Paraoxonase-1 is not associated with coronary artery calcification in type 2 diabetes: Results from the PREDICT study

Bharti Mackness^a, Judit Marsillach^b, Robert S. Elkeles^c, Ian F. Godsland^c, Michael D. Feher^d, Michael B. Rubens^e, Marcus D. Flather^f, Steve E. Humphries^g, Jackie Cooper^g and Mike Mackness^{a,*}

^a*Pontech Art, Calle De L'Argelagar, Tarragona, Spain*

^b*Medicine (Division of Medical Genetics) and Genome Sciences, University of Washington, Seattle, WA, USA*

^c*Endocrinology and Metabolic Medicine, Imperial College London, St Mary's Hospital, London, UK*

^d*Beta Cell Unit, Chelsea and Westminster Hospital, London, UK*

^e*CT Scanning Unit, Royal Brompton Hospital, London, UK*

^f*Clinical Trials and Evaluation Unit, Royal Brompton and Harefield NHS Foundation Trust and Imperial College, London, UK*

^g*The Rayne Institute, Royal Free and University College Medical School, London, UK*

Abstract. *Objectives:* To determine any association between serum paraoxonase-1 (PON1) activity, protein and coding region genetic polymorphisms and coronary artery calcification (CACS) and to determine factors which modulate serum PON1 in type 2 diabetes (T2DM).

Methods and results: 589 patients (419 Caucasian, 120 South Asian, 50 other) from the PREDICT Study were investigated. All patients were asymptomatic for coronary disease and had established T2DM. CACS, lipids, lipoproteins, inflammatory markers, insulin resistance and PON1 activity, concentration and Q192R and L55M genotypes were measured. Independent associations were: 1) PON1 activity negatively with insulin resistance, triglycerides and PON1-55 genotype and positively with PON1-192 genotype; 2) PON1 concentration negatively with Caucasian ethnicity, duration of diabetes and statin use and positively with plasma creatinine and PON1-192 genotype. There was no association between CACS and any of the PON1 activity, concentration or genotype and this finding was not different in the various ethnic groups within the PREDICT study.

Conclusion: PON1 is modulated by a number of factors, some of which are reported here for the first time, including ethnicity and insulin resistance in subjects with T2DM. No association between CACS and PON1 was found.

Keywords: Paraoxonase-1, coronary calcification, insulin resistance, Type 2 diabetes

1. Introduction

Coronary heart disease (CHD) is the most common complication and the major cause of death in type 2 diabetes mellitus (T2DM). The risk of CHD is 2–5 times greater in people with diabetes than those without even when other risk factors are equivalent [1]. Despite cur-

rent treatments there is still an excess of cardiovascular mortality in T2DM and current methods to assess CHD risk may not adequately detect risk in asymptomatic individuals.

Electron beam computed tomography (EBCT) enables high resolution quantitative images of coronary artery calcification to be rapidly acquired. Coronary artery calcium is a well established index of atherosclerosis [2]. Coronary artery calcium score (CACS) predicts CHD in non-diabetic groups [3]. The primary aim of the Prospective Evaluation of Diabetic Ischaemic

*Corresponding author: Mike Mackness, Pontech Art, Calle De L'Argelagar, 43840 Salou, Tarragona, Spain. Tel.: +34 977374310; E-mail: mike.mackness@gmail.com.

Disease by Computed Tomography (PREDICT) Study was to assess the role of EBCT derived CACS in predicting CHD and stroke in asymptomatic patients with T2DM. CACS was found to be a powerful predictor of cardiovascular events in this study [4].

The paraoxonase (PON) multi-gene family comprises 3 members, PON1, PON2 and PON3 [5]. The genes for all 3 members of the family are widely expressed in mammalian tissues [6], however, PON1 and PON3 are predominantly located in the plasma associated with high-density lipoprotein (HDL) while PON2 is not found in the plasma but has a wide cellular distribution [7]. PON1, PON2 and PON3 all retard the proatherogenic oxidative modification of low-density lipoprotein (LDL) and cell membranes and are therefore considered to be antiatherogenic [8]. PON1 is now considered to be a major factor in the antioxidative activity of HDL [9].

The transgenic expression of human PON1, PON2 or PON3 in various mouse models of atherosclerosis has been shown to retard or reverse atherosclerosis by mechanisms which include a reduction in circulating and aortic oxidised-LDL (ox-LDL), a reduction in macrophage oxidative stress and foam cell formation, an increase in reverse cholesterol transport and a normalisation of endothelial function [10–14]. Interestingly, in human aortas, immunostaining for PON1 progressively increases as atherosclerosis develops [15] and the presence of both PON1 and PON3 in aortic macrophages indicates a cellular protective effect of these enzymes [16].

Low levels of serum PON1 have been associated with susceptibility to CHD development and low serum PON1 (which is independent of PON1 coding region polymorphisms) is a characteristic of T2DM [17]. The low PON1 in T2DM is believed to be a major cause of the dysfunctional HDL (less atheroprotective) found in this disease [18]. The purpose of the present study was to investigate whether any significant relationship exists between CACS as a marker of atherosclerosis susceptibility and serum PON1 parameters in T2DM in the various ethnic groups within the PREDICT study.

2. Methods

2.1. Study participants

The protocol of the PREDICT study has been published previously [19]. In summary, 589 patients with T2DM were recruited from diabetes clinics in Cen-

tral and West London, UK between November 2000 and November 2003 and were reviewed annually until November 2006. Median follow-up time was 4 years as per the original protocol [19]. Ethics committee approval was obtained from each of the participating centres and all participants gave written informed consent.

Participants had T2DM diagnosed by WHO criteria and were on standard diabetic therapy including diet, tablets or insulin. They were of either gender, aged 50–75 years and were Caucasian or Asian. People of Black African origin were excluded because of their known low rate of CHD in the UK at the time of the study [20]. Other exclusion criteria were, known CHD or other cardiac disease, congestive heart failure, uncontrolled hypertension (systolic BP > 160 mmHg or diastolic BP > 95 mmHg, with or without anti-hypertensive treatment), pregnancy, inability to provide informed consent or other medical conditions likely to limit life expectancy or requiring extensive medical treatment.

