Paraoxonase 2 (*PON2*) polymorphisms and development of renal dysfunction in type 2 diabetes: UKPDS 76

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Abstract

Aims/hypothesis Identification of variants predicting development of renal dysfunction would offer substantial clinical benefits. There is evidence that coding non-synonymous variants in the gene encoding paraoxonase 2 (*PON2*) are associated with nephropathy in both type 1 and type 2 diabetes.

Methods We examined the relationship between variation at the C311S and A148G polymorphisms (together with *PON2* intronic variant rs12704795) and indices of renal dysfunction (progression to micro- and macroalbuminuria, plasma creatinine increases) in 3,374 newly diagnosed type 2 diabetic subjects from the UK Prospective Diabetes Study followed prospectively (median 14.0 years), using proportional hazards models, adjusted for sex, ethnicity and other known or putative risk factors.

Results rs12704795 genotypes were associated with differing rates of development of microalbuminuria (relative risk [RR] for CC vs AA homozygotes 0.68 [95% CI 0.54–0.87], $p=0.002$) but not other measures of worsening renal function. Heterozygotes for C311S were more likely to develop microalbuminuria (RR=1.31 [95% CI 1.11–1.54], $p=0.001$) but less likely to double creatinine levels during follow-up (RR=0.49 [95% CI 0.27–0.89], $p=0.02$). There was no corroboration of this latter association for related outcomes and no prior evidence supports heterosis effects at this locus.

Conclusions/interpretation We conclude that the *PON2* variants typed in this study have, at best, a small effect on the risk of renal dysfunction in type 2 diabetes.

Keywords Diabetic nephropathy · *PON2* · Type 2 diabetes · UKPDS

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Abbreviations

CVD	cardiovascular disease
FPG	fasting plasma glucose
HR	hazard ratio
LD	linkage disequilibrium
PON	paraoxonase
RR	relative risk
SBP	systolic blood pressure
SNP	single nucleotide polymorphism
UKPDS	UK Prospective Diabetes Study

Introduction

Diabetic nephropathy associated with type 2 diabetes is the most frequent cause of end-stage renal disease in the

USA, Europe and Japan. In the USA, the incidence of diabetic nephropathy has increased by 150% in the past ten years and, in North America, 40% of incident dialysis subjects in 1998 had diabetic nephropathy [1]. Published results suggest that genetic factors contribute to the risk of development of diabetic nephropathy, independently of known risk factors such as hyperglycaemia and hypertension [2], but studies designed to identify the variants responsible are often hampered by small sample size, their cross-sectional nature and insufficient duration of follow-up.

The gene encoding paraoxonase 2 (*PON2*) is a member of the PON gene cluster on chromosome 7q21.3, which also includes the homologous genes for PON1 and PON3 [3, 4]. Ubiquitously expressed, the function of PON2 remains unclear [4], although, like PON1, it is known to have antioxidant properties [5]. Cells over expressing PON2 show significantly reduced intracellular oxidative stress in response to treatment with hydrogen peroxide or oxidised phospholipids [5]. Oxidative stress has been implicated in the pathophysiology of diabetic microvascular complications including nephropathy [6–8].

Recently, there have been reports of associations between non-synonymous coding variants within the *PON2* gene and diabetic nephropathy, in both type 1 and type 2 diabetes [9, 10]. However, these studies had small sample sizes, raising doubts about the robustness of these findings [11]. We have used the UK Prospective Diabetes Study (UKPDS) patient cohort to examine the relationship between indices of renal dysfunction and the two coding non-synonymous *PON2* single nucleotide polymorphisms (SNPs) (A148G/rs11545942 and C311S/rs6954345) which have been the focus of previous association studies in diabetic nephropathy and cardiovascular disease (CVD). We report also data for one intronic SNP (rs12704795) selected because of its capacity to tag additional haplotypes within the gene.

