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Serum paraoxonase-1 activity is inversely related to free thyroxine in euthyroid subjects: The PREVEND Cohort Study

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Abstract

Background: Low-normal thyroid function within the euthyroid range has been suggested to enhance atherosclerosis susceptibility. Paraoxonase-1 (PON-1), may protect against atherosclerotic cardiovascular disease development by attenuating oxidative stress. We evaluated relationships of PON-1 with TSH, free T_4, free T_3, lipids and apolipoprotein (apo)A-I in euthyroid subjects, and assessed whether such relationships are modified in the context of the metabolic syndrome (MetS).

Materials and Methods: Serum PON-1 activity (arylesterase activity), TSH, free T_4, free T_3, lipids and apoA-I were measured in 2206 euthyroid subjects (aged 28 to75 years; 1138 men (age 49 ± 13 years) and 1068 women (age 46 ± 12 years), recruited from the general population (PREVEND cohort).

Results: In age- and sex-adjusted analysis, PON-1 activity (divided into tertiles) was positively related to TSH (β=-0.045, *P*=0.036) and inversely to free T_4 (β=-0.042, *P*=0.050), but not to free T_3 (β=-0.027, *P*=0.20). PON-1 activity was positively related to total cholesterol, non-HDL cholesterol and triglycerides, as well as to HDL cholesterol and apoA-I (*P*<0.01 to <0.001). The inverse relationship of PON-1 activity with free T_4 remained present after adjustment for lipids and other potential confounders (β=-0.066, *P*=0.002), but the positive relationship with TSH lost significance (β=0.034, *P*=0.11). The inverse relationship of PON-1 activity with free T_4 was not different in subjects with vs. without MetS (*P*=0.94), nor modified by the presence of its individual components (*P*≥0.22 for each).

Conclusions: Serum PON-1 activity is inversely associated with free T_4 in euthyroid subjects, suggesting that low-normal thyroid function may affect PON-1 regulation.

Introduction

Low-normal thyroid function, as indicated by a higher thyroid stimulating hormone (TSH) or lower thyroid hormone levels within the euthyroid reference range, may contribute to the development of atherosclerotic cardiovascular disease (CVD) [1-4]. The mechanisms responsible for the association of (subclinical) atherosclerosis with low-normal thyroid function are still incompletely

understood. Low-normal thyroid function is associated with a modest increase in plasma total cholesterol, low density lipoprotein (LDL) cholesterol and triglycerides [4-7]. Low-normal thyroid function may also attenuate high density lipoproteins (HDL) function, such as its ability to protect against oxidative stress [4,8].

Accumulating evidence supports the hypothesis that systemic oxidative stress, as at least in part reflected by enhanced oxidative modification of LDL, may contribute to the development of atherosclerosis [9-11]. In this context, it is relevant that LDL oxidation *in vitro* is exaggerated in both hypothyroidism and hyperthyroidism [12,13]. Moreover, increased circulating oxidized LDL levels have been demonstrated in euthyroid subjects with higher TSH levels [14]. Paraoxonase-1 (PON-1) is a HDL-associated hydrolytic enzyme with important anti-oxidative properties [15]. PON-1 hydrolyzes lipid peroxides, thereby preventing their accumulation in LDL particles [15,16]. Studies in rodent models and humans have suggested that the anti-atherogenic effects of the HDL fraction are to a considerable extent attributable to PON-1 activity [16]. PON-1 activity has been shown to be impaired in patients with metabolic syndrome (MetS), type 2 diabetes mellitus (T2DM) and hypercholesterolemia [15,17-19]. Furthermore, lower serum PON-1 activity may predict increased risk of coronary events [20-21] , although the association of PON-1 activity with increased CVD risk was not independent of HDL cholesterol [22].

The effect of thyroid dysfunction on serum PON-1 activity has only been determined in a limited number of studies [23-27]. Remarkably, PON-1 activity was found to be impaired in both hypothyroidism and hyperthyroidism [23]. In addition, PON-1 activity was decreased in (subclinical) hypothyroidism in some [24, 25], but not in other studies [26-28]. No data are currently available concerning the association of serum PON-1 activity in the context of variations in thyroid hormone levels within the euthyroid range.