2.2. Electron beam computed tomography

As previously described [19], EBCT was carried out on an Imatron C-150 EBCT scanner (Imatron Inc, San Francisco, CA, USA). Total procedure time was 15 min with a radiation dose of 0.5–0.9 mSv (UK annual background radiation is 2.5–7.5 mSv). Quantification of CACS was in Agatston units (AU) [21].

2.3. Biochemical measurements

After an overnight fast blood samples were taken for baseline measurement of plasma glucose, HbA_{1c}, insulin, total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, apolipoproteins (apo) A1 and B, creatinine, fibrinogen, homocysteine, high sensitivity CRP and urine creatinine: albumin ratio as previously described [22,23]. Insulin resistance was calculated from the fasting plasma glucose and insulin concentrations, according to the homeostasis model assessment (HOMA) formula: $HOMA-IR = (\text{fasting plasma insulin concentration (mU/L)} \times \text{fasting plasma glucose concentration (mmol/L)}) / 22.5$ [24]. Presence of the Metabolic Syndrome was determined according to International Diabetes Federation criteria [25].

Serum PON1 activity towards paraoxon was measured spectrophotometrically at 405 nm [17], PON1 concentration was measured using our in-house ELISA [17] and the PON1 Q192R and L55M polymorphisms were analysed by PCR and restriction fragment length polymorphism analysis using DNA isolated from peripheral blood leukocytes as described [17]. Those patients taking insulin had no insulin since the previous day.

2.4. Statistical analysis

For normally distributed variables results have been presented as mean $\pm$ standard deviation. Non-normally distributed variables were log-transformed or square root transformed to give a normal distribution and standard deviations are approximate for these variables. Where this was not possible results have been presented as medians with inter quartile ranges and non-parametric tests have been used.

Differences between ethnic groups were assessed with ANOVA (parametric) or the Kruskal-Wallis test (non-parametric). For categorical variables differences were tested by chi-squared or Fisher's exact tests. Associations with PON1 activity were assessed using Spearman rank correlation. For PON1 concentration partial correlations were obtained after adjustment for ethnic group.

Independent predictors of PON1 parameters were selected using stepwise models in conjunction with bootstrap resampling. Variables with $p < 0.25$ on univariate analysis were considered for inclusion into the models and stepwise regression with backwards elimination and a threshold of $p = 0.05$ was run on 1000 bootstrap samples. Variables selected in at least 60% of the bootstrap samples were included in the final model. An additive genetic model was used in these models. For PON1 activity an ordinal logistic regression model was used based on quintiles due to the non-normal distribution. For analysis of CACS score, tobit regression of $\ln(\text{CACS} + 1)$ was used, modelling zero as a censored threshold (no detectable CACS) and assuming a normal distribution for the non-censored scores. Results were also analysed using ordinal logistic regression with categories reflecting the presence of no, minimal, mild, moderate and severe CACS (scores of 0, 1–10, 101–400, > 400). Results from this model are the odds ratio for a higher category CACS category.

The study was powered to detect an R^2 of 1.4% with 80% power at the 5% significance level using an additive genetic model. The study was also powered with > 90% power to detect a difference of 0.5 standard deviations between the Caucasian and Other ethnic group at the 5% significance level. Results were not adjusted for multiple comparisons and should therefore be interpreted in light of the number of tests made.

3. Results

The population comprised 419 Caucasians, 120 South Asians and 50 others, there were a majority

of males and patients with metabolic syndrome and were mostly moderate alcohol drinkers. Fasting plasma glucose was elevated (Supplemental Tables 1 and 2). There were significant differences between the ethnic groups with respect to age, duration of diabetes, BMI, alcohol intake, HbA1c, fasting plasma glucose, serum creatinine, triglycerides, apo B, fibrinogen, CRP, the percentage of males, ex-smokers, subjects taking oral hypoglycaemic therapy and those with metabolic syndrome. Median CACS was significantly higher in the Caucasian population than the other 2 groups (119 (14–469) vs. 102.5 (14.5–350) vs. 58 (2–22) respectively ($P = 0.02$)).

There was no difference in PON1 activity between the ethnic groups (Table 1), however PON1 concentration was significantly lower in the Caucasian group ($P < 0.001$). There were no significant differences in PON1 specific activity (SP) (activity divided by concentration) between the ethnic groups which were 1.50 ± 1.12 , 1.30 ± 0.84 and 1.50 ± 1.14 in the Caucasian, South Asian and Other groups respectively ($P = 0.14$). However, SP was affected by both the PON1-55 and -192 genotypes with $LL > LM > MM$ and $RR > QR > QQ$. SP was significantly higher in the PON1-55LL genotype in Caucasians compared to South Asians and Others (1.92 ± 1.22 , 1.42 ± 0.74 and 1.60 ± 1.29 respectively, $P = 0.003$). SP was also higher in Caucasians compared to South Asians and Others in the PON1-192RR (3.07 ± 1.93 , 1.93 ± 0.64 and 2.71 ± 1.32 respectively, $P = 0.002$) and PON1-192QR genotypes (2.21 ± 1.03 , 1.67 ± 0.78 and 1.78 ± 0.93 respectively, $P = 0.0003$). Differences in SP were due to differences in activity as concentration was not affected by genotype (result not shown).

The frequency of the PON1-55M allele was significantly higher and that of the PON1-192R allele significantly lower in the Caucasian group ($P < 0.0001$ and $P = 0.04$ respectively). PON1 activity was highest in PON1-192R and 55L homozygotes and lowest in PON1-192Q and 55M homozygotes as expected from previous studies (supplemental Table 3). Both polymorphisms were in Hardy Weinberg equilibrium.

PON1 activity was higher in those subjects who drank alcohol ($P = 0.05$) or did not have metabolic syndrome ($P = 0.07$) (Table 2). PON1 concentration was significantly higher in those not on statin treatment ($P = 0.001$) or insulin therapy ($P = 0.05$).

