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The Haptoglobin genotype predicts cardio-renal mortality in type 1 diabetes

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Abstract

Aims—The Haptoglobin (*HP*) 2-2 genotype increases cardiovascular diabetes complication incidence. In type 1 diabetes, *HP* 2-2 also predicts declining kidney function and end-stage renal disease. We investigated whether *HP* 2-2 predisposes to cardio-renal mortality, while considering other causes of death as competing risks.

Methods—Individuals with type 1 diabetes and *HP* data available (n=486; mean baseline age, 27 and duration, 19 years) were selected for study. Vital status was assessed as of 8/31/2014. The underlying cause of death was determined and classified based on standardized procedures.

Results—During 25 years of follow-up, 79 (16.3%) cardio-renal deaths and 43 (8.8%) deaths related to other causes occurred. Although total mortality did not differ by *HP* (25.4% with *HP* 1 versus 24.6% with *HP* 2-2, p=0.84), a greater proportion of *HP* 2-2 carriers exhibited a cardio-renal death (19.0 versus 14.2, p=0.05). In time-to-event analyses, *HP* 2-2 was associated with a statistically significant increase of the sub-distribution hazard ratio for cardio-renal mortality (HR=1.64, p=0.03), although this effect was somewhat attenuated after multivariable adjustment (HR=1.58, p=0.05).

Conclusions—Our results suggest that in addition to predicting the incidence of cardio-renal complications, *HP* 2-2 also increases susceptibility for cardio-renal mortality in type 1 diabetes. These findings require validation in other cohorts.

Keywords

Type 1 Diabetes; Mortality; Cardiovascular mortality; Kidney disease mortality; Haptoglobin genotype; Competing risk

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Duality of interest

The authors have no conflicts of interest.

Author Contributions

T.C. researched data and wrote the manuscript; T.J.O. researched data and reviewed/edited the manuscript. T.C. is the guarantor of this work.

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Introduction

Haptoglobin is an acute phase plasma glycoprotein whose name (from the Greek verb “ἅπτειν”, haptain, to bind) conveys its major function, binding free hemoglobin, thereby inhibiting heme iron release and thus reducing free hemoglobin’s oxidative potential (1). The haptoglobin-hemoglobin complex formed through this binding is subsequently primarily cleared from circulation by attaching to the CD163 receptor on monocytes/macrophages while a secondary pathway for excretion is through the kidneys (1). Two major haptoglobin alleles exist in humans: the *HP* 1 and the *HP* 2, the latter formed from the duplication of exons 3 and 4 of the *HP* 1 allele (1). As *HP* is a copy number variant (CNV), currently available GWAS versions cannot tag *HP* CNV (2–4). *HP* is a known functional polymorphism, however, with well described structural and functional differences across the genotypes. Thus, the *HP* 1-1 protein (dimer) has been ascribed superior antioxidant and anti-inflammatory properties compared to the other two genotypes (*HP* 2-1 and *HP* 2-2), whereas the *HP* 2 allele is a more efficient angiogenic factor (1, 5–6).

Several prospective studies of type 2 diabetes have provided evidence that the *HP* 2-2 genotype directly predicts the development of cardiovascular events (4, 7–10), likely owing to its inferior antioxidant capacity and inefficient clearance of hemoglobin (1, 5). To date, the role of this polymorphism in the pathogenesis of cardiovascular disease in type 1 diabetes remains largely unexplored. Our finding in the Epidemiology of Diabetes Complications (EDC) study of increased susceptibility to coronary artery disease (CAD) conferred by the *HP* 2 allele (11) has yet to be validated in other prospective cohorts, although a similarly increased risk was observed for the incidence of coronary artery calcification in the Coronary Artery Calcification in Type 1 Diabetes (CACTI) study (12). Despite the abundance of data rendering *HP* 2 as a risk factor for CAD, other EDC findings suggest a protective role for this allele against the extent of white matter hyperintensities (13) and stroke incidence (14). This contrasting finding may be mediated by the *HP* 2 allele’s enhanced angiogenic activity (1).