Against this background, we performed the present study to evaluate the relationships of serum PON-1 activity with thyroid function in euthyroid subjects. In view of decreased PON-1 activity in MetS [15] and potential alterations in thyroid hormones in MetS [4], we also determined the extent to which such a relationship is modified by the presence of MetS and its individual components.

Subjects and Methods

Subjects

Reporting of the study conforms to STROBE (STrengthening the Reporting of OBservational studies in Epidemiology) statement along with references to STROBE statement and the broader EQUATOR guidelines [29].

The study population consisted of a random subset of participants of the PREVEND (Prevention of Renal and Vascular End Stage Disease) cohort, aged 28–75 years, living in the city of Groningen, The Netherlands. Participants were predominantly of Caucasian origin (94.2%). The protocol of this study has been described in detail elsewhere [30,31]. The local medical ethical committee approved the study; all participants gave written informed consent. For the current analysis, we excluded subjects not being euthyroid, subjects using thyroid hormones, anti-thyroid drugs, amiodarone and lithium carbonate. Euthyroidism was defined as TSH, free T_4 and free T_3 levels each within the respective reference range as provided by the manufacturer (see Laboratory Analyses). We additionally excluded subjects with positive anti-thyroid peroxidase autoantibodies (cut-off value: see Laboratory Analyses). Information on self-reported medication use was combined with information from a pharmacy-dispensing registry, which has complete information on drug of >95% of subjects in the PREVEND study. Applying these selection criteria 2206 subjects were eligible for the current analyses. The presence of a self-reported history of myocardial infarction, percutaneous coronary intervention, coronary artery bypass surgery, stroke or the diagnosis of narrowing of one or both carotid arteries was defined as CVD. Type 2 diabetes mellitus (T2DM) was defined as a fasting serum glucose concentration >7.0 mmol/L, a nonfasting plasma glucose concentration >11.1 mmol/L, a self-report of a physician diagnosis, or the use of glucose-lowering drugs. In order to categorize subjects with the metabolic syndrome (MetS) 3 or more of the following criteria were required: waist circumference > 102 cm for men and > 88 cm for women, hypertension (blood pressure ≥ 130⁄85 mmHg or use of anti-hypertensive drugs), fasting plasma triglycerides ≥

1.70 mmol/L, fasting glucose ≥ 5.6 mmol/L (or use of glucose lowering drugs), and HDL cholesterol < 1.03 mmol/L for men and < 1.29 mmol/L for women applying NCEP ATPIII criteria [32]. Patient characteristics including age, sex, alcohol use, smoking status, body mass index (BMI), waist circumference, systolic and diastolic blood pressure were obtained. The participants were instructed to let venous blood samples being drawn after an overnight fast for measurement of, glucose, total cholesterol, high density lipoprotein (HDL) cholesterol, triglycerides, TSH, free T_4 and free T_3 and PON-1 activity. Urinary albumin excretion (UAE) was documented as the mean of two 24-hour urine collections. Body mass index (BMI) was defined as weight (kg) by height (m) squared. Waist circumference (WC) was measured on bare skin between the 10th rib and iliac crest. Alcohol consumption was recorded with one drink being assumed to contain 10 grams of alcohol. Smoking was categorized into current, former and never. Estimated glomerular filtration rate (eGFR) was calculated with the use of the combined creatinine-cystatin C-based Chronic Kidney Disease Epidemiology Collaboration equation [33].