Subjects and methods

Subjects

The UKPDS recruitment and selection process has been reported previously [12]. The study received ethical committee approval in all 23 clinical centres and conformed to the guidelines of the Declarations of Helsinki (1975 and 1983). Of 7,616 subjects referred with newly diagnosed type 2 diabetes, 5,102 entered the study between 1977 and 1991, all of whom gave informed consent to participate. The main reasons for exclusion were: (1) ketonuria >3.0 mmol/l; (2) serum creatinine >175 µmol/l; and (3) severe previous illness that would limit life or require

extensive systemic treatment. The 5,102 recruited were aged 25–65 years and had fasting plasma glucose (FPG) levels >6.0 mmol/l on two occasions after referral. Of these, 81% were self-reported white Caucasians, 10% Indian Asian and 9% Afro-Caribbean. After a 3-month dietary run-in period, therapies for diabetes were allocated according to the UKPDS protocol [12] and subjects followed 3-monthly in UKPDS clinics for up to 20 years (median 14.0 years). DNA samples were available for 4,134 of 5,102 UKPDS subjects representative of the full UKPDS cohort at baseline. For 760 of these we have been consistently unable to generate valid genotypes in this study; thus there were 3,374 subjects from whom we were able to obtain at least one valid genotype passing quality control.

Biochemical and clinical measurements

FPG was measured quarterly after an overnight fast from 22.00 h, HbA_{1c} annually by HPLC (Biorad Diamat Automated Glycosylated Haemoglobin Analyser, Hemel Hempstead, UK; normal reference range 4.5–6.2%) and plasma triacylglycerol annually by the UKPDS central laboratory as reported previously [13]. Urinary albumin was measured annually on single early morning urine samples [14]. Local NHS clinical chemistry laboratories undertook annual plasma creatinine measurements and triennial white cell counts.

UKPDS blood pressure measurements were made with an automatic sphygmomanometer (mercury sphygmomanometer in subjects with atrial fibrillation) with a medium or large cuff, as appropriate, taking the mean of the last three readings (of a minimum of four) with a CV <15%. Baseline data are those recorded at the end of the dietary run-in period. If an item was missing, a value recorded at entry to the study or during the dietary run-in period was used. For the purposes of this study, baseline is taken to be equivalent to the time of diagnosis of type 2 diabetes.

Retinopathy was defined as an Early Treatment for Diabetic Retinopathy Study ‘191’ score ≥20, a history of retinal photocoagulation or previous vitreous haemorrhage [15]. Neuropathy was defined as both ankle jerks or both knee jerks absent or a vibration perception threshold >25 V for both great toes. Where information was missing from a limb because of a diabetes-related amputation, worst-case values were imputed. Where information was missing from a limb because of a non-diabetes-related amputation, the values available for the other limb were substituted. CVD was defined as Minnesota-coded ECG evidence of a myocardial infarction or a history of myocardial infarction, angina or a transient ischaemic attack.

Hypertension was defined as a systolic blood pressure (SBP) ≥140 mmHg and/or diastolic ≥90 mmHg and/or on antihypertensive therapy. Patients were classified according

to whether they had ever taken antihypertensive therapy during the course of the study. Microalbuminuria was defined as urine albumin values ≥ 50 but < 300 mg/l and macroalbuminuria as values ≥ 300 mg/l. Progression to microalbuminuria included subjects who moved directly from normoalbuminuria to macroalbuminuria. Glomerular filtration rate was estimated using the Cockcroft–Gault formula. Event times for microalbuminuria, macroalbuminuria and plasma creatinine doubling were taken as the first year at which they occurred, provided the next assessment confirmed the same or worse.

