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The author's reply

To the Editor: We read with interest the negative study of Canani et al. regarding the association between *PON2* polymorphisms and diabetic nephropathy in patients with Type I (insulin-dependent) diabetes mellitus, as well as the related letter. We have a few observations about their conclusions. As suggested by the authors [1], the results of association studies could be discordant because of the vulnerability of case-control studies to various biases and phenotype definitions. This is partly illustrated by the discordant results of these two case-control studies. First, our study, was carried out on patients with Type II (non-insulin-dependent) diabetes mellitus [2]. Second, the definition of diabetic nephropathy is very different: cases had persistent proteinuria or end-stage renal disease in the study of Type I diabetic patients and in our study, cases had either micro-albuminuria (83 % of the cases) or persistent proteinuria. Third, we observed that the susceptibility to diabetic nephropathy was enhanced by the degree of obesity. Unfortunately, information about the body mass index is not available in the study of Canani et al. The interaction between the body mass index and the risk of diabetic nephropathy-associated *PON2* polymorphisms in Type II diabetic patients could be the most important message of our study. Fourth, population stratification seems very unlikely because classical risk factors associated with diabetic nephropathy are present in our study (Table 1). Furthermore, microvascular and macrovascular complications in diabetes mellitus are both related to endothelial dysfunction. The definitions of these diabetic vascular complications remain controversial. The absence of consensus concerning definitions for these phenotypes is well illustrated by the recent article regarding the value of albumin excretion rate as predictor of diabetic nephropathy [3]. Initial studies have shown an approximate 80 % rate of progression from microalbuminuria to proteinuria in Type I diabetic patients. More recent studies have observed only a 30 to 45 % progression to proteinuria over 10 years and about a 40 % progression during the same period from normoalbuminuria to macroalbuminuria. Similar findings have been reported in Type II diabetes mellitus. Microalbuminuria seems to be more a marker of endothelial dysfunction than a risk factor for diabetic nephropathy. This marker is associated with macrovascular, and micro-vascular diabetic complications. Our observations suggest that *PON2* gene polymorphisms could be more closely related to endothelial dysfunction than to diabetic nephropathy and that PON cluster play a part in the oxidative stress pathway.

In conclusion, the discordant results between these two case-control studies could be explained by the different definitions of the diabetic nephropathy and the type of diabetes rather than a selection bias. Replication of these findings on other populations of Type II diabetic patients with the same definition for diabetic nephropathy is required to determine whether the *PON2* gene is a susceptibility factor for endothelial dysfunction or micro-albuminuria.

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Table 1. Clinical and biological characteristics of Type II diabetic patients with and without nephropathy

	Nephropathy + (n = 147)	Nephropathy – (n = 152)	p value
Age (years)*	63.0 (61.0–65.0)	56.0 (53.0–58.0)	0.0001
Diabetes duration (years)*	11.0 (8.1–15.0)	5. 2 (5.0–5.5)	0.0001
Male sex (%)	70.5	53.6	0.003
Smoker (%)	48.3	42.8	NS
HBP (%)	72.2	46.0	0.001
BMI (kg/m^2)*	29.1 (28.4–29.8)	28.0 (27.1–29.1)	0.0834
$HbA1_c$ (%)*	8.30 (7.90–8.73)	7.35 (7.10–7.74)	0.0001
HDL-chol (mmol/l)*	1.00 (0.94–1.08)	1.13 (1.07–1.20)	0.0022
Triglycerides (mmol/l)*	2.07 (1.90–2.27)	1.59 (1.47–1.80)	0.0003
LDL-chol.*	3.14 (2.83–3.34)	3.21 (2.96–3.44)	0.109
NS > 0.1			

* Findings given as median (95 % CI)

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Observation(s)

A novel mutation in islet amyloid polypeptide (IAPP) gene promoter is associated with Type II diabetes mellitus

To the Editor: Islet amyloid polypeptide (IAPP) is thought to be involved in the normal regulation of glucose metabolism because it is synthesized and co-released with insulin from pancreatic islet beta cells [1]. IAPP could have an important physiological role to play because it is the main constituent peptide of islet amyloid deposits, which are a characteristic feature of human Type II (non-insulin-dependent) diabetes mellitus [2]. The mechanisms responsible for the conversion of IAPP to insoluble fibrils, a biochemical characteristic of amyloidogenesis, are not known. An S20G missense mutation in exon 3 of the *IAPP* gene has been reported in 4.1 % of Japanese subjects with Type II diabetes [3]. Studies in COS-1 cells have shown that the S20G mutant amylin displays increased amyloidogenity and increased intracellular cytotoxicity compared with the wild-type amylin [4]. Overexpression of IAPP is also thought to be involved in islet amyloidogenesis and Type II diabetes. We previously demonstrated a higher stimulatory effect of glucose on IAPP than on insulin mRNA levels in human isolated islets [5]. Moreover, the secretory response of both peptides was dissociated, suggesting that overexpression is involved in secretory defects and could contribute to islet IAPP deposition. In addition, some models of transgenic mice overexpressing the human *IAPP* gene are able to form intracellular IAPP fibrils with the subsequent development of hyperglycaemia [6, 7].