In contrast to heart disease for which abundant evidence exists in type 2 diabetes, prospective data for an association between the *HP* genotype and kidney disease appear thus far limited to studies in type 1 diabetes, where our observation of a twofold increased risk of declining kidney function and end-stage renal disease with *HP* 2-2 (15) was recently confirmed in the Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications (DCCT/EDIC) study (16). However, whether *HP* has the ability to predict cardio-renal – specific mortality in diabetes has yet to be evaluated. We therefore assessed the presence of such an association in a childhood-onset type 1 diabetes cohort recruited in the late 1980s and followed prospectively for 25 years.

Material and Methods

The Pittsburgh EDC, on which the present investigation was based, is a representative, historical, cohort of incident cases of childhood-onset type 1 diabetes, diagnosed or seen within one year of diagnosis (1950–80) at Children’s Hospital of Pittsburgh (17). A first clinical EDC assessment took place in 1986–88 (mean age, 28 and diabetes duration, 19

years). Surveys (biennial) and clinical examinations (biennial for 10 years and again at 18 and 25 years) followed for over 25 years. The University of Pittsburgh IRB approved the study protocol.

At each assessment, participants provided demographic, health care, self-care, and medical history information. During the clinical examinations, blood pressure was measured with a random zero sphygmomanometer, after a five minute rest. Hypertension was defined as >140/90 mmHg or antihypertensive medication use. Stable glycosylated hemoglobin (HbA1) was measured by ion exchange chromatography (Isolab, Akron, OH) and subsequently by automated high-performance liquid chromatography (Diamat, BioRad, Hercules, CA). The two assays were highly correlated ($r=0.95$). HDL cholesterol (HDLc) was determined by a precipitation technique (18); cholesterol and triglycerides enzymatically (19–20); and non-HDLc was calculated as total minus HDLc. Urinary albumin was measured by immunonephelometry (21) and creatinine was assayed by an Ectachem 400 Analyzer (Eastman Kodak Co., Rochester, NY). Glomerular filtration rate was estimated (eGFR) (22). High molecular weight genomic DNA was isolated using the PureGene kit (Gentra Systems, Minneapolis, MN), and *HP* was genotyped by an amplification method. Genotypes were assigned visually by comparison to controls of known genotype (23). We have previously shown good agreement between our genotyping and a sandwich enzyme immunoassay (EIA) for the haptoglobin gene product ($\kappa=0.92$, $p<0.0001$) (24).

Vital status was assessed as of August 31, 2014. In addition to death certificates, medical records, autopsy/coroner's reports, and/or interviews with next-of-kin regarding the circumstances surrounding the death were also obtained when possible. The underlying cause of death was determined and classified (i.e. cardiovascular, renal, acute complication, accident/suicide, infection, cancer, other diabetes related, or other non-diabetes related) by a Mortality Classification Committee of at least two physician epidemiologists using all available data, based on standardized procedures as previously published (25). For the present analysis, cardio-renal deaths comprised all cases for which the underlying cause was due to either cardiovascular or renal complications. The distribution of the underlying causes of death among deceased participants is displayed in Table 1.

Statistical analysis

Analyses were conducted using the SAS statistical software version 9.4. Differences in participant baseline characteristics by subsequent mortality status were assessed with simple descriptive statistics. Univariate associations were determined using parametric (linear regression) or non-parametric (the Kruskal-Wallis test) approaches for normally and non-normally distributed continuous variables, respectively, and the χ^2 -test for categorical variables. To evaluate the association between the *HP* genotype and cardio-renal mortality risk, deaths from causes other than cardiovascular or renal were treated as competing events. Although prior evidence suggested an inverse *HP* 2-2 – stroke association (14), six of the seven stroke deaths occurred among *HP* 2-2 carriers in this population and, therefore, stroke was not isolated from other cardiovascular causes of death. To analyze these competing risks data, procedure PHREG was used to model the cumulative incidence function, by

defining the sub-distribution hazard according to a method developed by Fine and Gray (26). To better account for the strong effect of diabetes duration on the risk of death, duration of diabetes was used as the time axis. Variables that were univariately associated with the outcome were included as covariates and a final model was produced after backward elimination. A graphic presentation of cardio-renal – specific cumulative mortality hazards was also obtained from the final multivariable competing risk model.

