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Serum paraoxonase-1 activity and risk of incident cardiovascular disease: The PREVEND study and meta-analysis of prospective population studies

Running Title: Paraoxonase-1 activity and CVD risk

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"These authors take responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation"

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ABSTRACT

Background: Paraoxonase-1 (PON-1) has been suggested to be associated with cardiovascular disease (CVD) risk, however, aspects of the association, such as shape and independence from conventional risk factors are still uncertain. We aimed to assess the association of PON-1 with CVD risk and determine its potential utility for CVD risk prediction.

Methods: PON-1 was measured as its arylesterase activity at baseline in the PREVEND prospective study of 6902 participants.

Results: During a mean follow-up of 9.3 years, 730 CVD events were recorded. Serum PON-1 was weakly correlated with several cardiovascular risk markers including high-density lipoprotein cholesterol (HDL-C) (r=0.18; $P < 0.001$) and was approximately log-linearly associated with CVD risk. In analyses adjusted for conventional risk factors, the hazard ratio (95% CI) for CVD per 1 standard deviation (SD) increase in $\log_e$ PON-1 was 0.92 (0.85 to 0.99; P=0.020), which remained persistent after additional adjustment for potential confounders 0.93 (0.86 to 0.99; P=0.037). The association was attenuated on further adjustment for HDL-C 0.95 (0.88 to 1.02; P=0.153). In a meta-analysis of 6 population-based prospective studies involving 15 064 participants and 2958 incident CVD outcomes, the pooled multivariable adjusted (including HDL-C) relative risk (95% CI) for CVD was 0.95 (0.90 to 1.02; P=0.138) per 1 SD increase in PON-1 values. Adding PON-1 to a CVD risk prediction model containing conventional risk factors did not improve the C-index or net reclassification.

Conclusions: There is an approximately log-linear inverse association between PON-1 activity and CVD risk, which is partly dependent on HDL-C levels. In addition, serum PON-1 activity provides no significant improvement in CVD risk assessment beyond conventional CVD risk factors.

Keywords: paraoxonase-1; cardiovascular disease; risk factor; risk prediction; meta-analysis

1. Introduction

The inverse and independent association of high-density lipoprotein cholesterol (HDL-C) with atherosclerotic cardiovascular disease (CVD) is well known,[1] and is commonly ascribed to the functional properties of this lipoprotein fraction.[2] HDL promotes the reverse cholesterol transport pathway, by stimulating efflux of cholesterol out of macrophages in the arterial wall and exerts antioxidant, anti-inflammatory, antithrombotic, and endothelial cell maintenance properties.[3-6] Paraoxonase-1 (PON-1) is a HDL-bound esterase enzyme with well-established antioxidant functions, which is synthesized by the liver.[7] It hydrolyses lipid peroxides, thereby protecting low-density lipoproteins (LDLs) from oxidative modifications. Recent studies in rodent models and humans have suggested that the anti-atherogenic and cardioprotective effects of the HDL fraction are primarily attributable to PON-1 activity.[8] Serum PON-1 activity has been shown to be reduced in patients with stable coronary artery disease, post-myocardial infarction patients, and in patients at increased CVD risk such as hypercholesterolaemia, metabolic syndrome, and type 2 diabetes.[9-11]

Emerging evidence indicates that PON-1 activity may be linked to CVD risk. Two published literature-based meta-analyses have assessed the relationship between PON-1 activity and coronary heart disease (CHD) risk.[12, 13] However, because they only compared PON-1 activity in CHD cases and non-CHD controls, the nature, shape, and magnitude of the prospective association could not be addressed. A number of population-based prospective studies have generally reported inverse associations between PON-1 activity and CVD outcomes.[14-19] Some of these reports were relatively small or did not adjust for potential confounders. Data on the prospective associations in approximately healthy ("general") populations are also sparse. Among the limited studies that have been based in general populations, some reported inverse associations,[15, 17] with majority reporting null associations.[14, 15, 20, 21] For these reasons, the nature of the shape, magnitude, and independence of the association between PON-1 activity and CVD risk in general population settings remains uncertain. There are suggestions that PON-1 might be a better atherosclerotic CVD risk factor compared to HDL-C,[22] and there is a growing interest in the potential

value of PON-1 in CVD risk prevention. There is therefore a requirement for an adequately powered and comprehensive assessment of the association of baseline PON-1 activity with risk of future first-ever CVD events using long-term observational evidence in the general population. Our primary aim was to characterize the shape and assess the magnitude and independence of the prospective association between PON-1 activity and CVD risk, using a large population-based sample of 6902 participants free from pre-existing CVD at baseline. A secondary aim was to investigate the extent to which PON-1 activity could improve the prediction of first-onset CVD outcomes in general population settings when added to a conventional CVD risk prediction model**.** Finally, to contextualize these findings, we report a meta-analysis of available published prospective evidence on the associations of baseline PON-1 activity and risk of CVD in general population settings.

2. Materials and methods

2.1. Participants

The study population consisted of inhabitants living in the city of Groningen in the Netherlands. Subjects were participants in the Prevention of Renal and Vascular End-stage Disease (PREVEND) study, an observational, general population-based prospective cohort study designed to investigate the natural course of urinary albumin excretion and its relationship to renal disease and CVD and which began in 1997. The description of the study design and recruitment processes have been described in detail previously.[23] The baseline cohort (n=8592) was recruited from participants who were aged 28-75 years and had baseline measurements performed between 1997 and 1998. For the present analyses, participants with a prevalent history of CVD, liver disease, renal disease, or malignancy were excluded and the final cohort comprised of 6902 subjects with non-missing information on serum PON-1 activity, several CVD risk markers, and cardiovascular outcomes. The medical ethics committee of the University Medical Center Groningen approved the PREVEND study and which was conducted in accordance with the Declaration of Helsinki. Informed written consent was provided by each participant and was documented in a consent form approved by the medical ethics committee.

2.2. Risk factor assessment

Baseline data on demographics, physical measurements (including anthropometrics), and cardiovascular risk factors were assessed during two outpatient visits by study participants. After an overnight fast and 15 minutes of rest, venous blood was obtained from participants. Plasma samples were prepared by centrifugation at 4 ºC. Sera was stored at -20 ºC and heparinized plasma samples stored at -80 ºC from baseline collection until analysis. The analysis of serum PON-1 enzymatic activity was conducted in 2014 and was measured as its arylesterase activity, i.e. as the rate of hydrolysis of phenyl acetate into phenol, which has been described previously.[24] The inter-assay CV was 8%. Arylesterase activity, measured with this assay, is positively correlated PON-1 enzymatic activity toward paraoxon.[25] Given the virtual absence of free PON-1 in serum, as determined by gelfiltration,[26] its activity was essentially measured as HDL-bound PON-1 activity, which was consistent with several large-scale epidemiological studies of this nature. Plasma glucose, total cholesterol, apolipoproteins (Apo) A-1 and B, HDL-C, high sensitivity C-reactive protein (hsCRP), triglycerides, total bilirubin, serum creatinine, and serum cystatin C were measured using standard methods described previously.[27-31] Urinary albumin excretion (UAE) was calculated as the mean of two 24-hour urine collections. Estimated glomerular filtration rate (eGFR), was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) combined creatinine-cystatin C equation.[32] Hypertension and type 2 diabetes mellitus were defined as previously reported.[30, 31, 33]

2.3. Endpoint ascertainment

The primary endpoint in our study was first-onset CVD, with subsidiary analyses of incident CHD and stroke endpoints. The dates and causes of death were obtained by record linkage with the Dutch Central Bureau of Statistics. Information on hospitalization for cardiovascular morbidity was received from PRISMANT, the Dutch national registry of hospital discharge diagnoses.[34] Until 01-01-2009, all data were coded according to the *International Classification of Diseases*, Ninth Revision (ICD-9). After this

date, data were coded according to ICD-10 codes. First-onset CVD was defined as the combined endpoint of acute myocardial infarction, acute and subacute ischemic heart disease, coronary artery bypass grafting or percutaneous transluminal coronary angioplasty, subarachnoid hemorrhage, intracerebral hemorrhage, other intracranial hemorrhage, occlusion or stenosis of the precerebral or cerebral arteries, and other vascular interventions such as percutaneous transluminal angioplasty or bypass grafting of peripheral vessels and aorta. Coronary heart disease events were defined as fatal or nonfatal myocardial infarction, ischemic heart disease, coronary artery bypass grafting, and percutaneous transluminal coronary angioplasty. Stroke events were defined as subarachnoid hemorrhage, intracerebral haemorrhage, other and unspecified intracranial haemorrhage, and occlusion and stenosis of precerebral or cerebral arteries. Further details on the definition of cardiovascular outcomes are also provided in **Appendix Supplement 1**.