Pearson's correlation coefficients (Table 3) indicated PON1 activity was significantly positively associated with HDL-cholesterol and apo A1 and significantly negatively associated with triglycerides, HbA1c, glu-

Table 1
PON1 activity, concentration and genotype frequency by ethnic group

	Caucasian <i>N</i> = 419	South Asian <i>N</i> = 120	Other <i>N</i> = 50	P value
PON1 activity (nmol/min/ml) ¹	122.1 [62.1–186.4]	121.9 [70.5–169.7]	141.6 [73.2–193.3]	0.34
PON1 concentration (μg/ml) ²	73.2 ± 26.7	84.7 ± 28.3	84.5 ± 30.7	< 0.0001
PON1 55 genotype, N(%)				
LL	169 (42.3)	73 (62.9)	30 (62.5)	
LM	187 (46.8)	38 (32.8)	16 (33.3)	< 0.0001
MM	44 (11.0)	5 (4.3)	2 (4.2)	
m allele freq (95% CI)	0.344 (0.311–0.378)	0.207 (0.157–0.265)	0.208 (0.132–0.303)	< 0.0001
PON1 192 Genotype, N(%)				
QQ	179 (45.1)	48 (40.7)	19 (38.8)	
QR	184 (46.4)	55 (46.6)	17 (34.7)	0.004
RR	34 (8.6)	15 (12.7)	13 (26.5)	
r allele freq (95% CI)	0.317 (0.285–0.351)	0.360 (0.299–0.425)	0.439 (0.339–0.543)	0.04

¹Median [Interquartile range];

²transformed back from square root transformation with approximate SD.

Table 2
Differences in PON1 parameters

		N	PON1 activity (nmol/min/ml) ¹	P	PON1 concentration (μg/ml) ²	P*
Gender	Female	212	116.4 (66.3–170.6)		73.6 ± 25.9	
	Male	369	128.4 (67.2–183.6)	0.31	78.0 ± 28.9	0.12
Ever smoker	Yes	325	127.0 (67.2–183.6)		74.6 ± 26.2	
	No	256	121.5 (66.8–177.6)	0.57	78.6 ± 29.7	0.37
Alcohol use	No	259	116.8 (61.2–170.6)		75.1 ± 28.2	
	Yes	322	129.4 (71.4–186.4)	0.05	77.4 ± 27.6	0.11
Oral hypoglycaemic therapy	No	112	139.3 (66.6–196.5)		78.2 ± 25.5	
	Yes	469	121.5 (67.2–177.5)	0.16	75.9 ± 28.4	0.18
Insulin therapy	No	438	125.9 (67.7–182.2)		77.6 ± 27.5	
	Yes	143	113.1 (62.1–181.7)	0.28	72.6 ± 28.5	0.05
Statin therapy	No	358	126.8 (66.8–179.4)		79.3 ± 28.5	
	Yes	223	120.5 (67.2–183.6)	0.82	71.8 ± 26.2	0.001
Fibrate therapy	No	532	123.1 (67.2–182.0)		76.5 ± 28.2	
	Yes	49	122.4 (59.3–169.7)	0.63	74.5 ± 24.1	0.80
BP-lowering therapy	No	214	125.2 (66.3–181.7)		75.3 ± 26.1	
	Yes	367	122.4 (67.2–182.2)	0.98	77.0 ± 28.8	0.50
Metabolic syndrome	No	149	140.9 (71.4–187.3)		76.3 ± 28.8	
	Yes	432	119.6 (65.6–178.7)	0.07	76.4 ± 27.5	0.54

*adjusted for ethnic group;

¹Median [Interquartile range];

²transformed back from square root transformation with approximate SD.

cose, insulin, HOMA-IR and the albumin/creatinine ratio. On the other hand, PON1 concentration was significantly positively associated with plasma creatinine and significantly negatively associated with duration of diabetes, HbA_{1c} and fasting plasma glucose.

In multiple regression analysis we sought to determine the effects of those factors associated with PON1 in the above analyses on PON1 activity (Table 4a). The factors that showed significant association with PON1 activity are HOMA-IR ($P = 0.02$), triglycerides ($P = 0.001$), apo A1 ($P < 0.0001$) and both PON1 genotypes

(both $P < 0.001$). Factors associated with PON1 concentration (Table 4b) were ethnicity ($P < 0.001$), duration of diabetes ($P = 0.02$), creatinine ($P = 0.006$), statin use ($P = 0.002$) and PON1-192 genotype ($P = 0.05$).

We could find no association between CACS, either as a continuous variable or as quintiles and any of the PON1 parameters (PON1 genotype (supplemental Tables 4), or by tertiles of PON1 activity or concentration (supplemental Table 5), when analysed either in the total population or divided by ethnicity.

Table 3
Correlation coefficients for association with PON1 activity and concentration

	PON1 activity		PON1 concentration	
	rho	P	r*	P
Age	−0.05	0.24	0.06	0.13
Duration of diabetes	−0.01	0.74	−0.10	0.02
BMI	−0.06	0.16	−0.04	0.38
SBP	−0.06	0.14	−0.03	0.50
DBP	−0.01	0.88	−0.03	0.55
CACS	0.03	0.53	0.02	0.70
HbA1c	−0.13	0.002	−0.10	0.02
Glucose	−0.10	0.02	−0.09	0.04
Insulin	−0.12	0.0003	0.05	0.23
HOMA-IR	−0.15	0.0004	0.02	0.68
Albumin/creatinine	−0.14	0.002	0.06	0.17
Creatinine	−0.03	0.50	0.11	0.007
Cholesterol	−0.01	0.84	0.04	0.37
Triglyceride	−0.13	0.003	0.01	0.90
LDL	0.05	0.28	0.03	0.42
HDL	0.13	0.002	0.001	0.98
Apo A1	0.12	0.003	−0.04	0.34
Apo B	0.01	0.88	−0.03	0.46
Fibrinogen	−0.01	0.89	0.02	0.60
CRP	−0.05	0.23	0.05	0.23
homocysteine	−0.04	0.33	0.04	0.39
PON1 concentration	0.08	0.07	—	—

*Partial correlation adjusted for ethnic group.