Results

Of 658 study participants, 486 had DNA available for *HP* genotyping. Compared to those without DNA, individuals with DNA available had shorter diabetes duration, lower HbA_{1c}, blood pressure, non-HDL, and AER levels, and higher eGFR levels. Approximately 12.1% and 43.4% were homozygous for the *HP* 1 and *HP* 2 allele, respectively. Given the small proportion of *HP* 1-1 carriers and previous observations that the greatest complication risk is associated with *HP* 2 homozygosity, all analyses were conducted comparing *HP* 2-2 carriers to individuals with at least one *HP* 1 allele.

During 25 years of follow-up, 122 (25.1%) deaths occurred: 79 attributed to cardio-renal complications and 43 due to other causes. Differences in baseline participant characteristics by survival status are shown in Table 2. As expected, deceased individuals were more likely to have been older at study initiation, and to have had longer diabetes duration, greater waist to hip ratio, and elevated non-HDLc, as well as increased albumin excretion rate and lower eGFR. They were also more likely to have been smokers, with a hypertension diagnosis. With the exception of a greater proportion of *HP* 2-2 carriers among individuals whose death was attributed to cardio-renal causes, no other differences were observed by cause of death. Thus, although total mortality did not differ by *HP* genotype [25.4% (11.8 per 1,000 person-years) in *HP* 1 allele versus 24.6% (11.4 per 1,000 person-years) in *HP* 2-2 carriers, $p=0.84$], a greater proportion of *HP* 2-2 carriers exhibited a cardio-renal death [19.0% (8.8 per 1,000) vs. 14.2% (6.6 per 1,000), $p=0.05$].

To further explore these findings, we assessed differences in the proportion of participants with “any mention” of cardiovascular or renal complications as playing a role in the death (whether as the primary cause of death or a secondary cause) by *HP* genotype (Table 1). Strikingly, all but 3.8% of deaths among *HP* 2-2 carriers had a cardio-renal component to their death, whereas the proportion without a cardio-renal complication among carriers of at least one *HP* 1 allele exceeded 27% ($p=0.0006$).

Complete covariate information for time-to-event analyses was available in 463 participants. Treating death from other causes as a competing event, the *HP* 2-2 genotype was associated with a 64% (95% CI: 1.05–2.57) increased risk of cardio-renal mortality (Table 3), whereas carrying at least one *HP* 1 allele was associated with a 39% (95% CI: 0.39–0.95) risk reduction. Allowing for univariately significant risk factors, a 58% increased risk of cardio-renal mortality was still apparent among *HP* 2-2 carriers (with a concomitant 37% lower risk among *HP* 1-1/2-1 carriers); however, the statistical significance of this effect was reduced ($p=0.05$). In contrast, carrying at least one *HP* 1 allele was associated with a twofold greater risk of death from other causes, while an inverse association was observed for *HP* 2-2

(HR=0.47, $p=0.06$). Figure 1 depicts the adjusted cumulative incidence of death due to cardio-renal causes, treating death due to other causes as competing events, by *HP* genotype.

To exclude the possibility that survival bias may have led to a spurious association, we repeated the analysis restricting the sample to individuals having been diagnosed with type 1 diabetes after 1965. This diagnosis year was selected because mortality before study initiation was only 13% for those diagnosed with type 1 diabetes after 1965 versus 40% when the participant's diagnosis of diabetes occurred before 1965. Despite the considerably smaller number of events (27 cardio-renal deaths and 21 due to other causes), a 79% increased hazard of cardio-renal mortality was observed among *HP* 2-2 carriers, although results did not reach statistical significance (95% CI: 0.83–3.87, $p=0.14$).

Discussion

In this study of individuals with childhood-onset type 1 diabetes followed for a period of 25 years, the *HP* 2-2 genotype was associated with a 64% greater risk of mortality due to cardio-renal complications of diabetes. In contrast, carrying at least one copy of the *HP* 1 allele increased the risk of death from other causes, mainly comprising of acute complications and non-diabetes related causes. These associations were somewhat weakened after adjustment for factors relating to cardiovascular and renal disease outcomes, although, a 58% increased hazard of cardio-renal mortality associated with the *HP* 2-2 genotype remained apparent. Similar (though non-significant likely reflecting the small number of events), findings were obtained when the analyses were restricted to the more recently diagnosed EDC cohort, which would be less subject to survival bias.