2.4. Statistical analyses

Prospective cohort analyses Variables that were skewed (e.g., PON-1 activity, hsCRP, triglycerides, creatinine, and total bilirubin) were natural logarithm ($\log_e$) transformed to achieve approximately normal distributions. Baseline characteristics of participants were summarized using descriptive analyses. Partial correlation coefficients (adjusted for age and sex) were calculated to assess the cross-sectional associations of serum PON-1 activity with CVD risk markers. Analyses of the association between serum PON-1 activity and CVD risk involved Cox proportional hazards models after confirmation of assumptions of proportionality of hazards.[35] Shapes of the association of PON-1 activity with CVD risk were assessed by plotting hazard ratios (HRs) estimated within quintiles of baseline PON-1 values relative to the bottom quintile against the mean $\log_e$ PON-1 value in each quintile using floating absolute risks (FARs).[36] Given the log-linear shape of the association, hazard ratios (HRs) were calculated per 1 standard deviation (SD) higher $\log_e$ serum PON-1 values. The SD of baseline $\log_e$ serum PON-1 was 0.31 (equivalent to approximately one-fold higher PON-1 activity, as $e^{0.31}=1.36$). To assess the independence of the association, HRs were calculated with progressive adjustment for age and sex, other established

CVD risk factors [smoking status, history of diabetes, SBP, and total cholesterol], potential confounders [body mass index (BMI), alcohol consumption, fasting glucose, triglycerides, eGFR, UAE, hsCRP, and total bilirubin], and finally HDL-C. Effect modification by individual characteristics, such as age, sex, and other cardiovascular risk markers was assessed using formal tests of interaction. To limit potential biases due to pre-existing undiagnosed CVD (i.e. reverse causation), subsidiary analyses included excluding the first two years of follow-up, participants on regular anti-hypertensive medication, and participants on regular lipid-lowering medication (as these patients are supposed to have at least a 20% 10-year risk of CVD[37]).

We calculated measures of discrimination for censored time-to-event data (Harrell's C-index[38]) and reclassification, to assess whether adding information on serum PON-1 values to a model that included conventional cardiovascular risk factors[39] is associated with improvement in CVD risk prediction. To investigate the change in C-index, we added PON-1 to a model based on risk factors included in the Framingham CVD Risk Score (i.e., age, sex, smoking status, SBP, total cholesterol, and HDL-C).[40] For participants with at least 10 years of follow-up, reclassification was assessed using the net-reclassification-improvement (NRI).[41] Reclassification analysis was based on predicted 10-year CVD risk categories of low (<5%), intermediate (5 to <7.5%), and high (≥7.5%) risk.[42] In subsidiary analyses, we also performed risk discrimination and reclassification analyses based on the Reynolds Risk Score (RRS).[43, 44] Risk prediction analysis was restricted to participants without a known history of diabetes or CVD at baseline.

Literature review and meta-analysis We conducted a meta-analysis using a predefined protocol and in accordance with the PRISMA (**Appendix Supplement 2**) and MOOSE guidelines[14,15] (**Appendix Supplement 3**). We sought prospective cohort studies that have assessed the association between PON-1 activity and CVD risk published up to July 2015 using MEDLINE, EMBASE, and Science Citation Index databases as well as scanning reference lists from the included articles. The computer-based searches combined free and MeSH search terms and combination of key words related to the exposure (e.g., "paraoxonase-1", "arylesterase", etc.) and outcome (e.g., "cardiovascular disease", "coronary heart

disease", "stroke", etc.). Further details on the search strategy are presented in **Appendix Supplement 4**. Studies were eligible if they had at least one year of follow-up and recruited participants from approximately general populations as previously described.[45] The primary endpoint of this analysis was any CVD event (i.e., composite CVD, CHD, or stroke). A pooled estimate was also assessed for CHD outcomes. **Appendix Supplement 1** provides details of study-specific endpoint definitions. Study quality was assessed based on the Newcastle–Ottawa Scale (NOS) method.[46]

Summary measures were presented as relative risks (RRs) with 95% confidence intervals (CIs). We assumed hazard ratios and odds ratios to approximate the same measure of RRs. To enhance consistency, reported study-specific RRs were transformed to per 1SD change in baseline PON-1 values using standard statistical methods[47, 48] which have been described in detail previously.[45] The summary RR (including the estimate from the present study) was calculated using random effects meta-analysis. All statistical analyses were conducted using Stata version 14 (Stata Corp, College Station, Texas).

3. Results

Results from the PREVEND cohort

3.1. Baseline Characteristics and Correlates of paraoxonase-1 Activity

Data were available for 6902 participants without a known history of CVD at baseline. The mean age of overall participants at baseline was 48 (SD 12) years and 52% were women. (**Table 1**). Mean (SD) $\log_e$ PON-1 value was 3.97 (0.31) U/L. Log_e PON-1 values were weakly correlated with physical measures (BMI, waist-to-hip ratio, and blood pressure), as well as several lipid markers, but not with total bilirubin. The strongest correlations were observed for HDL-C (r = 0.18) and Apo-A1 (r = 0.17). Baseline PON-1 values were higher by 6% in current alcohol consumers compared with non-consumers. Values were lower by 6% in people with diabetes compared with people without diabetes and by 14% in people on regular glucose lowering medication compared with non-users (**Table 2**).

3.2. Paraoxonase-1 Activity and Risk of Incident CVD

During an average follow-up of 9.3 years (64 407 person-years at risk), 730 incident CVD events (annual rate 11.2/1000 person-years at risk, 95% confidence interval [CI]: 10.4 to 12.1) were recorded. An approximately log-linear relationship was observed between PON-1 activity and CVD risk, in analyses adjusted for established CVD risk factors and potential confounders (**Figure 1**). The HR for CVD per 1 SD increase in $\log_e$ PON-1 was 0.90 (95% CI, 0.84 to 0.97; *P*=0.004) in age- and sex-adjusted analyses, which remained consistent in analyses adjusted for established factors 0.92 (95% CI, 0.85 to 0.99; *P*=0.020) and additional adjustment for BMI, alcohol consumption, glucose, $\log_e$ triglycerides, eGFR, $\log_e$ UAE, and $\log_e$ total bilirubin 0.93 (95% CI: 0.86 to 0.99; *P*=0.037). The results were somewhat attenuated after additional adjustment for HDL-C 0.95 (95% CI, 0.88 to 1.02; *P*=0.153) (**Table 3**). In an age- and sex-adjusted analysis, the initial association 0.90 (95% CI, 0.84 to 0.97; *P*=0.004) was attenuated after single additional adjustment for HDL-C 0.96 (95% CI, 0.89 to 1.03; *P*=0.249). Substituting total cholesterol and HDL-C for apolipoproteins B and A-1 respectively resulted in similar associations (data not shown). HRs were similar in analyses that excluded CVD outcomes recorded in the first two years of follow-up, participants on regular antihypertensive medication, and participants on regular lipid-lowering medication (**Table 4**). The associations did not vary significantly by levels or categories of several clinically relevant individual characteristics, except for suggestion of effect modification by BMI (*P* for interaction = 0.026) and smoking (*P* for interaction = 0.053) (**Figure 2**). In separate analyses for CHD and stroke, the approximately log-linear inverse association of PON-1 with CHD in analyses adjusted for several established risk factors and potential confounders was somewhat attenuated on additional adjustment for HDL-C (**Table 3**; **Figure 3**) and there was no statistically significant evidence of an association with stroke (**Table 3**). To put the strength of the association of PON-1 activity with CVD risk into context, direct comparisons were made to associations of HDL-C with CVD, CHD, and stroke outcomes. Serum HDL-C concentrations were inversely and independently associated with CVD and CHD outcomes. The associations were also independent of serum PON-1

activity. There was no evidence of an association with stroke outcomes (**Table 5**). Similar findings were found in analysis with Apo A-I instead of HDL-C (data not shown).