Table 4
Multiple Regression analysis for PON1 activity

a) Multiple ordinal logistic regression analysis for PON1 activity				
		OR (95% CI)	P	
HOMA	1 SD increase	0.82 (0.69–0.96)	0.02	
Triglyceride	1 SD increase	0.75 (0.63–0.89)	0.001	
Apo A1	1 SD increase	1.38 (1.16–1.64)	< 0.0001	
PON1-55	Per M allele	0.59 (0.45–0.77)	< 0.0001	
PON1-192	Per R allele	20.62 (14.04–30.28)	< 0.0001	
b) Multiple linear regression analysis for PON1 concentration				
		B (se)	P	Partial R ²
Ethnicity	Caucasian	0	< 0.0001	4.2%
	S. Asian	0.67 (0.17)		
	Other	0.77 (0.24)		
Duration of diabetes	1 SD increase	−0.16 (0.07)	0.02	1.1%
creatinine	1 SD increase	0.19 (0.07)	0.006	1.5%
Statin use	Yes: No	−0.41 (0.14)	0.002	1.2%
PON1-192	Per R allele	0.20 (0.10)	0.05	0.7%

4. Discussion

Several studies have previously shown prospectively that PON1 activity is a risk factor for CHD development independently of HDL concentration [26–28] including a study in T2DM [29] although the finding is not universal [30,31]. Low PON1 concentration predicts cardiovascular mortality in haemodialysis patients [32]. Many studies have also investigated several PON1 polymorphisms as risk factors for CHD with positive associations being seen in some but not all

studies. Meta analyses have shown at best a marginal significance of the PON1-Q192R polymorphism as a risk factor for CHD but no relationship of other PON1 polymorphisms and CHD [33,34]. In this context therefore, it is perhaps not altogether surprising that no relationship between PON1 and CACS was found in our study. It is entirely possible that the protective effects of PON1 are manifest much earlier in atherosclerosis development through the inhibition of LDL oxidation preventing oxidised LDL induced MCP-1 production and macrophage oxidative stress and thus the prevention of

atherosclerosis initiation [10–12]. Two previous studies have investigated the relationship between PON1 and CACS. In the Coronary Artery Risk Development in Young Adults Study, there was no association between PON1 activity and CACS [35], while in the Diabetes Heart Study there were modest associations between CACS and the PON1-Q192R ($P = 0.002$) and PON2-S311C genotypes ($P = 0.037$) [36]. Further, much larger studies are required to determine any relationships between PON1 parameters and CACS.

Although we could find no differences in PON1 activity or PON1 specific activity between the different ethnic groups, serum PON1 concentration and the PON1-192R in the Caucasian group, in line with previously published population studies [26–34]. The higher prevalence of the PON1-55M genotype would result in the lower PON1 concentration found in the Caucasians as it is associated with lower PON1 mRNA levels [7]. Differences in specific activity found in different PON1-55 and 192 genotypes and higher specific activity found in PON1-55LL, 192RR and 192QR genotypes in Caucasians are harder to understand. The increased inflammatory environment found in Caucasian blood in this study would appear not to be conducive to higher PON1 activity but rather should be inhibitory [10–12,27]. The data suggest that in Caucasians the PON1-55L and 192R genes code for a more active enzyme than they do in South Asians or Others, which could be an evolutionary adaptation to some environmental stimulus. Large scale comparative studies of PON1 in different ethnic groups living in similar environments are warranted to confirm this finding.

The CACS was also significantly higher in the Caucasian group, however, none of the PON1 parameters were related to the CACS. It seems more likely that either the significantly older age of the Caucasians had allowed more atherosclerosis development or the increased prevalence of raised risk factors for atherosclerosis (triglycerides and apo B) and inflammation (fibrinogen and CRP) were responsible.

Recently it has been shown that human PON1 can prevent diabetes development in mice through its antioxidant properties and the stimulation of beta-cell insulin release, suggesting a possible role for PON1 in insulin biosynthesis [37,38]. PON2 also has an important role in hepatic insulin signalling [39] which may suggest a role for the PON family in energy metabolism which requires further investigation. PON1 activity in type 1 diabetes is inversely correlated with blood glucose levels and also PON1 is lower in subjects with the metabolic syndrome, suggesting modulation of

PON1 by factors associated with insulin resistance [40]. The finding in this study of inverse correlations of PON1 with blood glucose, insulin and insulin resistance (HOMA-IR) to our knowledge for the first time in T2DM, add further support to this but requires more detailed molecular analysis. *In vitro* studies have indicated that the PON1 gene is upregulated by high glucose concentrations in HepG2 hepatocytes [41]. However, PON1 is extremely susceptible to oxidative inactivation [42] and the high levels of oxidative stress which accompany hyperglycaemia [43] may well counteract increased hepatic PON1 production. The association of PON1 with duration of diabetes, which has not been reported previously in T2DM, may also be explained by this mechanism.

The negative relationship between PON1 activity and insulin resistance offers interesting possibilities. The relationship between insulin resistance, the metabolic syndrome and the subsequent progression to type 2 diabetes could indicate that the measurement of PON1 activity may provide an early indicator of metabolic disturbances before the onset of measurable arterial changes including CACS. Further work in this area is warranted.

Previous studies have also shown that moderate alcohol consumption increases PON1 [44], a finding confirmed by the present investigation.

Patients recruited for PREDICT were free from clinical cardiovascular disease. Nevertheless, it is possible that those receiving statins were perceived to be at increased risk and may indeed, have had increased sub-clinical CVD. In support of this we have previously reported a significant positive association between statin use and CACS in the cohort at baseline [22]. Therefore, the negative association between statin use and PON1 concentration could reflect underlying CVD. However, these relationships were not apparent with regard to PON1 activity and statin use.

With regard to the metabolic syndrome, among 225 statin users 80.8% had metabolic syndrome whereas of 364 non users 70.9% had metabolic syndrome ($P = 0.007$). PON1 activity but not concentration was lower in those with metabolic syndrome (Table 2) but with only borderline significance. Since associations between PON1 and metabolic syndrome were only apparent with activity and associations between PON1 and statin use were only apparent with concentration, it is difficult to draw any firm conclusions regarding interactions between statin use (as a proxy for underlying CVD), metabolic syndrome and PON1.