The two classes of the haptoglobin protein are distinct in terms of their structure and function (1), differences which are thought to underlie the *HP* genotype association with diabetes-related vascular complications. Thus, the ability of the *HP* 2-2 – hemoglobin complex to structurally and functionally modify HDL, leading to impaired reverse cholesterol function (27–30), is thought as one of the pathways by which *HP* 2-2 increases the incidence of CAD in diabetes. In addition, the greater likelihood that the *HP* 2-2 – hemoglobin complex is cleared from the kidneys rather than by the usual CD163 monocyte/macrophage receptor, may lead to a concomitant increase in iron accumulation in renal proximal tubule cells, oxidative stress, and hypertrophy (1, 31). This is a suggested mechanism for the increased risk of declining kidney function and end-stage renal disease incidence among *HP* 2-2 carriers (15–16). In both cases, the main biologic mechanism triggering the greater *HP* 2-2 associated risk appears to relate to the potential for oxidative damage caused by the *HP* 2-2 – hemoglobin complex both because it contains redox active iron as well as because its steady state plasma concentration is increased compared to complexes formed by *HP* 1-1 (32).

Indeed, in the past decade, studies in humans have identified the *HP* 2-2 genotype as a major contributor to cardiovascular complications of both type 1 and type 2 diabetes (7–12). In type 1 diabetes, it has also been previously demonstrated that the *HP* 2 allele increases susceptibility to declining kidney function and is further associated with progression to end-stage renal disease (15–16). The strength and consistency of the observed associations

between the *HP* genotype and cardiovascular and kidney complications of diabetes undoubtedly generate questions regarding the ability of this genetic marker to predict risk of death. Herein, we provide evidence for the first time that the *HP* 2-2 genotype predicts cardio-renal mortality in a type 1 diabetes population.

The recognition of *HP* as a functional polymorphism, discerns these findings as of immense importance. Thus, the established structural and functional differences between the two *HP* allelic protein products, which are thought to mediate the *HP* association with vascular disease outcomes (1, 5), provide an opportunity for the development of targeted prevention strategies. To this point, three clinical trials in type 2 diabetes have suggested that supplementation with vitamin E reduces the increased cardiovascular susceptibility associated with the *HP* 2-2 genotype (33–35), potentially by ameliorating the noted HDL dysfunction (5) in this population subgroup. We have similarly shown in type 1 diabetes, that HDL-mediated cholesterol efflux appeared to worsen incrementally with the number of *HP* 2 alleles in a small type 1 diabetes intervention trial, while the daily administration of 400 IU α -tocopherol was shown to improve cholesterol efflux in the genetically susceptible *HP* 2-2 group (30). Given the lack of robust clinical trial data, however, no conclusions can be currently drawn on whether α -tocopherol supplementation may lower the incidence of cardiovascular disease in type 1 diabetes.

Prospective data on the *HP* genotype – kidney function association are also limited to results from the EDC and DCCT/EDIC studies in type 1 diabetes (15–16) while similar data from prospective studies in type 2 diabetes are completely lacking in humans. Nevertheless, investigators have previously reported significantly increased glomerular and proximal tubular hypertrophy in mice with diabetes and the *HP* 2-2 genotype (31, 36). Interestingly, administration of vitamin E was shown to yield benefits in terms of kidney disease in diabetic mice carrying the *HP* 2-2 genotype, slowing disease progression (31) and reducing the oxidation of the lysosomes (36). However, supplementation had no effects among diabetic mice carrying the *HP* 1-1 genotype.

A limitation of most studies having thus far focused on *HP*, including the present investigation, lies on the relatively small sample analyses of genetic data are based upon. It should, however, be pointed out that the EDC (11, 15), CACTI (12) and DCCT/EDIC (16), comprise three of the largest type 1 diabetes cohorts currently available in the United States. Another weakness is the availability of DNA for *HP* typing in only 75% of the study population. However, a significantly greater proportion of those with DNA available were diagnosed with type 1 diabetes after 1965, where mortality before study initiation was significantly lower (13%) compared to those diagnosed before 1965 (40%). We therefore believe this does not represent a major weakness, as a greater *HP* 2-2 effect on cardio-renal mortality was observed in the subgroup diagnosed with type 1 diabetes post 1965, despite the reduced sample size. An additional limitation may pertain to the assessment of the *HP* genotype. Although the optimal way of determining genotyping and phenotyping has not definitively been established, in our internal analysis there was good agreement between our genotyping and an EIA assay for *HP* (24). DCCT/EDIC investigators typing for *HP* genotype both by polyacrylamide gel electrophoresis (PAGE) and ELISA also observed

good agreement, with differences noted in a small proportion (0.38%) of samples analyzed (16).