3.3. Paraoxonase-1 Activity and CVD Risk Prediction

Addition of HDL-C measurements to a prognostic model for first cardiovascular events that included data on age, sex, smoking status, blood pressure, and total cholesterol increased the C-index by 0.0034 (0.0003 to 0.0064; *P*=0.02). The C-index increased non-significantly by 0.0003 (-0.0005 to 0.0011; *P*=0.49) on further addition of information on serum PON-1 to this model, yielding a NRI of 0.75% (-0.02 to 1.52%; *P*=0.06) for the predicted 10-year CVD risk categories (**Appendix Supplement 5**). There were no differences in the C-index according to clinically relevant individual level characteristics (data not shown). There was no significant improvement in risk discrimination and reclassification when RR risk score components were used (**Appendix Supplement 6**).

Literature-based meta-analyses

3.4. Literature Search, Characteristics, and Quality of Eligible Studies

The initial search identified 1986 potentially relevant citations. After screening and detailed assessment, 5 articles based on 5 unique population-based studies were included (**Figure 4**). Therefore we meta-analyzed estimates from 6 studies (including the current study) involving a total of 15 064 unique participants with 2958 incident CVD outcomes reporting on the association between PON-1 activity and CVD risk. Detailed characteristics of these studies and quality assessment have been summarized in **Appendix Supplements 7-8**

3.5. Paraoxonase-1 Activity and CVD Risk in Pooled Analysis

Of the six studies, three studies (including the current study) provided risk estimates for PON-1 as measured by its arylesterase activity (using phenyl acetate as substrate)[17, 20] and the other three reported estimates for PON-1 as measured by paraoxonase activity (using paraoxon as substrate).[15, 16, 21] Both assay

systems were used in two studies[17, 20] (**Appendix Supplement 7**). Including the current study, the overall pooled unadjusted or age-adjusted RR (95% CI) for any CVD event per 1 SD higher PON-1 values was 0.87 (0.80 to 0.96; *P*=0.005). There was substantial heterogeneity between studies (I^2>70%), but this could not be explored in detail because of the limited number of studies. However, exclusion of any single study at a time from the meta-analysis had minimal effect on the pooled RRs, which ranged from 0.85 (0.77-0.94) to 0.91 (0.84-0.98). The combined RR excluding the single largest study was 0.86 (0.76-0.97), very similar to the main finding. The pooled unadjusted or age-adjusted RRs were 0.77 (0.65 to 0.93 *P*=0.006) and 0.95 (0.91 to 1.00; *P*=0.041) for PON-1 measured as its arylesterase activity and paraoxonase activity respectively (*P* for meta-regression = 0.023). The overall pooled RR in analyses typically adjusted for several established CVD risk factors (including HDL-C) was 0.95 (0.90 to 1.02; *P*=0.138) and the corresponding RRs were 0.90 (0.75 to 1.07; *P*=0.223) and 0.98 (0.93 to 1.03; *P*=0.368) for PON-1 measured as its arylesterase activity and paraoxonase activity respectively (*P* for meta-regression = 0.495) (**Figure 5**). The pooled corresponding fully adjusted RR for CHD (5 studies; 14,032 participants and 2,541 CHD cases) was 0.98 (0.94 to 1.02; *P*=0.265). There was no evidence of publication bias (*P*-value for Egger test=0.201).

4. Discussion

4.1. Summary of main findings

Our findings in the PREVEND cohort comprising individuals without a history of CVD at baseline, provide several important findings that have not been previously addressed. PON-1 activity was correlated with several cardiovascular risk markers and more strongly with HDL-C and Apo A-1. In analyses adjusted for established CVD risk factors, we observed an approximately log-linear association of PON-1 activity with risk of CVD. The association remained consistent on further adjustment for a comprehensive panel of potential confounders, but was attenuated after single adjustment for HDL-C in both analyses initially only adjusted for age and sex and further for established and potential risk factors, consistent with previous reports.[15, 16] In contrast, the association between HDL-C and CVD was

independent of PON-1 in analyses conducted in the same participants, which suggests HDL-C as a stronger risk indicator than PON-1. Mackness and colleagues[16] have demonstrated some similar findings. However, additional analyses in their study suggested PON-1 as a stronger risk indicator for CVD than HDL-C in men with pre-existing CHD. Indeed, it has been shown that populations with pre-existing cardiometabolic disease have marked reductions in serum PON-1 activity without significant lower concentrations of HDL-C.[9] The associations remained generally similar across several clinically relevant subgroups, except for the suggestion of effect modification by BMI and smoking status; characterized by an inverse association in individuals with a normal BMI category compared to a statistically non-significant association in individuals with higher BMIs and an inverse association in smokers compared to a statistically non-significant association in non-smokers. Given the multiple statistical tests for interaction conducted, results of the subgroup analyses should be interpreted with caution and may require replication in further studies. The associations were similar in analyses that excluded first two years of follow-up, participants on regular antihypertensive medication, or participants on regular statin medication. Similar associations were observed for CHD risk. However, there was no statistically significant evidence of an association with stroke, consistent with the findings for HDL-C in the same set of participants. Uncertainties still exist for the association between HDL-C and stroke, particularly for stroke subtypes;[49] however, there is a possibility that the current findings may be attributed to the low event rate for stroke in our study. There is also a possibility that the null findings for stroke could be due to the combined endpoints of different stroke subtypes, with different aetiologies. We were however unable to conduct separate analyses for the subtypes of stroke because of the very low event rates in each endpoint. Given that no previous study has evaluated the association of PON-1 activity and stroke-specific outcomes, larger-scale studies are warranted to confirm or refute these findings. Results from assessments of improvements in measures of risk discrimination and reclassification, indicate that additional information on PON-1 measurements provides no significant improvement in CVD risk prediction.

Pooled findings from the systematic meta-analysis of the six available studies showed that PON-1 was inversely associated with CVD risk in unadjusted or age-adjusted analysis. In the PREVEND study, we measured PON-1 activity as its arylesterase activity (with phenyl acetate as substrate) which was driven by previous documentation that arylesterase activity is less variable between subjects compared to its activity towards paraoxon, and is closely correlated with PON-1 mass.[7, 25] In view of this, we also grouped the studies included in the meta-analysis according to the type of PON-1 activity assay. Indeed, in unadjusted or age-adjusted analysis, the risk estimate for PON-1 as arylesterase activity was stronger than that of PON-1 as paraoxonase activity. Further adjustment for established risk factors (including HDL-C) showed a less robust association of PON-1 activity with incident CVD, confirming the suggestion that the inverse association between PON-1 activity and CVD risk is partly dependent on HDL-C concentrations.