Laboratory analyses

Heparinized plasma samples were stored at -80ºC until analyses. Sera were stored at -80ºC until analyses. Serum TSH (Architect; Abbott Laboratories, Abbott Park, IL, USA; reference range 0.35 - 4.94 mU/L), free T_4 (AxSYM; Abbott Laboratories, Abbott Park, IL, USA; reference range 9.14 – 23.81 pmol/L) and free T_3 (AxSYM; Abbott Laboratories, Abbott Park, IL, USA; reference range; 2.23 - 5.35 pmol/L) were measured by microparticle enzyme immunoassays. Anti-thyroid peroxidase autoantibodies were determined using commercially available automated enzyme linked immunoassays (Abbott Laboratories, Abbott Park, IL, USA; kit number 5F57). Anti-thyroid peroxidase autoantibodies were considered positive using a cut-off value as indicated by the supplier (≥12 kU/L).

Serum PON-1 enzymatic activity was measured as its arylesterase activity, i.e. as the rate of hydrolysis of phenyl acetate into phenol, as described [34]. The inter-assay CV was 8%. Arylesterase activity, measured with this assay, is positively correlated with PON-1 enzymatic activity toward paraoxon as well as with PON-1 mass [35].

Total serum cholesterol and plasma glucose were measured using Kodak Ektachem dry chemistry (Eastman Kodak, Rochester, NY, USA). Serum triglycerides were measured enzymatically. HDL cholesterol was measured with a homogeneous method (direct HDL, AEROSET system; Abbott Laboratories, Abbott Park, IL, USA; no. 7D67). Non-HDL cholesterol was calculated as the difference between total cholesterol and HDL cholesterol. Serum apoA-I was determined by nephelometry applying commercially available reagents for Dade Behring nephelometer systems (BN II; Dade Behring, Marburg, Germany; apoA-I test kit, code no. OUED).

Serum creatinine was measured by an enzymatic method on a RocheModular analyzer (Roche Diagnostics, Mannheim, Germany). Serum cystatin C was measured by Gentian Cystatin C Immunoassay (Gentian AS, Moss, Norway) on a Modular analyzer (Roche Diagnostics). Urinary albumin concentration was measured by nephelometry with a threshold of 2.3 mg/l (Dade Behring Diagnostic, Marburg, Germany).

2.4 Statistical analyses

Data analysis was performed using IBM SPSS software (version 23.0, SPSS Inc. Chicago, IL, USA). Normally distributed data are given as mean ± SD and non-parametrically distributed data are presented as median (interquartile range). Categorical variables are given as percentages. Differences in PON-1 activity between men and women were determined by Mann-Whitney U-test. Clinical and laboratory characteristics of the study population are presented according to sex-stratified tertiles of PON-1 activity. Differences in proportions of dichotomous variables across tertiles of PON-1 activity were determined by multinomial χ-square tests. Multivariable linear regression analyses, adjusted for age and sex, were used to test for linear trends between tertiles of PON-1 activity. Age- and sex-adjusted multivariable linear regression analyses were also used to determine the extent to which PON-1 activity (as continuous variable) was related to thyroid function parameters (TSH, free T_4, free T_3) taking clinical and laboratory covariates into account. PON-1 activity, TSH, triglycerides and UAE were natural logarithm ($\log_e$) transformed in order to achieve approximately normal distributions. Interaction terms were calculated as the product term of TSH or free T_4 with sex or the presence of MetS or its component of interest. To account for outliers the individual TSH or free T_4

values were centered to the mean by subtracting the group mean value from individual values [36,37]. Interaction terms were considered statistically significant at *P*-values <0.10, as recommended by Selvin [38]. Otherwise, two-sided *P*-values <0.05 were considered significant.