Undeniably, further mechanistic data, as well as evidence from prospective studies, are required to confirm the role of the *HP* genotype in the pathophysiology of cardiovascular and, especially, renal complications of diabetes. Such evidence must be urgently attained given the large proportion of individuals with diabetes affected (greater than 40% of the type 1 and ~36% of the type 2 diabetes population are homozygous for the *HP* 2 allele) and, importantly, given the potential this functional polymorphism offers for prevention.

Conclusion

In conclusion, findings from the present investigation suggest that in addition to predicting the incidence of cardio-renal complications, the *HP* 2-2 genotype also increases susceptibility for cardio-renal mortality among individuals with type 1 diabetes. These results require validation in other cohorts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CACTI	Coronary Artery Calcification in Type 1 Diabetes
CNV	Copy number variant
CAD	Coronary artery disease
DCCT/EDIC	Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications
EDC	Epidemiology of Diabetes Complications
eGFR	Estimated Glomerular Filtration Rate
GWAS	Genome-Wide Association Study
HDLc	High Density Lipoprotein cholesterol
HP	Haptoglobin genotype
HR	Hazard Ratio

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Highlights

- The Haptoglobin (*HP*) 2-2 genotype predicts cardiovascular disease incidence among individuals with type 1 diabetes.
- The *HP* 2-2 genotype predicts declining kidney function and end-stage renal disease among individuals with type 1 diabetes.
- The *HP* 2-2 genotype also increases susceptibility for cardio-renal mortality in type 1 diabetes.

Figure 1.

Cumulative incidence of death due to cardio-renal causes by Haptoglobin genotype, treating death due to other causes as competing events and allowing for waist to hip ratio, smoking status, HbA1c, insulin per weight, hypertension, HDL and non-HDL cholesterol, albumin excretion rate, and estimated glomerular filtration rate

Table 1

Distribution of the underlying and/or secondary causes of death among deceased (n=122)

	Total cohort (n=122)	HP 1-1/2-1 (n=70)	HP 2-2 (n=52)
<u><i>Underlying cause (% , n)</i></u>			
Cardio-renal complications	64.7 (79)	55.7 (39)	76.9 (40)
Cardiovascular (n)	63	30	33
Renal (n)	16	9	7
Acute complications	9.8 (12)	14.3 (10)	3.8 (2)
Diabetic ketoacidosis (n)	5	5	
Diabetic coma (n)	2	2	
Hypoglycemia (n)	5	3	2
Accident/suicide	4.9 (6)	5.7 (4)	3.8 (2)
Infection	10.7 (13)	10.0 (7)	11.5 (6)
Cancer	1.6 (2)	2.9 (2)	0.0 (0)
Other/Non-diabetes related/Unknown	4.9 (6)	7.1 (5)	1.9 (1)
Hemorrhagic gastritis/ulcer (n)	1	0	1
Respiratory failure (n)	1	1	0
Multiple sclerosis (n)	1	1	0
Unknown (n)	3	3	0
Other diabetes related	3.3 (4)	4.3 (3)	1.9 (1)
<u><i>Underlying or secondary cause (% , n)</i></u>			
Cardio-renal complications	82.8 (101)	72.9 (51)	96.1 (50)
Cardiovascular	75.4 (92)	70.0 (49)	82.7 (43)
Renal	45.1 (55)	35.7 (25)	57.7 (30)
Other (i.e. no mention of cardio-renal complications)	17.2 (21)	27.1 (19)	3.8 (2)

Table 2

Baseline participant characteristics by mortality status during 25 years of follow-up in the Pittsburgh EDC study