4.2. Possible explanations for findings

Our results indicate that PON-1 is inversely associated with CVD (including CHD) risk, independent of several established risk factors except for HDL-C. Though the majority of previous studies conducted in the general population have failed to demonstrate an inverse independent association,[14-16, 21] the dependency of the association on HDL-C has previously been reported.[15, 16] The attenuation of the association on adjustment for HDL-C is expected, given that PON-1 is an important component of HDL,[50-53] which acts as its carrier and site of action. Plausible biologic mechanisms by which PON-1 contributes to reduced CVD risk is through the inhibition of LDL oxidation,[54] which is believed to play a central role in the development of atherosclerosis.[55] PON-1 is also involved in the destruction of pro-inflammatory oxidised phospholipids (involved in the development and progression of atherosclerotic lesions[56]) which are present in oxidised LDLs,[57] decreases the lipid peroxide content in arterial lesions, could promote cholesterol efflux, and also prevents HDL from being oxidized.[58]

4.3. Implications of findings

The findings of our study may have several implications. Irrespective of whether addition of information on PON-1 to conventional risk factors provides no improvement in CVD risk prediction, its potential utility for CVD risk prevention and treatment has been debated. Given the potential role of PON-1 in the detoxification of lipid peroxides, the overall evidence supports the possibility that elevated PON-1 activity might protect from CVD events in the general population. Conversely, individuals with low PON-1 activity may have a greater risk of developing atherosclerotic CVD. Indeed, significantly higher rates of atherosclerosis have been demonstrated in genetic rodent models deficient in PON-1 and who were fed a high cholesterol diet.[59] Futhermore, PON-1 overexpression inhibits atherosclerosis development in mice.[60] Therefore, there remains a possibility that dietary or pharmacological interventions that preserve or increase PON-1 activity might form the basis of CVD prevention and treatment strategies. Dietary consumption of red wine flavonoids has been shown to preserve serum PON-1 activity in atherosclerotic ApoE deficient mice[61] and pomegranate juice (rich in flavonoids) has been shown to cause significant elevation in PON-1 activity in human volunteers and also substantially reduce the size of atherosclerotic lesions in ApoE deficient mice.[62] Rigorous intervention studies in humans are warranted to investigate these potential therapeutic implications of PON-1.

4.4. Strengths and limitations

The strengths of the current study deserve consideration. Our study included analyses of primary data as well as a meta-analysis of all available published prospective cohorts in a single comprehensive investigation. It provides the most comprehensive investigation, to date, of the observational epidemiological association between PON-1 activity and CVD risk. PREVEND participants were selected from a sample that was nationally representative, were well characterized, involved a high response and follow-up rates and had been prospectively monitored using established hospital admissions databases and disease endpoints. The PREVEND database had measurements on a comprehensive panel of lifestyle and biological markers that permitted adequate adjustment for potential confounders, hence enabling

reliable assessments of the magnitude of associations. The current study had higher statistical power to examine the associations in greater detail, including comparison of the associations at different levels or categories of vascular risk factors and sensitivity analyses to supplement and contextualize the principal findings. Individuals with prevalent CVD at baseline were excluded from the analyses, therefore limiting any possibilities of reverse-causation bias. The PREVEND study participants were suitable for risk prediction analyses because they were followed for a long duration and had complete set of measurements used in standard risk prediction scores. We also carried out several sensitivity analyses to avoid potential bias due to undiagnosed disease at baseline. Despite these, residual biases (i.e., reverse causation) may still remain because of the possibility of subclinical or undetected prevalent disease. On the other hand, the present analysis also has some limitations. In the PREVEND cohort, given the nature of the observational data available, there is a possibility of potential residual confounding due to errors in measurements of risk marker and other unmeasured confounders. Measurements of PON-1 in the PREVEND study involved prolonged serum storage, which could have resulted in under-estimation of the associations due to sample degradation. However, PON-1 is known to show no loss of activity by prolonged storage in frozen samples (-70°C) and on repeated freeze-thawing over two years, with an approximate loss in activity by 20% over seven years.[63] In our analyses, we were unable to show the extent to which baseline PON-1 measurements may underestimate the association between long-term PON-1 activity and CVD outcomes, because of the absence of repeat measurements on PON-1. The analyses were based on a predominantly white-European population, therefore the findings may not be generalisable to individuals of different ethnicities, given that interpopulation differences in serum paraoxonase specific activity (which are independent of differences in serum lipids and lipoprotein concentrations) have been demonstrated in healthy populations.[64] There was substantial heterogeneity between the contributing studies for the meta-analysis and the limited number of studies precluded detailed exploration of heterogeneity; however, our results remained robust in several sensitivity analyses.

5. Conclusions

In conclusion, there is an approximately log-linear inverse association between PON-1 activity and CVD risk, which is partly dependent on HDL-C levels. In the general population, adding information on PON-1 to conventional CVD risk factors provides no significant improvement in CVD risk prediction.