Genotyping

The two coding non-synonymous SNPs (A148G/rs11545942 and C311S/rs6954345) were typed using fluorescence polarisation (FP) assays. After initial PCR (primer sequences and amplification conditions available from the authors), amplicons were incubated with shrimp alkaline phosphatase and exonuclease I, followed by a final primer extension reaction using AcycloPrime-FP SNP detection kits (Perkin-Elmer) with slight modifications. Reactions were incubated for 10–40 cycles until sufficient differences between the negative controls and samples were achieved and the clearest fluorescent signal was obtained for the assignment of genotypes. The remaining SNP (rs12704795 in intron 1) was genotyped using TaqMan assays (primers and conditions available from the authors) detected using an ABI PRISM 7900HT Sequence Detector (Applied Biosystems) following the manufacturer's instructions for an endpoint plate read. Based on data from 23 European subjects deep-resequenced for *PON2* (Seattle SNPs, <http://pga.mbt.washington.edu/>), these SNPs capture at least 68% of all common ($> 5\%$ frequency) haplotypes in the gene. Note that one of these typed SNPs (rs11545942) is not represented in the Seattle SNP data. Based on duplicate genotype data for these samples, we estimate a genotype error level $< 1\%$.

Statistical analysis

SNPs were confirmed as being in Hardy–Weinberg equilibrium in each ethnic group separately ($p > 0.01$) using the HelixTree package (C.G. Lambert, HelixTree Genetics Analysis Software, Golden Helix, Inc. <http://www.goldenhelix.com/>). r^2 and D' measures of pairwise linkage disequilibrium (LD) were calculated for all SNP pairs. Haplotype frequencies for all three SNPs were estimated within ethnic groups using the expectation–maximisation algorithm implemented in HelixTree; haplotype (and diplotype) assignments were made for each individual, weighted according to their probabilities.

Statistical analyses were performed using SAS v8.2 and v9.1.3 (SAS Institute, Cary, NC, USA). Missing data values were interpolated from immediately preceding and subse-

quent values using linear methods for continuous data and last observation carried forward for categorical data to provide complete sequences of data for analysis. Data are reported as mean (with SD), geometric mean (with 1 SD interval), median (and interquartile range) or percentages. For continuous variables, comparison of multiple means was by ANOVA with a linear contrast test for trend across genotypes. For categorical variables, we used a chi-squared Cochran–Armitage test for trend across genotype categories. Where necessary, exact p values were obtained using Monte Carlo estimation.

Kaplan–Meier analysis of time-to-event with a log-rank test was used to assess differences in survival between genotypes. Proportional hazards models with discrete time intervals were used to estimate relative risks (RRs) as hazard ratios (HRs), reported with 95% CIs. Unadjusted models and models adjusted for associated risk factors and potential confounding variables using a stepwise selection procedure were used. Only variables with univariate p values < 0.05 were included in the stepwise model. Significance of the overall model was tested using a likelihood ratio test. Tests for heterogeneity of HRs were performed using a Wald test of the hypothesis of no difference between HRs.

Analyses were conducted in the entire data set (including ethnicity as a covariate) and in white Caucasians alone (81% of the 3,374). Models excluding prior retinopathy or CVD were also examined, since these could be interpreted as either risk factors or associated outcomes. Analyses stratified for variables differing at baseline were used in addition to investigate confounding.

To investigate the effects of SNPs acting in combination with one another on disease outcome, the stepwise proportional hazards modelling process was undertaken using the individual-specific haplotype assignments (made as described above), explicitly considering only those diplotypes with estimated frequencies $\geq 1\%$. Absolute risk rates are expressed as events per 1,000 person years. Throughout, we use a threshold of $p < 0.05$ to denote nominal significance. All significance values are quoted without correction for multiple testing since the complex correlation structure of both genotypes and outcome phenotypes makes appropriate correction difficult.

Given an average sample size of 3,200 and assuming a disease allele frequency of 30%, this study (with a median follow-up of 14 years and event rate of 40% [microalbuminuria] in the control group) would have a power of approximately 75% to detect an HR of 1.1 for a dominant gene effect. For an HR of 1.25 this study design has in excess of 90% power. These power calculations were performed using nQuery Advisor v4.0 assuming exponential survival distributions with unequal sample sizes.