We hypothesized that mutations in the *IAPP* promoter region could lead to abnormal regulation or expression of the gene and could, therefore, be associated with Type II diabetes. This study aimed to investigate the presence of mutations in the promoter region of the *IAPP* gene in a Spanish cohort of Type II diabetic patients. The total study population consisted of 316 unrelated Caucasian Spanish subjects. Altogether 186 Type II diabetic patients (98 men and 88 women, mean age 63 ± 10 years, with BMI 28.5 ± 5.3 Kg/m^2, duration of diabetes of 12 ± 9 years) were consecutively selected from our outpatient clinic. These patients fulfilled the World Health Organization criteria for Type II diabetes. The control group consisted of a sample of 130 healthy non-diabetic subjects (65 men and 65 women, mean age 50 ± 14 years, with BMI 26.6 ± 5.3 Kg/m^2), without a family history of diabetes, recruited from among patients' spouses and hospital staff. The study protocol was approved by the Ethics Committee of the Hospital Clinic of Barcelona and all subjects gave their informed consent to their participation in this study.

Mutations were screened by polymerase chain reaction (PCR) and single strand conformation polymorphism analysis (SSCP) and then confirmed by DNA sequence analysis. Four sets of primers were used to amply overlapping regions of the *IAPP* promoter from –571 bp to + 163 bp of the transcription start site. Primers A (-571 to –375): forward 5'-TCCCTGTCATATCTCTGGTA-3', reverse 5'-CCAAGTGACCTCAATGGCTG-3', primers B (-418 to –172): forward 5'-TATTCCTGAAGCTTCATGGG-3', reverse 5'-CGTAGCAAATACACAGTGT-3', primers C (-229 to + 38): forward 5'-ACTTCTGCTGTGTATGACACACCA-3', reverse 5'-GAGTCCAAGCTTGTATCCACTGGA-3', primers D (-85 to + 163): forward 5'-ATGACAGAGGCTCTCTGAGCT-3', reverse 5'-ACACCAAGTGTGCATTTCTCT-3'. We performed PCR in a 50 l volume containing 1.5 mmol/l $MgCl_2$, 0.2 mmol/l dNTPs, 1 μmol/l of each primer, 2.5 units of Taq DNA polymerase and 100 ng of genomic DNA. The PCR conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 1 min; annealing at 50–54 °C for 30 sec, and extension at 72 °C for 1 min; and a final extension at 72 °C for 10 min. The amplification products were examined by SSCP analysis and the samples showing an electrophoretic variant pattern were sequenced on a ABI 377 DNA sequencer.

Table 1. Allele and genotype frequencies of the G-to-A mutation in the promoter region of the IAPP gene according to glucose tolerance status

	Allele frequencies		Genotype frequencies	
	G	A	GG	GA
Control subjects	0.930	0.007	0.985	0.015
Type II diabetic patients	0.952	0.048[a]	0.903	0.097[b]

[a] A-allele between Type II diabetic vs control subjects, $p < 0.001$
[b] GA genotype between Type II diabetic vs control subjects, $p < 0.005$

We detected a single heterozygous mutation consisting of a G-to-A substitution at position –132 bp upstream from the transcription start site in 18 patients with Type II diabetes and in 2 control subjects. The frequency of the *GA* genotype was higher in the diabetic population than in control subjects: 9.7 % vs 1.5 %, $p < 0.005$, odds ratio: 6.85, 95 %-Confidence interval: 1.56–30.08 (Table 1). The distribution of genotypes did not differ from Hardy-Weinberg law expectations in the study group as a whole, including patients and control subjects. We did not find any clinical difference between diabetic subjects with and without the promoter variant. The two non-diabetic carriers, aged 26 and 35, did not have clinical antecedents of a family history of diabetes. Unfortunately, they did not consent to an OGTT.

To evaluate the possible contribution of the G-to-A mutation to the development of Type II diabetes, the carriers' families were studied. Altogether 14 first-degree relatives (mean