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Conflicts of interest

None

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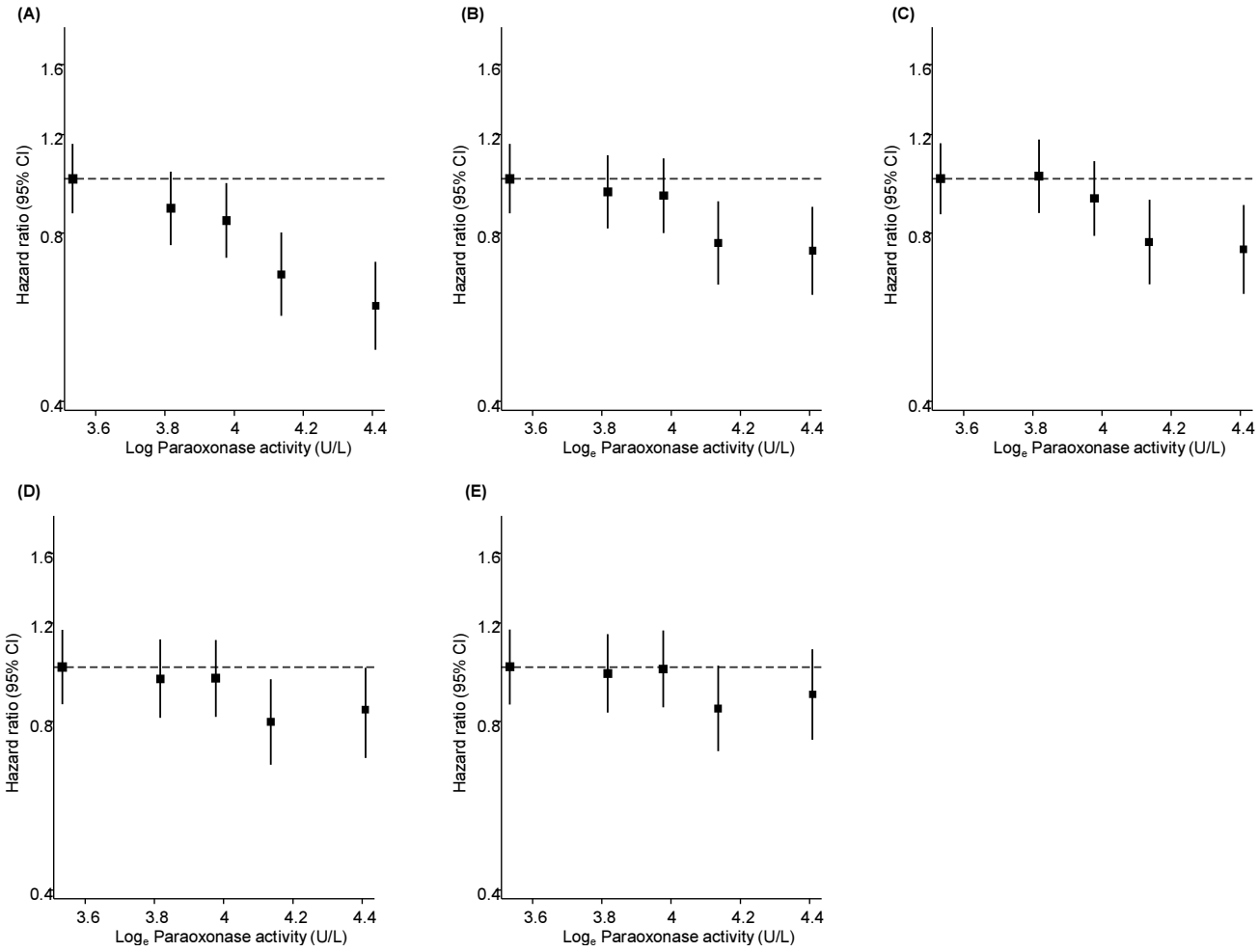
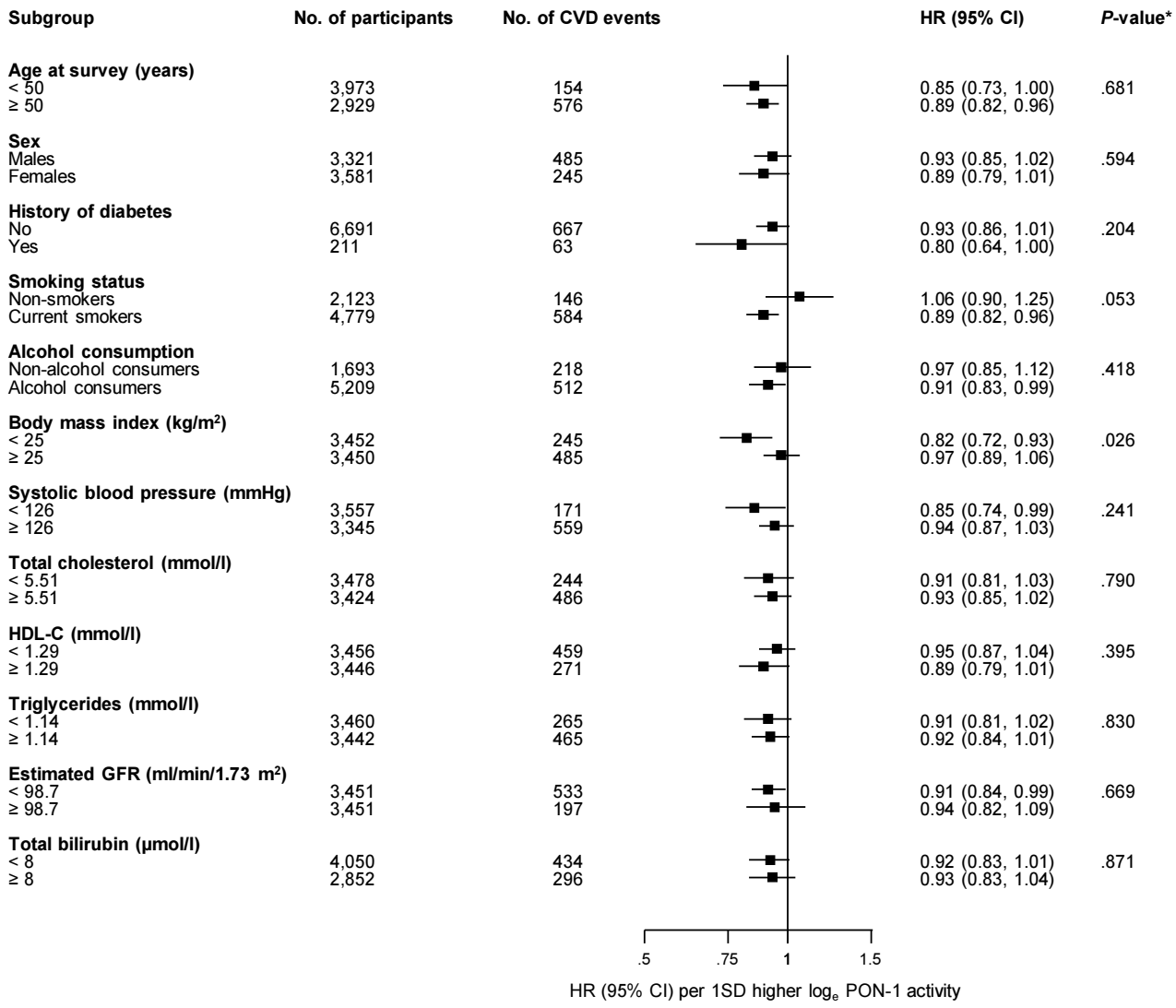
Figure 1. Hazard ratios for incident cardiovascular disease by baseline values of $\log_e$ paraoxonase-1 using floating absolute risks



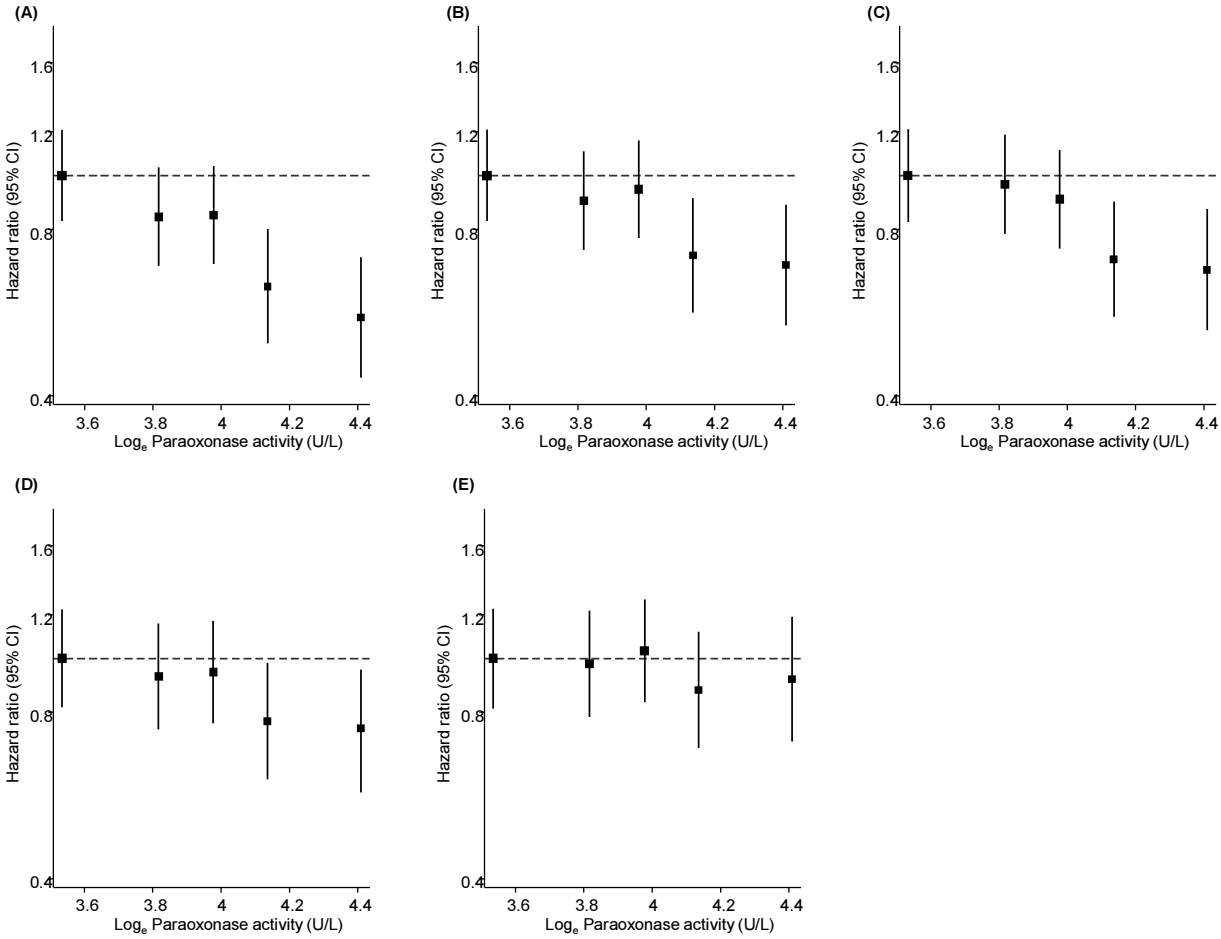
A, Unadjusted; **B**, adjusted for age and sex; **C**, adjustment as in B plus smoking status, history of diabetes, systolic blood pressure, and total cholesterol; **D**, adjustment as in C plus body mass index, alcohol consumption, glucose, $\log_e$ triglycerides, estimated glomerular filtration rate (as calculated using the Chronic Kidney Disease Epidemiology Collaboration combined creatinine-cystatin C equation), $\log_e$ urine albumin excretion, and $\log_e$ total bilirubin; **E**, adjustment as in D plus high-density lipoprotein cholesterol; the size of the box is proportional to the inverse of the variance of hazard ratio