Results

Mean age of the 2206 participants was 48 ± 13 years. 1138 participants (52.6 %) were men (age 49 ± 13 years) and 1068 (47.4 %) were women (age 46 ± 12 years). Serum PON-1 activity amounted to 56.1 (46.1 - 68.1) U/L in the whole population, and was 60.0 (46.7 - 70.5) U/L in women vs. 54.9 (45.8 - 66.0) U/L in men (*P*=0.002). Clinical and laboratory characteristics of the study population are, therefore, shown according to sex-stratified tertiles of PON-1 activity (Table 1). One hundred two participants (4.6 %) reported a previous cardiovascular event, 65 subjects (2.9 %) had T2DM and 418 (19%) subjects fulfilled the criteria for MetS. A history of CVD (*P*= 0.002) and the presence of MetS (*P*=0.013) was more prevalent in subjects categorized in the lowest tertile of PON-1 activity, but diabetes status did not significantly vary according to the PON-1 categories (*P*=0.096) (Table 1). Oral glucose lowering drugs were used by 36 subjects, lipid modifying drugs (mainly statins) by 113 participants and antihypertensives by 274 subjects. Oral contraceptives were used by 283 women. The use of oral glucose lowering drugs (*P*<0.002, antihypertensives (*P*<0.001) was more prevalent in the subjects belonging to the lowest tertile of PON-1 activity, whereas the use of oral contraceptives was more prevalent in the women belonging to the highest tertile of PON-1 activity (*P*<0.001) (data not shown). The use of lipid lowering drugs did not vary across tertiles of PON-I activity (*P*=0.17). Accordingly, serum PON-1 activity was lower in subjects using oral glucose lowering drugs (45.9 (39.5-56.8 U/L) vs. 56.2 (46.3-68.4) U/L, *P*<0.001), in subjects using antihypertensives (52.6 (41.8-64.7 U/L) vs. 56.7 (46.8-68.8 U/L), *P*<0.001) and in women using oral contraceptives (62.9 (52.2-76.7 U/L) vs. 55.2 (45.7-66.7 U/L), *P*<0.001), but was not different in subjects using lipid lowering drugs compared to those who did not (56.5 (46.6-64.2 U/L vs. 56.1 (46.1-67.5) U/L, *P*=0.10). Serum PON-1 activity was inversely related to age. In age- and sex-adjusted analysis, PON-1 activity was positively related to systolic and diastolic blood pressure (Table 1). PON-1 activity was unrelated to BMI, waist circumference, glucose, eGFR and UAE, and did not

vary significantly according to smoking status and alcohol consumption. Additionally, PON-1 activity was positively related to total cholesterol, non-HDL cholesterol, triglycerides, as well as to HDL cholesterol and apoA-I (Table 1). Of note, in age- and sex-adjusted analysis, PON-1 activity was positively related to TSH and inversely to free T_4, but not to free T_3 (Table 1). Fig.1 shows TSH, free T_4 and free T_3 levels according to sex-stratified tertiles of PON-1activity. There were no interactions of sex with TSH, free T_4 or free T_3 on PON-1 activity (*P*=0.52 to *P*=0.64; data not shown).

We then tested whether the relationships of PON-1 activity (as continuous variable) with TSH and free T_4 remained present after adjustment for relevant clinical and laboratory covariates (Table 2). In age- and sex-adjusted multivariable linear regression analysis including free T_4 and TSH together PON-1 activity was positively associated with TSH and inversely with free T_4 (Table 2, model 1). In analysis with free T_4, free T_3 and TSH together, there was no significant independent association of PON-1 activity with free T_3 (β=-0.029, *P*=0.18; data not shown). The inverse relationship of PON-1 activity with free T_4 remained present after additional adjustment for non-HDL cholesterol, HDL cholesterol and triglycerides, although the positive relationship of PON-1 activity with TSH lost significance (Table 2, model 2). Likewise, PON-1 activity was inversely related to free T_4 in an alternative model which included apoA-I instead of HDL cholesterol (β= -0.055, *P*=0.01; data not shown). An inverse relationship of PON-1 activity with free T_4 was also found after additional adjustment for systolic and diastolic blood pressure, UAE, eGFR, alcohol consumption, smoking, a previous history of cardiovascular disease and diabetes status (Table 2, model 3), and finally after further adjustment for oral glucose lowering drugs, lipid lowering medication, antihypertensives and oral contraceptives (Table 2, model 4). The inverse relationship of PON-1 activity with free T_4 was not different in subjects with vs. without MetS (*P*=0.94), nor modified by the presence of its individual components (low HDL cholesterol: *P*=0.58); elevated triglycerides: *P*=0.96); enlarged waist circumference: *P*=0.57); elevated blood pressure: *P*=0.31; elevated glucose: *P*=0.22).