Previous studies in human and hepatocytes have indicated statins either to increase serum PON1 and its

hepatic synthesis or to have no effect [45,46], therefore the strong negative modulation of PON1 protein by statins in the PREDICT population was surprising. This may be unique to T2DM as it has not been reported in other populations, however, previous studies in humans tended to be short term interventions while it is likely that those taking statins in the PREDICT population would have been doing so for several years. Further large scale prospective studies of the effects of statin treatment on PON1 are warranted to determine if the effects on PON1 are detrimental to treatment outcome.

In conclusion, we could find no association between PON1 activity, protein or genetic populations with CACS. However, median CACS was higher in the older Caucasian population this was not related to PON1. In the PREDICT population of T2DM, PON1 is modulated by alcohol intake, statin therapy and insulin resistance.

Statement of author contributions: BM, MM researched data, contributed to discussion, wrote manuscript and reviewed/edited manuscript. JM researched data, wrote manuscript and reviewed/edited manuscript. RE, MR, MFI, JC researched data and reviewed/edited manuscript. IG, MFe reviewed/edited manuscript. SH research data, contributed to discussion and reviewed/edited manuscript.

Acknowledgements

The contribution of other members of the PREDICT study group is gratefully acknowledged: A Dunlop, A Widdowson, L Lyn, NK Suppiah, F Nugara and N Delahunty. Drs H Mather and A Dornhorst kindly allowed us to study their patients. MM is the guarantor of this work and, as such, had full access to all data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Conflict of interest

Nothing to declare.

Funding

Financial support for the PREDICT Study was provided by the Tomkins Foundation and British Heart Foundation (PG/03/112/16033). The study was also

supported by the North West London Diabetes Local Research Network. IFG is supported by the Heart Disease and Diabetes Research Trust and JM by a Beatriu de Pinós postdoctoral fellowship (2008 BP A 00166) from Comissionat per a Universitats i Recerca del Departament d'Innovacio, Universitats i Empresa, Catalunya, Spain. SHE and JC are supported by the British Heart Foundation (PG08/008).

References

- [1] Pyorala K, Laakso M. Macrovascular disease in diabetes, in: Mann J, Pyorala K, Teuscher A. (ed.), *Diabetes in Epidemiological Perspective*. Churchill Livingstone, 1983, 183-247.
- [2] Ardehali R, Nasir K, Kollandaivelu A, Budoff M, Blumenthal RS. Screening patients for subclinical atherosclerosis with non-contrast cardiac CT. *Atherosclerosis* 192 (2007), 235-242.
- [3] Arad Y, Goodman KJ, Roth M, Newstein D, Guerci AD. Coronary calcification, coronary disease risk factors, C-reactive protein and atherosclerotic cardiovascular disease events: The St Francis Heart Study. *J Am Coll Cardiol* 46 (2005), 158-165.
- [4] Elkeles RS, Godsland IF, Feher MD, Rubens MB, Roughton M, Nugara F, Humphries SE, Richmond W, Flather MD. Coronary calcium measurement improves prediction of cardiovascular events in asymptomatic patients with type 2 diabetes: the PREDICT study. *Eur Heart J* 29 (2008), 2244-2251.
- [5] Primo-Parma, SL, Sorenson, RC, Teiber, J, La Du, BN. The human serum paraoxonase/arylesterase gene (PON1) is one member of a multigene family. *Genomics* 33 (1996), 498-509.
- [6] Rodriguez-Sanabria F, Rull A, Beltran-Debon R, Aragonés G, Camps J, Mackness B, Mackness M, Joven J. Tissue distribution and expression of paraoxonases and chemokines in the mouse: the ubiquitous and joint localisation suggest a systemic and coordinated role. *J Mol Histol* 41 (2010), 379-386.
- [7] Mackness, B, Durrington, PN, Mackness, MI. The paraoxonase gene family and coronary heart disease. *Curr. Opin. Lipidol* 13 (2002), 357-362.
- [8] Reddy ST, Devarajan A, Bourquard N, Shih D, Fogelman AM. Is it just paraoxonase 1 or are other members of the paraoxonase gene family implicated in atherosclerosis? *Curr Opin Lipidol* 19 (2008), 405-408.
- [9] Liu, Y, Mackness, B, Mackness, M. Comparison of the ability of paraoxonases 1 and 3 to attenuate the in vitro oxidation of low-density lipoprotein and reduce macrophage oxidative stress. *Free Rad Biol Med* 45 (2008), 743-748.
- [10] Rozenberg, O, Shih, DM, Aviram, M. Paraoxonase 1 (PON1) attenuates macrophage oxidative status: studies in PON1 transfected cells and in PON1 transgenic mice. *Atherosclerosis* 181 (2005), 9-18.
- [11] Mackness, B, Quarck, R, Verreth, W, Mackness, M, Holvoet, P. Human paraoxonase-1 overexpression inhibits atherosclerosis in a mouse model of metabolic syndrome. *Arterioscler Thromb Vasc Biol* 26 (2006), 1545-1550.
- [12] Guns, P-J, Van Assche, T, Verreth, W, Franssen, P, Mackness, B, Mackness, M, Holvoet, P, Bull, H. Paraoxonase 1 gene transfer lowers vascular oxidative stress and improves vasomotor function in apolipoprotein E-deficient mice with pre-existing atherosclerosis. *Br J Pharmacol* 153 (2008), 508-516.
- [13] Ng CJ, Hama SY, Bourquard N, Navab M, Reddy ST. Adenovirus mediated expression of human paraoxonase 2 pro-