Results

Patient characteristics, numbers of events and LD structure

Table 1 shows the demographic, clinical and biochemical characteristics and numbers of endpoints for the 3,374 subjects categorised by SNP genotype. SNPs rs11545942 (exon 5) and rs6954345 (exon 9) were in tight LD with each other in all three ethnic groups (minimum pairwise r^2 values of 0.97 [$D' > 0.98$]). SNP rs12704795 (intron 1) was poorly correlated with the other SNPs in all ethnic groups ($r^2 < 0.22$, although $D' > 0.97$). For all three SNPs, there were ethnic differences in genotype distribution ($p < 0.0001$ for each). The only other nominally significant association between genotype and baseline characteristics was observed between rs6954345 (exon 9) and age ($p_{\text{trend}} = 0.01$). Call rates for the three SNPs were rs12704795 intron 1: 94%; rs11545942 exon 5: 86% and rs6954345 exon 9: 97%.

Progression to microalbuminuria or macroalbuminuria

Table 2 shows the RR, derived from the stepwise, adjusted proportional hazards model, for progression to microalbuminuria and macroalbuminuria with the most common genotype at each SNP as reference. Variables included in the models are listed in the footnotes. Significant associations between genotype and progression to microalbuminuria were evident for all three SNPs (p ranging from 0.0047 to 0.039). At rs12704795, there was a significantly reduced risk for microalbuminuria development for those with the CC genotype compared with AA homozygotes (0.68 [95% CI 0.54–0.87], $p = 0.002$) and CC compared with AC heterozygotes (0.72 [95% CI 0.38–1.00], $p = 0.047$), although the AA and AC groups did not differ significantly in risk ($p = 0.097$). When assessed in white Caucasian subjects only, the RR for CC vs AA was 0.74 (95% CI 0.57–0.96) ($p = 0.023$) and for AC vs AA was 0.88 (95% CI 0.73–1.06) ($p = 0.18$); heterogeneity $p = 0.068$. For the other two SNPs, the risk for microalbuminuria development was increased in heterozygote subjects compared with wild-type homozygotes but no significant differences between homozygote groups were evident. These associations were repeated in the analysis restricted to white Caucasian subjects only: at rs11545942 the RR for CG vs CC was 1.22 (95% CI 1.00–1.47) ($p = 0.045$), and at rs6954345 the RR for CG vs CC was 1.22 (95% CI 1.02–1.46) ($p = 0.030$). There were no significant relationships between genotypes at any of the SNPs and development of macroalbuminuria.

Doubling of plasma creatinine levels

Table 3 shows the RRs for plasma creatinine doubling by genotype. Variables included in the models are listed in

the footnotes. For plasma creatinine doubling, the two SNPs which were in strong LD (rs11545942 and rs6954345) showed a similar stepwise decrease in the point estimate of the RR with increasing numbers of copies of the rare allele (for example, 0.49 for heterozygotes and 0.18 for rare-allele homozygotes at rs6954345, both in comparison with the wild-type homozygote). However, in each case, only the comparison of wild-type homozygote with heterozygotes was close to or achieved the 5% threshold for nominal significance. When assessed in white Caucasian subjects only, the RRs for heterozygotes at the same SNP were 0.48 ($p = 0.034$) and for rare-allele homozygotes 0.22 ($p = 0.13$).

Reduced creatinine clearance

Analyses were undertaken using Cockcroft–Gault estimated creatinine clearance with a cut-off of 60 ml/min. As the results were essentially the same as for doubling of creatinine they are not shown.

Haplotype analyses

Not surprisingly, given the LD relationships described above, haplotypic analyses largely recapitulated the associations observed in the SNP analyses. There was no evidence to suggest that combined (or interactive) effects of alleles at multiple variants had any additional impact on the outcomes (data not shown).

Confounding factors

Examination of the variables included in the stepwise models revealed systematic differences in relation to the endpoints analysed. Ethnicity and sex were contributing factors for development of micro- and macroalbuminuria but not for plasma creatinine doubling. For albuminuria, the presence of diabetic complications was associated with risk for microalbuminuria, but not macroalbuminuria. Smoking status was associated only with albuminuria development. Blood pressure and glycaemic control, assessed by either FPG or HbA_{1c}, were significantly associated with all three endpoints. The RR for genotypes derived from the adjusted stepwise proportional hazards models (Tables 2 and 3) were not altered substantially by removing prior retinopathy and CVD from the models (results not shown).