Secondary analyses were performed after exclusion of subjects with a previous history of CVD and T2DM (n=2051, Table 3, model 1), as well as after exclusion of subjects using oral glucose lowering drugs, lipid lowering drugs, antihypertensives and oral contraceptives (n=1596, Table 3,

model 2). In both analyses, PON-I activity remained inversely associated with free T_4 (β= -0.047 to -0.049, *P*=0.035 to 0.049) taking account of age, sex, non-HDL cholesterol, HDL cholesterol, triglycerides, systolic and diastolic blood pressure, alcohol consumption and smoking status (data not shown). In these analyses PON-1 activity was also positively related to HDL cholesterol (β= 0.236 to 0.217, *P*<0.001).

Discussion

In this large population-based study among strictly euthyroid subjects, we have shown to our knowledge for the first time, that serum PON-1 activity is positively related to TSH and inversely to free T_4 in age- and sex-adjusted analysis. In multivariable logistic regression analysis in which we included TSH, free T_4 and free T_3 together and adjusted for lipoproteins and other potentially important covariates, the inverse association of PON-1 activity with free T_4 remained present. The inverse relationship of PON-1 activity with free T_4 was not different between subjects with and without MetS nor modified by the presence of its individual components. Our current results are, therefore, in agreement with the hypothesis that variations in thyroid function within the euthyroid range may affect serum PON-1 activity.

In the interpretation of the results it is relevant that serum PON-I activity was assayed with phenyl acetate as substrate. Arylesterase activity, as measured with this type of assay, is widely used in large scale studies, and has the advantage of an approximately normal distribution, making it suitable for multivariable modeling [22]. Moreover, PON-I activity towards phenyl acetate is less variable between subjects compared to its activity towards paraoxon [overviewed in 18]. As expected [22,39-41] , PON-1 activity was positively related to HDL cholesterol and apoA-I. Its correlation with non-HDL cholesterol and triglycerides is probably explained by an association of PON-1 with very low density lipoproteins which are able to act as a vector for its cellular secretion [42]. Such relations of PON-1 activity with circulating lipoproteins together with the effect of low-normal thyroid function to increase plasma cholesterol and triglycerides [4,5,43] underscore the necessity to adjust for (apo) lipoprotein levels when evaluating the relationship of PON-1 activity with variation in thyroid

function in euthyroid subjects. In the present study we only included euthyroid subjects using strict criteria, i.e. TSH, free T_4 and free T_3 each being within their respective reference range, as done in other reports [44,45]. Moreover, we excluded subjects with positive anti-thyroid peroxidase autoantibodies to avoid possible confounding of latent thyroid autoimmunity on inflammatory and oxidative stress as much as possible [25].

Inconsistent effects of thyroid function status on PON-1 activity have been reported so far [24-28]. The inverse relation of PON-1 activity with free T_4 as shown in the current study suggests that low-normal thyroid function could contribute to higher PON-1 activity, although it should be emphasized that this relationship was modest. The mechanisms responsible for this relationship are not yet known. It is unclear whether thyroid hormones are able to affect PON-1 gene expression. PON-1 is down regulated by interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) [46]. Hypothyroidism may increase IL-1 and TNF-α [47], whereas higher levels of TNF-α are also found in subjects with low-normal thyroid function [48]. In addition, oxidized lipids are recognized to inhibit PON-1 activity [49-51]. In this context, it is relevant that low density lipoprotein (LDL) oxidation *in vitro* is exaggerated in both hypothyroidism and hyperthyroidism [12,13]. Moreover, increased levels of oxidized LDL have been demonstrated in euthyroid subjects with high normal TSH levels [14]. Taken together, these data [14,46-48] make it unlikely that a higher PON-1 activity in relation to low normal thyroid function is to explained by thyroid hormone- mediated effects on IL-1 and TNF-α or on (systemic) oxidative stress.