Figure 2. Hazard ratios for cardiovascular disease per 1 SD increase in baseline $\log_e$ paraoxonase-1 values, by several participant level characteristics



Hazard ratios were adjusted for age, sex, smoking status, history of diabetes, systolic blood pressure, and total cholesterol; CI, confidence interval (bars); CVD, cardiovascular disease; Estimated GFR, glomerular filtration rate (as calculated using the Chronic Kidney Disease Epidemiology Collaboration combined creatinine-cystatin C equation); HDL-C, high-density lipoprotein cholesterol; HR, hazard ratio; SD, standard deviation; *, *P*-value for interaction; cut-offs used for body mass index, systolic blood pressure, total cholesterol, HDL-C, triglycerides, estimated GFR, and total bilirubin are median values.

Figure 3. Hazard ratios for incident coronary heart disease by baseline values of $\log_e$ paraoxonase-1 activity using floating absolute risks



A, Unadjusted; **B**, adjusted for age and sex; **C**, adjustment as in B plus smoking status, history of diabetes, systolic blood pressure, and total cholesterol; **D**, adjustment as in C plus body mass index, alcohol consumption, glucose, $\log_e$ triglycerides, estimated glomerular filtration rate (as calculated using the Chronic Kidney Disease Epidemiology Collaboration combined creatinine-cystatin C equation), $\log_e$ urine albumin excretion, and $\log_e$ total bilirubin; **E**, adjustment as in D plus high-density lipoprotein cholesterol; the size of the box is proportional to the inverse of the variance of hazard ratio

Figure 4. Selection of studies included in the meta-analysis

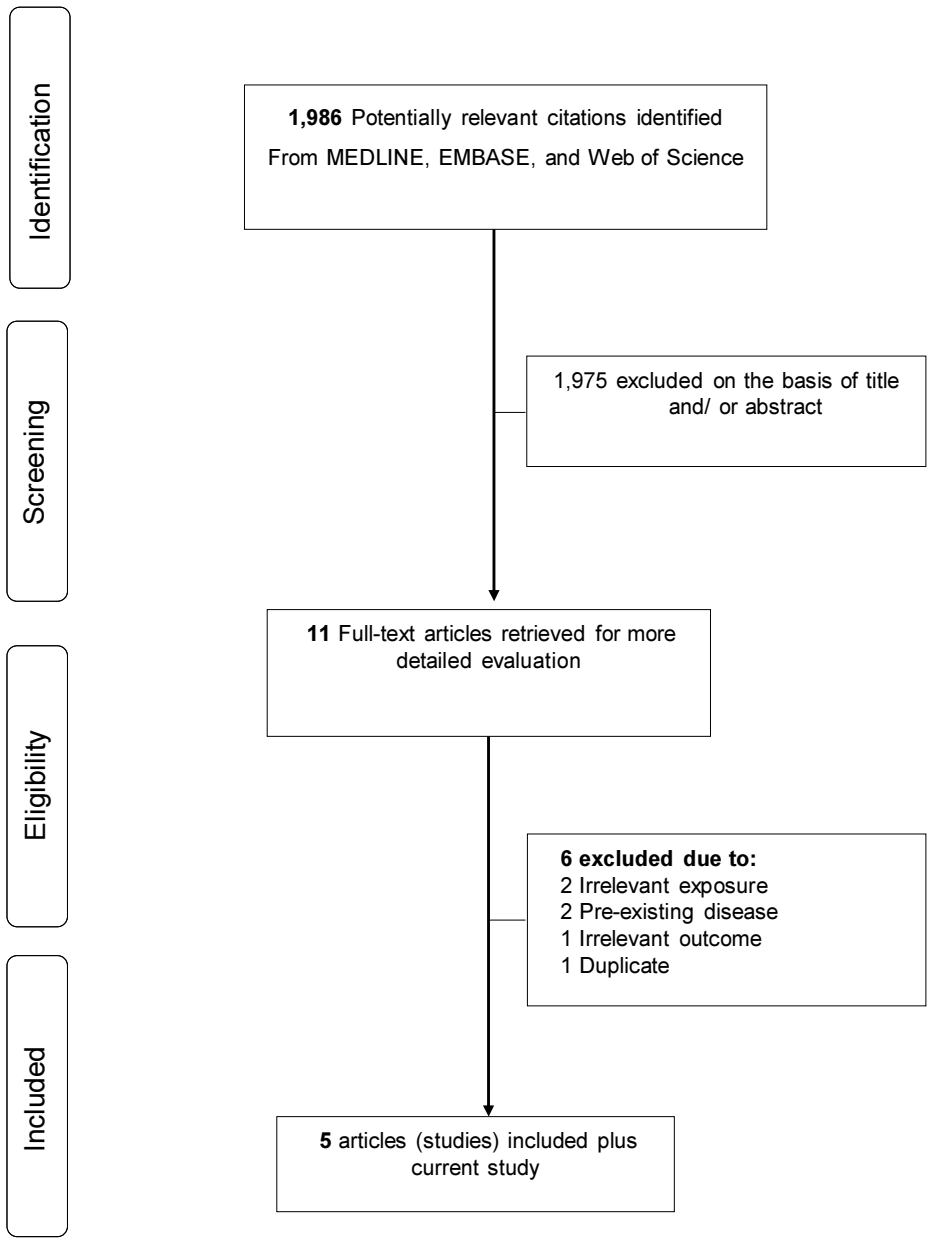
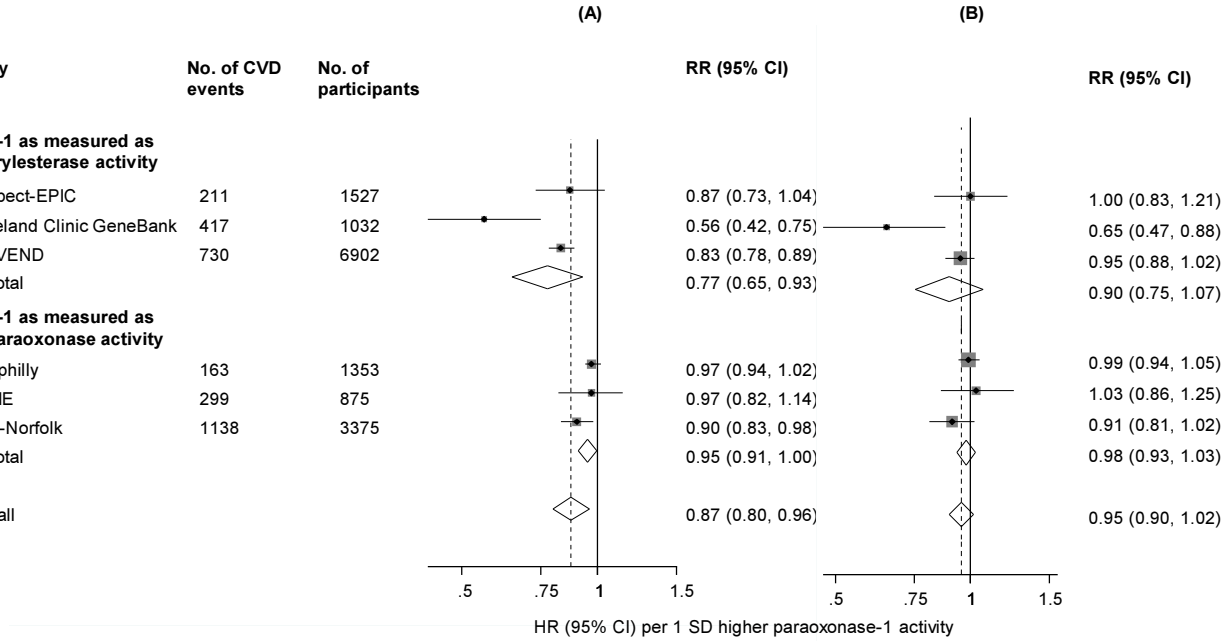