Discussion

In the present study, we examined the contribution made by variation in the *PON2* gene to individual susceptibility to renal dysfunction. This particular gene was selected given

Table 1 Baseline demographic and clinical characteristics of patients with genotype information for three SNPs in *PON2*

	SNP								
	rs12704795, intron 1			rs11545942, exon 5			rs6954345, exon 9		
Genotype	AA	AC	CC	CC	CG	GG	CC	CG	GG
<i>n</i>	1,230	1,449	498	1,656	1,062	167	1,879	1,174	231
Age (years) ^a	52±9	52±9	52±9	52±9	52±9	51±9	52±9	52±9	51±9
Ethnicity (%)									
White Caucasian	73	84	92	84	78	72	84	77	74
Afro-Caribbean	11	6	4	8	8	6	8	8	6
Indian Asian	15	9	4	7	13	22	7	14	20
Other	1	1	0	1	1	0	1	1	0
Sex (% male)	58	59	58	58	59	59	58	59	61
Weight (kg) ^a	76±15	77±16	77±16	77±16	76±16	77±15	77±16	77±16	76±15
BMI (kg/m ²) ^a	27.3±5.2	27.5±5.3	27.5±5.2	27.4±5.3	27.4±5.2	27.3±4.9	27.6±5.3	27.3±5.2	27.1±5.2
FPG (mmol/l) ^c	8.2	8.2	8.0	8.2	8.1	8.2	8.2	8.1	8.2
	(6.8–10.8)	(6.6–10.9)	(6.9–10.7)	(6.8–10.6)	(6.6–11.1)	(6.9–11.7)	(6.7–10.6)	(6.6–11.1)	(7.1–11.5)
<7.0 mmol/l (%)	26	29	27	26	30	26	27	30	24
≥7.0–9.9 mmol/l (%)	43	39	44	44	38	42	43	38	43
≥10.0 mmol/l (%)	31	32	29	30	32	32	30	32	33
HbA _{1c} (%)	6.9	6.9	6.7	6.9	6.8	7.1	6.9	6.8	7.1
	(6.0–8.1)	(5.9–8.1)	(5.8–7.8)	(5.9–8.0)	(5.9–8.0)	(6.0–8.5)	(5.9–8.1)	(5.9–8.0)	(6.0–8.5)
<6.0% (%)	24	25	28	25	26	24	25	26	23
≥6.0–7.9% (%)	47	46	48	48	46	41	47	46	43
≥8.0% (%)	29	29	24	27	28	35	28	28	34
Blood pressure (mmHg) ^a									
Systolic	134±19	135±20	133±18	135±19	133±20	132±18	135±19	134±20	132±18
Diastolic	83±11	82±10	82±10	83±10	82±11	83±10	83±10	82±11	82±10
Hypertensive (%)	43	42	40	43	42	40	43	42	40
On antihypertensive therapy (%)	19	19	17	18	19	20	18	19	16
Smoking status (%)									
Current	28	28	30	29	28	24	30	27	25
Ex-smoker	32	37	34	34	36	31	34	36	30
Never smoked	40	35	36	37	36	45	36	37	45
Urine albumin (mg/l) ^c	9 (4–22)	8 (4–18)	8 (4–16)	8 (4–19)	8 (4–19)	9 (4–18)	8 (4–18)	8 (4–19)	9 (4–19)
Plasma creatinine (μmol/l) ^c	81 (71–92)	81 (71–92)	80 (71–90)	81 (70–91)	81 (71–93)	81 (73–92)	81 (71–91)	81 (71–93)	81 (73–93)
Fasting plasma triacylglycerol (mmol/l) ^b	1.53	1.52	1.57	1.52	1.52	1.57	1.53	1.51	1.58
	(0.90–2.62)	(0.93–2.48)	(0.91–2.69)	(0.92–2.53)	(0.92–2.51)	(0.86–2.86)	(0.92–2.55)	(0.91–2.51)	(0.88–2.85)
White cell count (10 ⁹ /l) ^c	6.6	6.6	6.7	6.6	6.5	6.5	6.6	6.5	6.5
	(5.4–7.9)	(5.4–8.0)	(5.4–8.1)	(5.4–8.0)	(5.5–7.9)	(5.6–7.9)	(5.4–8.1)	(5.5–7.9)	(5.5–7.8)
Retinopathy, <i>n</i> =1,972 (%)	19	20	21	20	19	17	20	19	15
Neuropathy, <i>n</i> =2,808 (%)	20	21	19	21	19	21	21	20	20
Previous CVD, <i>n</i> =2,848 (%)	18	18	17	18	18	17	18	18	17
Development during study									
Microalbuminuria, <i>n</i> (%)	499 (41)	560 (39)	175 (35)	620 (37)	440 (41)	70 (42)	704 (37)	479 (41)	89 (39)
Macroalbuminuria, <i>n</i> (%)	153 (12)	175 (12)	51 (10)	194 (12)	123 (12)	20 (12)	221 (12)	141 (12)	25 (11)
Plasma creatinine doubling, <i>n</i> (%)	44 (4)	44 (3)	24 (5)	68 (4)	28 (3)	3 (2)	79 (4)	34 (3)	3 (1)