Given the inverse though modest relation of PON-1 activity with free T_4, it seems plausible that other mechanisms than effects of PON-1 on oxidative stress defense could contribute to the previously reported enhanced oxidative stress in the context of low normal thyroid function [14]. It also seems unlikely that changes in PON-1 activity play a major role in an attenuated ability of HDL to protect against LDL oxidation *in vitro* in subjects with low-normal thyroid function [8], a read-out of HDL functionality which is closely related to PON-1 activity [52]. In this regard, is relevant that other factors affecting oxidative stress such as superoxide dismutase [53] and circulating bilirubin levels [44] are also affected by thyroid function. Of note, it has been demonstrated recently that the

inverse relationship of bilirubin with free T4 is stronger in more insulin resistant individuals [44], and that the relationship of PON-1 activity with its activator, apoE, is impaired in subjects with MetS [41]. For this reason we also set out to determine whether the relationship of PON-1 activity was modified in the context of MetS. We found that the inverse relationship of PON-1 activity with free T_4 was not modified by the presence of MetS nor by its individual components.

The regulation of PON-1 is dependent on many genetic and environmental factors. Regarding environmental factors, several animal and human studies have shown that dietary lipids can influence PON-1 activity [54-56]. Furthermore it has been reported that physically active subjects have higher PON-1 activity [57]. A limitation of the present study is that detailed information on nutrient intake and data with respect to physical activity were not available. Statins may also increase PON-1 activity [58], although this has not been unequivocally reported [59]. In the current report, PON-1 activity was not affected by the use of lipid lowering drugs. However, PON-1 activity was inversely associated with the use of glucose lowering medication in analysis in which we also adjusted for the presence of T2DM. We explain this finding by assuming that the use of glucose lowering drugs preferentially labels diabetic patients with more severe hyperglycemia, requiring medical drug treatment. Further, PON-1 activity was elevated in women who used oral contraceptives. Although little information is available on this issue, it seems consistent with some other data suggesting that PON-1 activity is higher in women taking oral contraceptives, and may increase in response to ethinyl oestradiol and cyproterone acetate combination [60,61].

Several other methodological aspects and limitations of our study need to be considered. We performed a cross-sectional study, so that conclusions regarding cause-effect relationships cannot be drawn with certainty. However, we are not aware of any data underscoring a physiological role of PON-1 itself in thyroid hormone regulation. In addition owing to the observational nature of our study, residual confounding due to unmeasured confounders cannot be entirely ruled out. We performed secondary analyses after exclusion of subjects with a history of CVD and T2DM, and the use of glucose lowering, lipid lowering, antihypertensive medication and oral contraceptives. Reassuringly, these analyses showed the same inverse relationship of PON-1 activity with free T_4. Of

further note, PON-1 activity was assayed in sera that were stored for a prolonged period. However, loss of PON-1 enzymatic activity are minimal if samples stored frozen at -70 C^0 [62].

In conclusion, this large population-based cohort study demonstrates for the first time that serum PON-1 activity is inversely associated with free T_4 in euthyroid subjects. It is conceivable that low-normal thyroid function may influence PON-1 regulation.

Author Contributions: Conception and design of the study: LTW, EG, RJ, SB, RG and RD. Data collection and analysis: LTW, EG and RD. Interpretation of data: LTW, EG and RD. Drafting the manuscript: LTW, EB, and RD. All authors have revised and approved the submitted manuscript.

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Figure legend:

Figure 1. TSH, and free T_4 and free T_3 levels according to sex-stratified tertiles of serum paraoxonase-1 (PON-1) activity . *P*-values for linear trend (adjusted for age and sex): TSH: *P*=0.036, free T_4: *P*=0.050 and free T_3: *P*=0.20. Data are given in means and standard errors. TSH is logarithmically transformed.

Table 1. Clinical and laboratory characteristics in 2206 subjects according to sex-stratified tertiles of paraoxonase-1 (PON-1) activity.