- fects against development of atherosclerosis in apolipoprotein E-deficient mice. *Mol Genet Metab* 89 (2006), 368–373.
- [14] Ng CJ, Bourquard N, Hama SY, Shih D, Grijalva VR, Navab M, Fogelman AM, Reddy ST. Adenovirus mediated expression of human paraoxonase 3 protects against the progression of atherosclerosis in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol* 27 (2007), 1368–1374.
 - [15] Mackness, B, Hunt, R, Durrington, PN, Mackness, MI. Increased immunolocalisation of paraoxonase, clusterin and apolipoprotein AI in the human artery wall with progression of atherosclerosis. *Arterioscler Thromb Vasc Biol* 17 (1997), 1233–1238.
 - [16] Marsillach J, Camps J, Beltran-Debon R, Rull A, Aragones G, Maestre-Martinez C, Sabench F, Hernandez M, Del Castillo D, Joven J, Mackness M, Mackness B. Immunohistochemical analysis of paraoxonases 1 and 3 in human atheromatous plaques. *Eur J Clin Invest* 41 (2011), 308–314.
 - [17] Mackness, B, Mackness, MI, Arrol, S, Turkie, W, Julier, K, Abuashia, B, Miller, JE, Boulton, AJM, Durrington, PN. Serum paraoxonase (PON1) 55 and 192 polymorphism and paraoxonase activity and concentration in non-insulin dependent diabetes mellitus. *Atherosclerosis* 139 (1998), 341–349.
 - [18] Mastorikou, M, Mackness, M, Mackness, B. Defective metabolism of oxidised-phospholipid by high-density lipoprotein from people with type 2 diabetes. *Diabetes* 55 (2006), 3099–3103.
 - [19] Elkeles RS, Flather M, Feher MD, Godsland I, Richmond W, Humphries SE, Rubens MB, Underwood SR. Prospective evaluation of diabetic ischaemic heart disease by computed tomography: the PREDICT study. *Br J Diabetes Vasc Dis* 2 (2002), 69–72.
 - [20] Wild S, McKeigue P. Cross sectional analysis of mortality by country of birth in England and Wales 1970–92. *Br Med J* 314 (1997), 705–710.
 - [21] Agaston AS, Janowitz WR, Hilner FJ, Zusmer NR, Viamonte M, Detrano R. Quantification of coronary artery calcium using ultrafast computed tomography. *J Am Coll Cardiol* 15 (1990), 827–832.
 - [22] Elkeles RS, Feher MD, Flather MD, Godsland IF, Nugara F, Richmond W, Rubens MD, Wang D. The association of calcium score and conventional risk factors in type 2 diabetic subjects asymptomatic for coronary heart disease (The PREDICT study). *Diab Med* 21 (2004), 1129–1134.
 - [23] Godsland IF, Elkeles RS, Feher MD, Nugara F, Rubens MB, Richmond W, Khan M, Donovan J, Anyaoku V, Flather MD. Coronary calcification, homocysteine, C-reactive protein and the metabolic syndrome in type 2 diabetes: the PREDICT study. *Diab Med* 23 (2006), 1192–2000.
 - [24] Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and β -cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 28 (1985), 412–419.
 - [25] Alberti KG, Zimmet P, Shaw J, IDF ETFCG. The metabolic syndrome – a new worldwide definition. *Lancet* 366 (2005), 1059–1062.
 - [26] Mackness B, Durrington P, McEluff P, Yarnell J, Azam N, Watt M, Mackness, M. Low paraoxonase activity predicts coronary events in the Caerphilly Prospective Study. *Circulation* 107 (2003), 2775–2779.
 - [27] Bhattacharyya T, Nicholls SJ, Topol EJ, Zhang R, Yang X, Schmitt D, Fu X, Shao M, Brennan DM, Ellis SG, Brennan M-L, Allayee H, Lusis AJ, Hazen SL. Relationship of paraoxonase 1 (PON1) gene polymorphisms and functional activity with systemic oxidative stress and cardiovascular risk. *JAMA* 299 (2008), 1265–1276.
 - [28] van Himbergen TM, van der Schouw YT, Voorbij HAM, van Tits LJH, Stalenhoef AFH, Peeters PHM, Roest M. Paraoxonase (PON1) and the risk for coronary heart disease and myocardial infarction in a general population of Dutch women. *Atherosclerosis* 198 (2008), 408–414.
 - [29] Ikeda Y, Inoue M, Suehiro T, Arai K, Kumon Y, Hashimoto K. Low human paraoxonase predicts cardiovascular events in Japanese patients with type 2 diabetes. *Acta Diabetol* 46 (2009), 239–242.
 - [30] Troughton JA, Woodside JV, Yarnell JWG, Arveiler D, Amouyel P, Ferrieres J, Ducimetiere P, Patterson CC, Luc G. Paraoxonase activity and coronary heart disease in healthy middle-aged males: The PRIME study. *Atherosclerosis* 197 (2008), 556–563.
 - [31] Birjmohun RS, Vergeer M, Stroes ES, Sandhu MS, Ricketts SL, Tanck MW, Wareham NJ, Jukema JW, Kastelein JJ, Khaw KT, Boekholdt SM. Both paraoxonase-1 genotype and activity do not predict the risk of future coronary artery disease; the EPIC-Norfolk prospective population study. *PLoS One* 4 (2009), e6809.
 - [32] Ikeda Y, Suehiro T, Itahara T, Inui Y, Chikazawa H, Inoue M, Arai K, Hashimoto K. Human serum paraoxonase concentration predicts cardiovascular mortality in hemodialysis patients. *Clin Nephrol* 67 (2007), 358–365.
 - [33] Mackness B, Davies GK, Turkie W, Lee E, Roberts DH, Hill E, Roberts C, Durrington PN, Mackness MI. Paraoxonase status in coronary heart disease. Are activity and concentration more important than genotype? *Arterioscler. Thromb Vasc Biol* 21 (2001), 1451–1457.
 - [34] Wheeler JG, Keavney BD, Watkins H, Collins R, Danesh J. Four paraoxonase gene polymorphisms in 11212 cases of coronary heart disease and 12786 controls: meta-analysis of 43 studies. *Lancet* 363 (2004), 689–95.
 - [35] Thyagarajan B, Jacobs DR jr, Carr JJ, Alosie O, Steffes MW, Kailash P, Hayes JH, Gross MD. Factors associated with paraoxonase genotypes and activity in diverse, young, healthy population: the Coronary Artery Risk Development in Young Adults (CARDIA) study. *Clin Chem* 54 (2008), 738–46.
 - [36] Burdon KP, Langefeld CD, Beck SR, Wagenknecht LE, Carr JJ, Freedman BI, Herrington D, Bowden DW. Association of genes of lipid metabolism with measures of subclinical cardiovascular disease in the Diabetes Heart Study. *J Med Genet* 42 (2005), 720–4.
 - [37] Rozenberg O, Shiner M, Aviram M, Hayek T. Paraoxonase 1 (PON1) attenuates diabetes development in mice through its antioxidative properties. *Free Rad Biol Med* 44 (2008), 1951–1959.
 - [38] Koren-Gluzer M, Aviram M, Meilin E, Hayek T. The antioxidant HDL-associated paraoxonase-1 (PON1) attenuates diabetes development and stimulates β -cell insulin release. *Atherosclerosis* (2011) doi: 10.1016/j.atherosclerosis.2011.07.119.
 - [39] Bourquard N, Ng CJ, Reddy ST. Impaired hepatic insulin signalling in PON2-deficient mice: a novel role for the PON2/apoE axis on the macrophage inflammatory response. *Biochem J* 436 (2011), 91–100.
 - [40] Kordonouri O, James, RW, Bennetts JB, Chan A, Kao YL, Danne T, Silink M, Donaghue K. Modulation by blood glucose levels of activity and concentration of paraoxonase in young patients with type 1 diabetes mellitus. *Metabolism* 50 (2001), 657–660.