^a Mean (SD); ^b geometric mean (1 SD interval); ^c median (interquartile range)

Table 2 RRs for developing microalbuminuria or macroalbuminuria according to genotypes for three SNPs in *PON2*, adjusted for other risk factors or potentially confounding characteristics using a stepwise selection proportion hazards model

SNP	Microalbuminuria			Macroalbuminuria		
	No. events	RR (95% CI)	<i>p</i> value ^a	No. events	RR (95% CI)	<i>p</i> value ^a
rs12704795, intron 1 ^{b,d}						
Genotype AA (<i>n</i> =1,230)	499	1		153	1	
Genotype AC (<i>n</i> =1,449)	560	0.87 (0.74–1.03)	0.097	175	0.99 (0.74–1.33)	0.97
Genotype CC (<i>n</i> =498)	175	0.68 (0.54–0.87)	0.0020	51	0.66 (0.42–1.05)	0.078
<i>p</i> value for heterogeneity of RR			0.0069			0.18
rs11545942, exon 5 ^{c,e}						
Genotype CC (<i>n</i> =1,656)	620	1		194	1	
Genotype CG (<i>n</i> =1,062)	440	1.25 (1.05–1.48)	0.011	123	0.91 (0.67–1.24)	0.57
Genotype GG (<i>n</i> =1,67)	70	1.10 (0.79–1.54)	0.58	20	1.03 (0.58–1.85)	0.92
<i>p</i> value for heterogeneity of RR			0.039			0.83
rs6954345, exon 9 ^{b,f}						
Genotype CC (<i>n</i> =1,879)	704	1		221	1	
Genotype CG (<i>n</i> =1,174)	479	1.31 (1.11–1.54)	0.0011	141	0.96 (0.72–1.28)	0.78
Genotype GG (<i>n</i> =2,31)	89	1.10 (0.82–1.47)	0.53	25	0.78 (0.44–1.36)	0.38
<i>p</i> value for heterogeneity of RR			0.0047			0.67

