serial circulating levels of Prx4 with the future risk of outcomes of interest. The extent of within-individual variation over time might affect the statistical power of study ^{51,52}. For example, a higher variation can lead to less power and then the estimated risk is attenuated using a single Prx4 measurement. Although we accounted for confounding of traditional cardiovascular risk factors, a potential for uncontrolled or residual confounding can be plausible. Finally, we had no data on other oxidative stress markers such as oxidized low-density lipoprotein and homocysteine to compare their predictive values with that of Prx4.

In conclusion, our results suggest that higher serum Prx4 levels are associated with most cardiovascular risk factors including albuminuria and hs-CRP in a general population cohort study. Moreover, higher serum Prx4 levels were associated with significantly higher risk of incident of CVD events or CVD mortality, and all-cause mortality after adjustment for traditional cardiovascular risk factors. Prx4 marginally improve the 10-year risk prediction for CVD when added to the Framingham risk score.

Acknowledgments

We thank Prof. dr. L.T.W. de Jong-van den Berg and dr. S.T. Visser from the Department of Social Pharmacy, Pharmacoepidemiology and Pharmacotherapy, Groningen University Institute for Drug Exploration, University of Groningen, University Medical Center Groningen for providing the data on pharmacy-registered use of antidiabetic medication. This work was supported by the Netherlands Heart Foundation, Dutch Diabetes Research Foundation and Dutch Kidney Foundation. This research was performed within the framework of CTMM, the Center for Translational Molecular Medicine (www.ctmm.nl); project PREDICt (grant 01C-104-07).

Conflict of Interest Disclosures: Dr Struck and Ms Schulte hold patent rights to peroxiredoxin 4 assay and are employees of BRAHMS GmbH, the manufacturer of the peroxiredoxin 4 assay. The present study was not financed by BRAHMS GmbH. No other author has anything to potential conflicts of interest relevant to this article. None of the study sponsors had a role in study design; in data collection, analysis, or interpretation; in writing the report; or in the decision to submit for publication.

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