Figure 5. Relative risks for cardiovascular disease per 1 SD higher baseline paraoxonase-1 in published prospective studies



Study acronyms are provided in **Appendix Supplement 1**. The summary estimates were calculated using random effects models; **A**, results were unadjusted or adjusted for age only; **B**, results were adjusted for established cardiovascular risk factors including high-density lipoprotein cholesterol; Size of data markers are proportional to the inverse of the variance of the relative risk; CI, confidence interval (bars); CVD, cardiovascular disease; RR, relative risk; SD, standard deviation

Table 1. Baseline participant characteristics

	Overall (N=6,902) Mean (SD) or median (IQR) or n (%)	Without CVD (N=6,172) Mean (SD) median (IQR) or n (%)	With CVD (N=730) Mean (SD) or median (IQR) or n (%)
Paraoxonase-1 (U/L)	53.5 (43.5-65.3)	53.8 (43.7-65.7)	50.7 (41.3-61.6)
Questionnaire			
Males	3,321 (48.1)	2,836 (46.0)	485 (66.4)
Age at survey (years)	48 (12)	47 (12)	59 (10)
History of diabetes	211 (3.1)	148 (2.4)	63 (8.6)
Current smokers	4,779 (69.2)	4,195 (68.0)	584 (80.0)
Alcohol consumption			
None	1,693 (24.5)	1,475 (23.9)	218 (29.9)
1-4 drinks per month	1,073 (15.6)	974 (15.8)	99 (13.6)
2-7 drinks per week	2,387 (34.6)	2,154 (34.9)	233 (31.9)
1-3 drinks per day	1,389 (20.1)	1,251 (20.3)	138 (18.9)
≥ 4 drinks per day	360 (5.2)	318 (5.2)	42 (5.8)
History of hypertension	690 (10.0)	514 (8.3)	176 (24.1)
Regular use of anti-hypertensive medication	753 (10.9)	566 (9.2)	187 (25.6)
Regular use of diabetic medication	67 (1.0)	42 (0.7)	25 (3.4)
Regular use of lipid-lowering medication	173 (2.5)	121 (2.0)	52 (7.1)
Physical measurements			
BMI (kg/m^2)	26 (4)	26 (4)	27 (4)
WHR	0.88 (0.09)	0.87 (0.09)	0.93 (0.09)
SBP (mmHg)	128 (19)	126 (19)	144 (23)
DBP (mmHg)	74 (10)	73 (9)	80 (10)
Lipid markers			
Total cholesterol (mmol/l)	5.63 (1.12)	5.57 (1.11)	6.07 (1.04)
HDL-C (mmol/l)	1.34 (0.40)	1.35 (0.40)	1.21 (0.38)
Triglycerides (mmol/l)	1.13 (0.83-1.66)	1.11 (0.81-1.62)	1.40 (0.99-2.04)
Apo A-I (g/l)	1.39 (0.30)	1.39 (0.30)	1.34 (0.29)
Apo B (g/l)	1.02 (0.31)	1.01 (0.31)	1.15 (0.30)
Metabolic, inflammatory, and liver markers			
hsCRP (mg/l)	1.21 (0.53-2.82)	1.13 (0.50-2.68)	2.10 (1.00-4.70)
Glucose (mmol/l)	4.82 (1.09)	4.76 (0.95)	5.29 (1.82)
Creatinine (µmol/l)	82 (73-91)	81 (73-90)	88 (77-99)
Cystatine C (mg/dl)	0.79 (0.19)	0.78 (0.19)	0.89 (0.23)
eGFR (ml/min/1.73 m^2)	100.8 (39.0)	102.4 (39.7)	87.7 (29.6)
Total bilirubin (µmol/l)	7 (5-9)	7 (5-9)	7 (5-9)
UAE (mg/24 hours)	9.19 (6.25-16.59)	8.85 (6.14-15.26)	14.27 (8.13-38.70)
Framingham 2008 10-year CVD risk			
0%-< 10%	4,167 (60.4)	3,694 (59.9)	473 (64.8)
10%-< 20%	814 (11.8)	781 (12.7)	33 (4.5)
≥ 20%	1,921 (27.8)	1,697 (27.5)	224 (30.7)
RRS 10-year CVD risk			
0%-< 10%	6,225 (90.2)	5,749 (93.2)	476 (65.2)
10%-< 20%	510 (7.4)	340 (5.5)	170 (23.3)
≥ 20%	167 (2.4)	83 (1.3)	84 (11.5)

Apo A-I, apolipoprotein A-I; Apo B, apolipoprotein B; BMI, body mass index; CVD, cardiovascular disease; hsCRP, high sensitivity C-reactive protein; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate (as calculated using the Chronic Kidney Disease Epidemiology Collaboration combined creatinine-cystatin C equation); HDL-C, high-density lipoprotein cholesterol; IQR, interquartile range; RRS, Reynolds Risk Score; SBP, systolic blood pressure; UAE, urinary albumin excretion; WHR, waist-to-hip ratio

Table 2. Cross-sectional correlates of paraoxonase-1 activity

	Pearson correlation r (95% CI)†	Percentage difference (95% CI) in paraoxonase-1 values per 1 SD higher or compared to reference category of correlate‡
Log_e Paraoxonase-1 (U/L)	-	-
Questionnaire		
Sex		
Female	-	Ref
Male	-	-4% (-5, -3)***
Age at survey (years)	-0.08 (-0.10, -0.06)***	-2% (-3, -2)***
History of diabetes		
No	-	Ref
Yes	-	-6% (-10, -2)*
Smoking status		
Non-smokers	-	Ref
Current smokers	-	-1% (-3, 0)
Alcohol consumption		
Non-consumers	-	Ref
Current consumers	-	6% (4, 8)***
History of hypertension		
No	-	Ref
Yes	-	-2% (-4, 1)
Regular use of anti-hypertensive medication		
No	-	Ref
Yes	-	-2% (-5, 0)
Regular use of diabetic medication		
No	-	Ref
Yes	-	-14% (-20, -7)***
Regular use of lipid-lowering medication		
No	-	Ref
Yes	-	0% (-4, 5)
Physical measurements		
BMI (kg/m^2)	0.00 (-0.02, 0.02)	0% (-1, 1)
WHR	-0.02 (-0.04, 0.01)***	-1% (-2, 0)
SBP (mmHg)	0.05 (0.02, 0.07)	2% (1, 3)***
DBP (mmHg)	0.05 (0.03, 0.08)	2% (1, 3)***
Lipid markers		
Total cholesterol (mmol/l)	0.12 (0.10, 0.14)***	4% (3, 5)***
HDL-C (mmol/l)	0.18 (0.16, 0.20)***	6% (6, 7)***
Log triglycerides (mmol/l)	0.08 (0.06, 0.10)**	3% (2, 3)***
Apo A-I (g/l)	0.17 (0.15, 0.20)***	6% (5, 7)***
Apo B (g/l)	0.04 (0.02, 0.07)	1% (1, 2)**
Metabolic and inflammatory markers		
Log_e hsCRP (mg/l)	-0.03 (-0.05, -0.01)**	-1% (-2, -0)*
Glucose (mmol/l)	-0.04 (-0.07, -0.02)***	-1% (-2, -1)**
Log_e creatinine (μmol/l)	0.03 (0.00, 0.05)*	1% (0, 2)*
Cystatine C (mg/dl)	-0.05 (-0.08, -0.03)***	-2% (-3, -1)***
eGFR (ml/min/1.73 m^2)	0.02 (-0.00, 0.04)	1% (-0, 1)**
Log_e total bilirubin (μmol/l)	0.01 (-0.02, 0.03)	0% (-1, 1)
Log_e UAE (mg/24 hours)	0.02 (-0.00, 0.04)	1% (-0, 1)
Framingham 2008 10-year CVD risk		
0%-< 20%	-	Ref
≥ 20%	-	2% (0, 5)*
RRS 10-year CVD risk		
0%-< 20%	-	Ref
≥ 20%	-	-6% (-11, -1)*