^aWald chi-square *p* valueMicroalbuminuria: ^bcontrolling for sex, smoking status, SBP, BMI, FPG, previous CVD, ever being hypertensive, plasma triacylglycerol, previous retinopathy, white cell count; ^ccontrolling for ethnicity, sex, smoking status, SBP, weight, FPG, previous CVD, ever being hypertensive, plasma triacylglycerol, white cell countMacroalbuminuria: ^dcontrolling for ethnicity, sex, smoking status, BMI, SBP, HbA_{1c}, ever being hypertensive, plasma triacylglycerol; ^econtrolling for ethnicity, sex, smoking status, SBP, FPG, ever being hypertensive, plasma triacylglycerol;^fcontrolling for ethnicity, sex, smoking status, BMI, SBP, FPG, ever being hypertensive, plasma triacylglycerol

evidence that coding, non-synonymous variants within *PON2* were significantly associated with diabetic nephropathy in both type 1 and type 2 diabetes [9, 10]. However, as is the norm where complex trait associations have been sought in small sample sizes, these data remain far from conclusive and not all studies have succeeded in providing replication [16]. Here, we set out to make use of the large sample size and long duration of follow-up available in the UKPDS study to clarify the role of *PON2* variants in determining risk of renal dysfunction.

We focused our efforts on the two coding SNPs previously studied (A148G/rs11545942 and C311S/rs6954345). In addition, we typed a further intronic SNP (rs12704795), chosen on the basis of limited sequence data available at the time (prior to data release from the International HapMap Consortium), to provide additional capture (tagging) of common variation within the gene. In total, these three SNPs capture close to 70% of common variation within the gene. We recognise therefore that the present study does not represent a comprehensive assessment of variation with *PON2* and the immediate region.

In surveying the large number of tests performed, there are two main groups of findings that attain nominal significance ($p < 0.05$). One association involves rs12740795, at which individuals with the CC genotype have markedly reduced risk of microalbuminuria compared

with those with other genotypes. The data for progression to macroalbuminuria show similar point estimates of the effect size, but the numbers of events are small and these results did not approach thresholds for nominal significance. There is no substantiation of these findings from analyses of longitudinal changes in creatinine levels. When analyses were restricted to the white Caucasian subjects only, similar point estimates of the effect size were observed, but CIs were wider, and significance values less extreme, as compared with analyses of all subjects adjusted for ethnicity. These findings are consistent with homogeneity of effect size between the different ethnic groups, since the white Caucasian subset (81% of all subjects) generated an equivalent proportion of the incident events (81% for microalbuminuria, 77% for macroalbuminuria and 85% for doubling of creatinine), while the wider CIs and reduced significance levels reflect the smaller sample size. The association with development of microalbuminuria is novel (since other groups have not examined this SNP before) but relatively weak in the light of the number of tests performed. It is conceivable (although unlikely given the LD relationships) that both our finding at rs12740795 and the previous reports at C311S reflect associations with an aetiological SNP, as yet untyped, within *PON2* or the other adjacent PON genes. For all these reasons we do not regard these findings at rs12740795 as a replication of the

Table 3 RRs for doubling of plasma creatinine according to genotypes for three SNPs in *PON2*, adjusted for other risk factors or potentially confounding characteristics using a stepwise selection proportion hazards model

SNP	Plasma creatinine doubling		
	No. events	RR (95% CI)	<i>p</i> value ^a
rs12704795, intron 1 ^b			
Genotype AA (<i>n</i> =1,230)	44	1	
Genotype AC (<i>n</i> =1,449)	44	1.07 (0.61–1.89)	0.81
Genotype CC (<i>n</i> =498)	24	1.04 (0.48–2.28)	0.92
<i>p</i> value for heterogeneity of RR			0.97
rs11545942, exon 5 ^b			
Genotype CC (<i>n</i> =1,656)	68	1	
Genotype CG (<i>n</i> =1,062)	28	0.52 (0.27–1.01)	0.053
Genotype GG (<i>n</i> =167)	3	0.28 (0.04–2.06)	0.21
<i>p</i> value for heterogeneity of RR			0.084
rs6954345, exon 9 ^b			
Genotype CC (<i>n</i> =1,879)	79	1	
Genotype CG (<i>n</i> =1,174)	34	0.49 (0.27–0.89)	0.020
Genotype GG (<i>n</i> =231)	3	0.18 (0.02–1.30)	0.089
<i>p</i> value for heterogeneity of RR			0.020

^aWald chi-square *p* value

^bControlling for FPG and systolic BP

previous reports of positive associations at this gene. It is a distinct finding, itself deserving of attempted replication in other samples informative for these outcomes.