- [41] Ikeda Y, Suehiro T, Arai K, Kumon Y, Hashimoto K. High glucosa induces transactivation of the human paraoxonase 1 gene in hepatocytes. *Metabolism* 57 (2008), 1725-1732.
- [42] Aviram M, Rosenblat M, Scott B, Drogul J, Sorenson R, Bisgaier CL, Newton RS, La Du B. Human serum paraoxonase (PON1) is inactivated by oxidised low density lipoprotein and preserved by antioxidants. *Free Rad. Biol. Med.* 26 (1999), 892-904.
- [43] Giacco F, Brownlee M. Oxidative stress and diabetic complications. *Circ Res* 107 (2010), 1058-1070.
- [44] Van der Gaag MS, van Tol A, Scheek LM, James RW, Urgert R, Schaafsma G, Hendriks HF. Daily moderate alcohol consumption increases serum paraoxonase activity; a diet controlled, randomised intervention study in middle-aged men. *Atherosclerosis* 147 (1999), 405-410.
- [45] Mirdamadi HZ, Sztanek F, Derdak Z, Seres I, Harangi M, Paragh G. The human paraoxonase-1 phenotype modifies the effect of statins on paraoxonase activity and lipid parameters. *Br J Clin Pharmacol* 66 (2008), 366-374.
- [46] Deakin S, Leviev I, Guernier S, James RW. Simvastatin modulates expression of the PON1 gene and increases serum paraoxonase: a role for sterol regulatory element-binding protein-2. *Arterioscler Thromb Vasc Biol* 23 (2003), 2083-2089.

Supplemental material

Supplemental Table 1
Subject characteristics by ethnic group

	Caucasian	South Asian	Other	P value
Age (years)	64 (58–70)	60 (54–65)	58.5 (54–65)	< 0.0001
Duration of diabetes (years) ³	6.6 ± 6.6	9.7 ± 7.5	8.8 ± 7.4	< 0.0001
BMI (Kg/m ²) ²	29.8 ± 5.3	25.9 ± 3.7	28.1 ± 4.4	< 0.0001
Systolic blood pressure (mm Hg) ²	131.8 ± 15.5	128.8 ± 17.2	131.9 ± 17.7	0.19
Diastolic blood pressure (mm Hg)	77.9 ± 8.6	77.1 ± 8.9	77.2 ± 9.9	0.66
Alcohol (units per week) ¹	2 (0–12)	0 (0–3)	0.5 (0–2)	< 0.0001
CACS (AU) ¹	119 (14–469)	102.5 (14.5–350)	58 (2–2)	0.02
HbA1c (%) ²	7.9 ± 1.6	8.4 ± 1.6	7.8 ± 1.5	0.007
Fasting plasma glucose (mmol/L) ²	9.4 ± 3.1	8.5 ± 2.5	8.9 ± 3.1	0.009
Fasting plasma insulin (pmol/L) ¹	6.1 (2.7–15.2)	7.2 (3.1–12.1)	4.6 (2.1–17.1)	0.28
HOMA-IR ¹	0.35 (0.15–0.95)	0.38 (0.17–1.06)	0.30 (0.11–0.77)	0.38
Urine albumin/creatinine ¹	1.1 (0.7–2.9)	1.3 (0.7–4)	1.8 (0.8–4.2)	0.27
Serum creatinine (μmol/L) ¹	99 (91–109)	100 (92–111)	91 (84–100)	0.0002
Total cholesterol (mmol/L) ²	4.75 ± 0.94	4.54 ± 0.93	4.71 ± 0.68	0.10
Triglycerides (mmol/L) ²	1.69 ± 0.88	1.46 ± 0.70	1.58 ± 0.89	0.02
LDL cholesterol (mmol/L) ³	2.71 ± 0.84	2.66 ± 0.82	2.75 ± 0.74	0.79
HDL cholesterol (mmol/L) ²	1.14 ± 0.31	1.13 ± 0.25	1.12 ± 0.33	0.91
Apo A1 (mg/dl) ²	143.7 ± 27.7	138.6 ± 24.5	143.5 ± 30.4	0.19
Apo B (mg/dl) ³	96.9 ± 23.0	91.4 ± 21.7	98.5 ± 21.9	0.05
Fibrinogen (g/L)	3.38 ± 0.79	3.23 ± 0.87	3.08 ± 0.76	0.02
CRP (mg/dl) ²	0.26 ± 0.31	0.16 ± 0.20	0.17 ± 0.23	0.0002
Homocysteine (μmol/L) ²	10.6 ± 3.7	10.6 ± 3.8	9.5 ± 2.8	0.14

Values are mean ± SD except ¹which are median (interquartile range) ²geometric mean (approximate SD)
³transformed back from square root transformation with approximate Sd.