Apo A-I, apolipoprotein A-I; Apo B, apolipoprotein B; BMI, body mass index; hsCRP, high sensitivity C-reactive protein; CVD, cardiovascular disease; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate (as calculated using the Chronic Kidney Disease Epidemiology Collaboration combined creatinine-cystatin C equation); HDL-C, high-density lipoprotein cholesterol; Ref, reference; RRS, Reynolds Risk Score; SD, standard deviation; SBP, systolic blood pressure; UAE, urinary albumin excretion; WHR, waist-to-hip ratio

Asterisks indicate the level of statistical significance: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; †, Pearson correlation coefficients between $\log_e$ paraoxonase-1 and the row variables; ‡, Percentage change in paraoxonase-1 values per 1 SD increase in the row variable (or for categorical variables, the percentage difference in mean paraoxonase-1 values for the category versus the reference) adjusted for age and sex;

Table 3. Associations of serum paraoxonase-1 activity with incident CVD, CHD and stroke

Models	CVD		CHD		Stroke	
	Hazard ratio (95% CI) 6,902 participants and 730 cases	*P*-value	Hazard ratio (95% CI) 6,902 participants and 390 cases	*P*-value	Hazard ratio (95% CI) 6,902 participants and 153 cases	*P*-value
Model 1	0.83 (0.78 to 0.89)	< 0.001	0.82 (0.75 to 0.90)	< 0.001	0.79 (0.67 to 0.92)	0.002
Model 2	0.90 (0.84 to 0.97)	0.004	0.89 (0.81 to 0.98)	0.016	0.87 (0.74 to 1.01)	0.068
Model 3	0.92 (0.85 to 0.99)	0.020	0.90 (0.82 to 0.99)	0.029	0.89 (0.76 to 1.04)	0.142
Model 4	0.93 (0.86 to 0.99)	0.037	0.91 (0.83 to 1.00)	0.058	0.91 (0.78 to 1.07)	0.257
Model 5	0.95 (0.88 to 1.02)	0.153	0.96 (0.87 to 1.07)	0.471	0.89 (0.76 to 1.05)	0.158

Hazard ratios are reported per 1 standard deviation increase in $\log_e$ paraoxonase-1 activity; CHD, coronary heart disease; CVD, cardiovascular disease
Model 1: Unadjusted
Model 2: Age and sex
Model 3: Age, sex, smoking status, history of diabetes, systolic blood pressure, and total cholesterol
Model 4: Model 3 plus body mass index, alcohol consumption, fasting glucose, $\log_e$ triglycerides, estimated glomerular filtration rate (as calculated using the Chronic Kidney Disease Epidemiology Collaboration combined creatinine-cystatin C equation), $\log_e$ urinary albumin excretion, and $\log_e$ total bilirubin
Model 5: Model 4 plus high-density lipoprotein cholesterol

Table 4: Hazard ratios for cardiovascular disease with first two years of follow-up, participants on regular antihypertensive medication, and participants on regular lipid-lowering medication excluded

	Events/Total	**Model 1**		**Model 2**		**Model 3**		**Model 4**		**Model 5**	
		Hazard ratio (95% CI)	*P*-value	Hazard ratio (95% CI)	*P*-value	Hazard ratio (95% CI)	*P*-value	Hazard ratio (95% CI)	*P*-value	Hazard ratio (95% CI)	*P*-value
Excluding the first two years of follow-up	720 / 6,891	0.83 (0.78 to 0.89)	< 0.001	0.90 (0.84 to 0.96)	0.003	0.92 (0.85 to 0.98)	0.017	0.92 (0.86 to 0.99)	0.033	0.94 (0.88 to 1.02)	0.136
Excluding participants on regular antihypertensive medication	536 / 6,149	0.84 (0.77 to 0.91)	< 0.001	0.89 (0.82 to 0.97)	0.007	0.90 (0.83 to 0.97)	0.010	0.90 (0.83 to 0.98)	0.018	0.93 (0.86 to 1.02)	0.117
Excluding participants on regular lipid-lowering medication	670 / 6,729	0.83 (0.76 to 0.89)	< 0.001	0.90 (0.83 to 0.96)	0.003	0.91 (0.84 to 0.98)	0.014	0.92 (0.85 to 0.99)	0.024	0.94 (0.87 to 1.02)	0.125

Hazard ratios are reported per 1 standard deviation increase in $\log_e$ paraoxonase-1 activity
Model 1: Unadjusted
Model 2: Age and sex
Model 3: Age, sex, smoking status, history of diabetes, systolic blood pressure, and total cholesterol
Model 4: Model 3 plus body mass index, alcohol consumption, fasting glucose, $\log_e$ triglycerides, estimated glomerular filtration rate (as calculated using the Chronic Kidney Disease Epidemiology Collaboration combined creatinine-cystatin C equation), $\log_e$ urinary albumin excretion, and $\log_e$ total bilirubin
Model 5: Model 4 plus high-density lipoprotein cholesterol

Table 5. Associations of high-density lipoprotein cholesterol with incident CVD, CHD and stroke

Models	CVD		CHD		Stroke	
	Hazard ratio (95% CI)	*P*-value	Hazard ratio (95% CI)	*P*-value	Hazard ratio (95% CI)	*P*-value
	6,902 participants and 730 cases		6,902 participants and 390 cases		6,902 participants and 153 cases	
Model 1	0.68 (0.62 to 0.73)	< 0.001	0.56 (0.49 to 0.63)	< 0.001	0.90 (0.76 to 1.06)	0.205
Model 2	0.69 (0.64 to 0.76)	< 0.001	0.57 (0.50 to 0.64)	< 0.001	0.95 (0.80 to 1.12)	0.507
Model 3	0.83 (0.76 to 0.91)	< 0.001	0.71 (0.62 to 0.80)	< 0.001	1.12 (0.94 to 1.34)	0.202
Model 4	0.84 (0.76 to 0.94)	0.002	0.67 (0.57 to 0.78)	< 0.001	1.13 (0.91 to 1.39)	0.265
Model 5	0.86 (0.77 to 0.96)	0.006	0.68 (0.58 to 0.80)	< 0.001	1.16 (0.94 to 1.44)	0.161

Hazard ratios are reported per 1 standard deviation increase in high-density lipoprotein cholesterol; CHD, coronary heart disease; CVD, cardiovascular disease
Model 1: Unadjusted
Model 2: Age and sex
Model 3: Age, sex, smoking status, history of diabetes, systolic blood pressure, and total cholesterol
Model 4: Model 3 plus body mass index, alcohol consumption, fasting glucose, $\log_e$ triglycerides, estimated glomerular filtration rate (as calculated using the Chronic Kidney Disease Epidemiology Collaboration combined creatinine-cystatin C equation), $\log_e$ urinary albumin excretion, and $\log_e$ total bilirubin
Model 5: Model 4 plus $\log_e$ paraoxonase-1