The second set of nominally significant results involves the other two SNPs. Not surprisingly, given tight LD between these markers (as reflected in mutual r^2 values exceeding 0.97), both SNPs tell a similar story, best exemplified by the results at rs6954345 (C311S), one of the coding variants studied previously. Here, there is an association between the CG heterozygote and increased risk of microalbuminuria (RR=1.31, $p=0.0011$, compared with the CC common homozygote). However, no increase in RR was observed for the rare GG homozygote (the point estimate is very close to 1, albeit with wide CIs), nor was there any hint of a relationship between any of the genotypes at this SNP and progression to macroalbuminuria. There is a relatively weak, although nominally significant, relationship between rs6954345 genotype and doubling of creatinine, which again features heterozygotes as the outgroup, although these effects lie in the opposite direction (with respect to progression to nephropathy). Since the data from Pinizzotto et al. [9] indicated that nephropathy was more frequent amongst carriers of the minor allele at C311S and A148G (that is with the combined CG/GG genotype groups in the notation used in the present study), the nominal association with doubling of

creatinine runs counter to that which could be considered consistent with those previous findings.

These results have to be interpreted with caution. First, while there exist several established examples of heterosis in the genetics literature, e.g. the excessive role of HLA DR3/DR4 heterozygosity in susceptibility to type 1 diabetes [17], such instances are exceptional. In the absence of prior evidence supporting such a model, replication is essential before any claims of biological significance are made. Second, it is important to notice that heterozygotes for C311S have increased risk of developing microalbuminuria but reduced risk of creatinine increase. A possible post-hoc explanation of such a finding is that this genotype is enriched amongst individuals who are prone to development of proteinuria but protected from a decrease in renal function [18, 19] although no biological significance can be ascribed without clear directed replication.

The strengths of the current study are the large size of the cohort sampled combined with the strength of the prospective, longitudinal characterisation of the cohort for renal function and other variables. The clear and consistent relationships with known risk factors for renal dysfunction (glycaemic control, blood pressure, ethnicity) are testament to the quality and accuracy of the phenotypes available, and the likely generalisability of the findings.

Potential weaknesses include the genotyping success rates, which reflect the age of some of the DNA samples. However, the tight LD registered between several of the variants, as well as other measures of genotyping fidelity in these samples, indicates that typing was of good quality. The cohort design partly protects against the danger of biases arising from ‘informative missing data’, since these require the missing data to be non-random with respect to both genotype and phenotype. In this data set, we found no evidence of marked departures from Hardy–Weinberg equilibrium (within ethnic strata) and no evidence that untyped samples differed with respect to the outcome variables of interest.

In addition, this study focused on the two coding non-synonymous SNPs with previous associations, and has not attempted comprehensive tagging of all common variation within *PON2* (and adjacent homologues). Nevertheless, the three SNPs do tag around 70% of common variation within the gene. Finally, although two of the SNPs (rs11545942 and rs6954345) result in non-synonymous amino acid substitution in *PON2* protein sequence (and therefore reasonable prospects for a functional effect), absence of measurements of PON activity precludes the use of such direct intermediate trait information to assist with the inference of biological relationships and causation.

In summary, our data do not provide a clear pattern of association between *PON2* variation and risk of renal dysfunction which is consistent across different measures

of progression. Certain of the nominally significant findings are of interest, and these justify future efforts to obtain a comprehensive view of the role of local genome sequence variation on nephropathy risk. These studies should include not only *PON2*, but also *PON1* and any other adjacent candidates. They will require sample sizes in the thousands (at least as large as the current study) if robust findings are to be obtained. Pending such future studies, we can conclude that the *PON2* variants we have typed (and those that they tag) have at best only a modest effect on the development of renal dysfunction in subjects with type 2 diabetes.

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