	Sex stratified tertiles of PON-1 activity (U/L)				
	1 Men 19.7-48.8 Women 17.0-50.2	2 Men 48.9-62.2 Women 50.2-65.1	3 Men 62.2-130.7 Women 65.2-119.3	β	*P*-value
Participants, n	735	736	735		
Men, n (%)	379 (51.6)	380 (51.6)	379 (51.6)		
Women, n (%)	356 (48.4)	356 (48.4)	356 (48.4)		
Age (years)	49.8 ± 13.2	48.1 ± 12.6	45.7 ± 11.4	-0.132	<0.001
BMI (kg/m^2)	25.9 ± 4.3	26.1 ± 4.6	25.4 ± 3.9	-0.013	0.65
Waist circumference (cm)	87.8 ± 13.3	88.1 ± 13.5	86.3 ± 12.7	0.000	0.98
Systolic blood pressure (mmHg)	129 ± 20	129 ± 21	127 ± 18	0.038	0.037
Diastolic blood pressure (mmHg)	74 ± 9	74 ± 10	74 ± 10	0.053	0.004
Alcohol					0.43
< 10 gram per day (%)	538 (73.6)	521 (71.2)	518 (70.8)		
≥ 10 gram per day (%)	193 (26.4)	211 (28.8)	214 (29.2)		

Smoking					0.081
never (%)	188 (25.8)	221 (30.2)	221 (30.1)		
former (%)	246 (33.7)	245 (33.5)	263 (35.8)		
current (%)	296 (40.5)	266 (36.3)	250 (34.1)		
Glucose (mmol/L)	4.6±1.3	4.6±1.1	4.5±1.1	0.007	0.74
Total cholesterol (mmol/L)	5.5 ± 1.14	5.6 ± 1.22	5.7 ± 1.12	0.12	<0.001
Non-HDL cholesterol (mmol/L)	4.2 ± 1.25	4.2 ± 1.31	4.3 ± 1.22	0.069	<0.001
HDL cholesterol (mmol/L)	1.28 ± 0.39	1.36 ± 0.40	1.42 ± 0.42	0.131	<0.001
Triglycerides (mmol/L)	1.14 (0.81-1.63)	1.12 (0.82-1.62)	1.16 (0.85-1.69)	0.057	0.005
ApoA-I (g/L)	1.35 ± 0.32	1.40 ± 0.31	1.44 ± 0.33	0.127	<0.001
ApoB (g/L)	1.04 ± 0.35	1.04 ± 0.34	1.03 ± 0.33	0.034	0.099
CVD (n, %)	46 (6.3)	38 (5.2)	18 (2.4)		0.002
MetS (n, %)	161 (21.9)	140 (19.0)	117 (15.9)		0.013
T2DM (n, %)	29 (3.9)	21 (2.9)	15 (2.0)		0.096
eGFR (ml/min/1.73m^2)	95.1 (83.2-105.9)	97.3 (86.3-107.8)	98.7 (87.5-109.6)	0.020	0.23
UAE (mg/24 hrs)	9.0 (6.1-17.2)	9.0 (6.2-16.9)	8.6 (6.1-15.7)	0.028	0.18
TSH (mU/L)	1.28 (0.94-1.80)	1.29 (0.95-1.81)	1.39 (0.99-1.87)	0.045	0.036
free T_4 (pmol/L)	12.97 ± 1.83	12.93 ± 1.71	12.80 ± 12.8	-0.042	0.050
free T_3 (pmol/L)	3.75 ± 0.64	3.72 ± 0.61	3.73 ± 0.62	-0.027	0.20

Data in mean ± SD or in median (interquartile range). For continuous variables *P*-values for linear trend are adjusted for age and sex, except for age which was adjusted for sex only. Data with respect to smoking and alcohol consumption are missing in 10 (0.5%) and 11 (0.5%) of the subjects, respectively. Triglycerides, UAE and TSH are $\log_e$ transformed. For dichotomous variables *P*-values are by multinomial χ-square test. Apo, apolipoprotein; BMI, body mass index; CVD, cardiovascular disease; eGFR, estimated glomerular filtration rate, HDL, high density lipoproteins; MetS, metabolic syndrome; T2DM, type 2 diabetes mellitus; UAE, urinary albumin excretion. β: standardized regression coefficient.