Participant characteristics	Alive (n=364)	Mortality status Cardio-renal death (n=79)	Other death (n=43)	p-value
Age (years)	25.8 (7.4)	32.9 (6.5)*	30.3 (8.5)*	<0.0001
Age at onset (years)	8.4 (4.1)	8.1 (4.0)	8.7 (4.0)	0.72
Diabetes duration (years)	17.4 (6.9)	24.8 (6.8)*	21.6 (8.5)*	<0.0001
Percent female	50.8 (185)	44.3 (35)	41.9 (18)	0.36
Body mass index (kg/m ²)	23.4 (3.2)	24.2 (3.4)	23.5 (4.1)	0.15
Waist to hip ratio (n=362;79;42)	0.82 (0.76, 0.86)	0.86 (0.80, 0.92)	0.86 (0.80, 0.89)	<0.0001
Ever smoker (% , n)	30.8 (112)	51.9 (41)	51.2 (22)	0.0002
HbA _{1c} (n=363;78;42)				
(%)	8.6 (1.4)	8.9 (1.5)	8.9 (1.7)	0.10
(mmol/mol)	70 (15.3)	74 (16.4)	74 (18.6)	0.10
Insulin dose/weight (n=351;77;42)	0.79 (0.65, 0.94)	0.71 (0.57, 0.88)	0.65 (0.54, 0.86)	0.007
Systolic blood pressure (mmHg)	110.0 (12.2)	122.1 (20.2)*	119.9 (19.0)*	<0.0001
Diastolic blood pressure (mmHg)	70.8 (10.1)	76.9 (11.7)*	75.8 (11.3)	<0.0001
Hypertension (% , n)	9.3 (34)	34.2 (27)	30.2 (13)	<0.0001
HDL cholesterol (mmol/L, n=363;78;42)	1.4 (0.32)	1.3 (0.30)	1.4 (0.27)	0.09
Non-HDL cholesterol (mmol/L, n=363;78;42)	3.3 (0.95)	4.0 (1.1)*	3.9 (1.2)*	<0.0001
ACE/ARB use (% , n=354;78;43)	1.7 (6)	9.0 (7)	4.6 (2)	0.004
eGFR (mL/min/1.73 m ² , n=363;78;43)	108.9 (27.0)	85.8 (36.2)*	96.3 (35.7)*	<0.0001
Stages of chronic kidney disease (% , n)				
<30 mL/min/1.73 m ²	1.6 (6)	12.8 (10)	18.6 (8)	
30 – <60 mL/min/1.73 m ²	19.8 (72)	26.9 (21)	11.6 (5)	
60 – <90 mL/min/1.73 m ²	76.9 (279)	48.7 (38)	65.1 (28)	<0.0001
90 mL/min/1.73 m ²				
Albumin excretion rate (µg/min, n=364;78;43)	10.6 (6.4, 28.3)	282.9 (53.7, 742.6)	43.4 (8.4, 881.2)	<0.0001
HP genotype (% , n)				
1-1/2-1	74.5 (205)	14.2 (39)	11.3 (31)	
2-2	75.4 (159)	19.0 (40)	5.7 (12)	0.05

Note. Data presented are means (std), median (interquartile range) or percentages (n). ACE/ARB, Angiotensin Converting Enzyme/Angiotensin II Receptor Blockers; eGFR, estimated glomerular filtration rate; HDL, high density lipoprotein; HP, haptoglobin.

* Different from Alive at p<0.0167

† Different from Cardio-renal Death at p<0.0167

Table 3

Estimated effect of the *HP* 2-2 genotype on cardio-renal mortality using Fine and Gray sub-distribution proportional hazards models (n=463: 75 cardio-renal deaths; 39 deaths from other causes; 349 censored)

Haptoglobin genotype	Model 1 (unadjusted)		Model 2*	
	HR (95% CI)	p-value	HR (95% CI)	p-value
1-1/2-1	Referent		Referent	
2-2	1.64 (1.05 – 2.57)	0.03	1.58 (0.996 – 2.51)	0.05
AIC	774.317		737.644	

* Allowed for waist to hip ratio, smoking status, HbA1c, insulin per weight, hypertension, HDL and non-HDL cholesterol, albumin excretion rate (logarithmically transformed), and estimated glomerular filtration rate