Supplemental Table 2
Subject demographics by ethnic group

	Caucasian N (%)	South Asian	Other	P value
Male	256 (61.1)	88 (73.3)	29 (58.0)	0.04
Never-smoker	160 (38.2)	75 (62.5)	26 (52.0)	—
Ex-smoker	189 (45.1)	34 (28.3)	16 (32.0)	0.002
Current smoker	70 (16.7)	11 (9.2)	8 (16.0)	0.12
Alcohol use	251 (59.9)	50 (41.7)	25 (50.0)	0.001
Oral hypoglycaemic therapy	325 (77.6)	106 (88.3)	44 (88.0)	0.01
Insulin therapy	102 (24.3)	29 (24.2)	16 (32.0)	0.49
Statin therapy	161 (38.4)	51 (42.5)	13 (26.0)	0.13
Fibrate therapy	39 (9.3)	6 (5.0)	4 (8.0)	0.35
BP-lowering therapy	265 (63.3)	75 (62.5)	33 (66.0)	0.91
Metabolic syndrome	328 (78.3)	76 (63.3)	36 (72.0)	0.004

Supplemental Table 3
Difference in PON1 activity and concentration by PON1 genotype

	N	PON1 activity (nmol/min/ml) ¹	PON1 concentration (μg/ml) ²
PON-55			
LL	269	145.6 (94.6–195.2)	78.7 ± 27.6
LM	240	118.0 (63.7–173.9)	74.5 ± 28.6
MM	51	41.3 (32–58.9)	71.8 ± 25.5
P value		< 0.0001	0.42
PON-192			
QQ	243	62.6 (46.4–83.4)	73.4 ± 27.3
QR	256	162.3 (129.4–198.2)	78.5 ± 26.9
RR	61	201.7 (177.5–264.2)	80.3 ± 32.8
P value		P < 0.0001	0.11

¹ median (IQR), p value from Kruskal-Wallis test;

² mean ± SD, p value from ANOVA with adjustment for ethnic group.

Supplemental Table 4

CACS and genotype

Median CACS [IQR] by PON1 genotypes

		Caucasian		Asian		Other	
		N	CACS	N	CACS	N	CACS
PON-55	LL	169	136 [14–510]	73	107 [12–336]	30	67.5 [8–268]
	LM	187	119 [12–469]	38	71 [16–260]	16	4 [1.5–91]
	MM	44	154.5 [30.5–413.5]	5	243 [28–332]	2	1 [0–2]
	P value ^a		0.84		0.37		0.02
	P value ^b		0.77		0.34		0.02
PON192	QQ	179	104 [10–375]	48	66.5 [10–251.5]	19	67 [2–90]
	QR	184	148 [18–615]	55	152 [25–528]	17	171 [4–279]
	RR	34	46.5 [10–346]	15	40 [0–193]	13	8 [0–192]
	P value ^a		0.49		0.89		0.36
	P value ^b		0.80		0.74		0.74

^aunadjusted; ^badjusted for age and gender. From tobit regression model.**PON-55 genotype distribution by CACS category**

		Caucasians					Odds ratio (95% CI)	
		0	1–10	11–100	101–400	> 400	P value	
LL	9 (39.1)	27 (40.3)	42 (44.7)	42 (40.4)	49 (43.8)		1.00 (0.76–1.31) <i>P</i> = 0.998	
LM	11 (47.8)	34 (50.8)	43 (45.7)	47 (45.2)	52 (46.4)			
MM	3 (13.0)	6 (9.0)	9 (9.6)	15 (14.4)	11 (9.8)			

		S.Asians/Indians					Odds ratio (95% CI)	
		0	1–10	11–100	101–400	> 400	P value	
LL	10 (83.3)	7 (53.9)	18 (52.9)	21 (70.0)	17 (63.0)		1.11 (0.63–1.95) <i>P</i> = 0.71	
LM	2 (16.7)	6 (46.2)	14 (41.2)	7 (23.3)	9 (33.3)			
MM	0 (0)	0 (0)	2 (5.9)	2 (6.7)	1 (3.7)			

Result for combined ethnic groups is 0.96 (0.76–1.21) *p* = 0.71.**PON-192 genotype distribution by CACS category**

		Caucasians					Odds ratio (95% CI)	
		0	1–10	11–100	101–400	> 400	P value	
QQ	11 (50.0)	34 (51.5)	43 (45.3)	48 (46.2)	43 (39.1)		1.05 (0.79–1.39) <i>P</i> = 0.75	
QR	8 (36.4)	26 (39.4)	42 (44.2)	48 (46.2)	60 (54.6)			
RR	3 (13.6)	6 (9.1)	10 (10.5)	8 (7.7)	7 (6.4)			

		S.Asians/Indians					Odds ratio (95% CI)	
		0	1–10	11–100	101–400	> 400	P value	
QQ	6 (50.0)	6 (46.2)	16 (47.1)	12 (38.7)	8 (28.6)		1.08 (0.66–1.79) <i>P</i> = 0.76	
QR	2 (16.7)	7 (53.9)	13 (38.2)	16 (51.6)	17 (60.7)			
RR	4 (33.3)	0 (0)	5 (14.7)	3 (9.7)	3 (10.7)			

Result for combined ethnic groups is 1.06 (0.84–1.33) *p* = 0.64.

Supplemental Table 5
 CACS score by tertile of PON activity and concentration Median CACS [IQR]

	Caucasian		Asian		Other	
	N	CACS	N	CACS	N	CACS
Tertile of PON1 activity						
1	146	106 [12–469]	34	65 [13–243]	15	50 [2–86]
2	129	95 [10–451]	49	134 [29–306]	18	79 [3–590]
3	139	148 [27–495]	34	102.5 [12–518]	17	21 [4–202]
P value ^a		0.14		0.68		0.63
P value ^b		0.23		0.83		0.23
Tertile of PON1 concentration						
1	154	131 [16–490]	28	86.5 [11.5–427]	12	176 [12–469]
2	142	94 [10–383]	35	55 [13–243]	17	68 [3–171]
3	118	130.5 [20–548]	54	139 [13–518]	21	13 [1–171]
P value ^a		0.50		0.80		0.10
P value ^b		0.87		0.66		0.09

^aunadjusted; ^badjusted for age and gender. From tobit regression model.