Table 2. Multiple linear regression models demonstrating the independent association of free T_4 and TSH with paraoxonase-1 (PON-1) activity.

	Model 1		*Model 2*		*Model 3*		*Model 4*	
	β	*P-value*	*β*	*P-value*	*β*	*P-value*	*β*	*P-value*
Age (years)	-0.141	<0.001	-0.183	<0.001	-0.160	<0.001	-0.131	<0.001
Sex (men vs. women)	-0.050	0.018	-0.009	0.69	-0.005	0.85	0.029	0.276
free T_4 (pmol/L)	-0.064	0.003	-0.067	<0.001	-0.066	0.002	-0.0642	0.002
TSH (mU/L)	0.051	0.016	0.035	0.091	0.034	0.11	0.033	0.124
non-HDL cholesterol (mmol/L)			0.095	<0.001	0.089	0.001	0.097	<0.001
HDL cholesterol (mmol/L)			0.242	<0.001	0.230	<0.001	0.220	<0.001
Triglycerides (mmol/L)			0.116	<0.001	0.121	<0.001	0.103	<0.001
Systolic blood pressure (mm Hg)					-0.010	0.78	-0.005	0.878
Diastolic blood pressure (mm Hg)					0.013	0.70	0.006	0.870
eGFR (ml/min/1.73m^2)					0.029	0.28	0.030	0.271
UAE (mg/24 hrs)					0.033	0.15	0.028	0.226
CVD history (yes/no)					-0.021	0.34	-0.016	0.496

Diabetes status (yes/no)					-0.031	0.15	0.039	0.222
Alcohol consumption (< vs. ≥ 10 gram per day)					0.025	0.26	0.029	0.190
Smoking (never, former, current)					-0.049	0.024	-0.050	0.023
Glucose lowering drugs							-0.089	0.004
Lipid lowering drugs							0.007	0.766
Antihypertensives							-0.018	0.469
Oral contraceptives							0.092	<0.001

β: standardized regression coefficient. eGFR, estimated glomerular filtration rate; HDL, high density lipoproteins; UAE, urinary albumin excretion. PON-1 activity, TSH, triglycerides and UAE are $\log_e$ transformed. Alcohol consumption is categorized in per day. Smoking is categorized in never, former and current. Variables included in the models:

Model 1:age, sex, free T_4, TSH

Model 2: model 1 plus non-HDL cholesterol, HDL cholesterol and triglycerides

Model 3: model 2 plus systolic and diastolic pressure, alcohol consumption, smoking, plus cardiovascular disease (CVD) history and diabetes status.

Model 4: model 3 plus glucose lowering drugs, lipid modifying medication, antihypertensives and oral contraceptives.

Table 3. Multivariable linear regression analyses demonstrating relationships of paraoxonase-1 (PON-1) activity with after exclusion of subjects with a history of cardiovascular disease and type 2 diabetes mellitus (n=2051; model 1) or oral glucose lowering drugs, lipid lowering drugs, oral contraceptives and antihypertensives (n=1596; model 2).

	Model 1		**Model 2**	
	β	*P*-value	β	*P*-value
Age	-0.161	<0.001	-0.140	<0.001
Sex (men vs women)	-0.011	0.67	-0.026	0.37
TSH	0.028	0.21	0.047	0.06
free T_4	-0.047	0.035	-0.049	0.049

β: standardized regression coefficient. All models are adjusted for age, sex, non-high density lipoprotein (HDL) cholesterol, HDL cholesterol, triglycerides, systolic blood pressure, diastolic blood pressure, alcohol consumption and smoking. PON-1 activity, TSH and triglycerides are $\log_e$ transformed.