L. M. Maier · J. D. Cooper · N. Walker ·
D. J. Smyth · J. A. Todd

Comment to: Bionis-Lauber A, Boehm B, Lang-Muritano M et al. (2005) Association of childhood type 1 diabetes mellitus with a variant of *PAX4*: possible link to beta cell regenerative capacity. *Diabetologia* 48:900–905

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Abbreviations HWE: Hardy–Weinberg equilibrium · OR: odds ratio · SNP: single nucleotide polymorphism

To the Editor:

Bionis-Lauber et al. [1] recently reported evidence of an association between type 1 diabetes and a non-synonymous single nucleotide polymorphism (nsSNP) in *PAX4* (1168C>A, Pro321His, rs712701), in which the C allele was associated with susceptibility. The study was performed on a DNA collection from 249 type 1 diabetic patients and 424 control subjects from Switzerland, with allele frequencies for the C allele (Pro321) of 83 and 63% in type 1 diabetes cases and control subjects, respectively (derived C allele odds ratio [OR]=3.18, reported CC genotype OR=3.75, $p<0.0001$). The association found in this Swiss sample was replicated by Bionis-Lauber and colleagues in 130 type 1 diabetic patients and 646 control subjects from Germany, with allele frequencies for the C allele of 82% in cases and 65% in control subjects (derived C allele OR=2.42, reported CC genotype OR=3.75, $p=0.0001$). Based on their results from the genetic association study and functional experiments of the two alleles of the *PAX4* variant, including transrepression experiments and beta cell proliferation studies, the authors suggested that the *PAX4* locus may represent a link between susceptibility to development of type 1 diabetes and beta cell regenerative capacity.

We first noted that the genotype ORs may have been miscalculated by Bionis-Lauber and colleagues as we obtained different values from those reported (see Table 1 of [1]): according to our calculations, the ORs for the CC ge-

notype compared with the CA and AA genotypes were 7.28 ($p=3.4\times10^{-30}$) in the Swiss collection and 4.90 ($p=1.5\times10^{-15}$) in the German collection, and the ORs for the C allele compared with the A allele were 3.17 ($p=8.3\times10^{-17}$) in the Swiss collection and 2.42 ($p=1.1\times10^{-7}$) in the German collection.

Secondly, we noted that the genotypes deviated from Hardy–Weinberg equilibrium (HWE), although this was not reported. Using the genotyping data (see Table 1 of [1]) we estimated the p values against HWE to be in the order of 1×10^{-13} and 5.5×10^{-11} in the control subjects and type 1 diabetic patients from Switzerland, respectively, and 1.5×10^{-18} and 1.3×10^{-4} in the control subjects and type 1 diabetic patients from Germany, respectively. A χ^2 test was used to calculate HWE.

Deviation from HWE in control subjects/unaffected parents is often considered to indicate a problem with genotyping, such as genotyping errors (e.g. systematic misgenotyping of heterozygotes as homozygotes or vice versa, or non-randomness of missing data) and assay non-specificity, and violation of the HWE assumptions in a randomly mating population [2, 3]. However, deviation from HWE in cases/affected offspring could be evidence of a disease association of the variant. Assuming a sufficiently large random mating population, HWE testing provides a straightforward way to test for genotyping errors [2, 3]. However, HWE testing is frequently neglected—about 40% of published studies do not report whether the genotypes meet or violate HWE assumptions [4]. This is of particular concern in the study of complex disease genetics in which ORs are likely to be small and even modest HWE deviations/genotyping errors may severely impact inferences [4].

We attempted to replicate the association between type 1 diabetes and the *PAX4* variant (1168C>A, Pro321His, rs 712701) in large family collections (458 multiplex families from the UK, 318 multiplex families from the USA, 200 simplex families from Norway and 233 simplex families from Romania [<http://www-gene.cimr.cam.ac.uk/todd/dna-refs.shtml>, last accessed in July 2005]) and case–control collections (3,418 control subjects from the 1958 British

L. M. Maier (✉) · J. D. Cooper · N. Walker ·
D. J. Smyth · J. A. Todd
Juvenile Diabetes Research Foundation/Wellcome Trust
(JDRF/WT) Diabetes and Inflammation Laboratory,
Cambridge Institute for Medical Research,
Addenbrooke's Hospital, University of Cambridge,
Cambridge, CB2 2XY, UK
e-mail: lisa.maier@cimr.cam.ac.uk
Tel.: +44-1223-762105

Birth cohort [<http://www.cls.ioe.ac.uk/>, last accessed in July 2005] and 3,199 type 1 diabetes cases from the UK Genetic Resource Investigating Diabetes [GRID] study [<http://www-gene.cimr.cam.ac.uk/ucdr/grid.shtml>, last accessed in July 2005]). Genotyping was performed using TaqMan MGB probes (Applied Biosciences, Foster City, CA, USA) [5]. All genotyping data were double-scored by an independent researcher. The data were assessed for, and found to be in, HWE in parents and control subjects ($p>0.05$).

We found no evidence of an association between type 1 diabetes and the *PAX4* variant in either the family or case-control collections (Table 1). For the family-based analyses, we analysed 1,558 parent-child trios and obtained a genotype relative risk (CC vs AA and AC genotypes) of 0.92 (95% CI 0.79–1.06, $p=0.23$) and an allele relative risk (C) of 0.94 (95% CI 0.85–1.09, $p=0.58$). The analysis of 3,199 type 1 diabetes cases and 3,418 controls yielded a genotype OR (CC vs. AA and AC genotypes) of 1.08 (95% CI 0.98–1.21, $p=0.11$) and an allele OR (C) of 1.05 (95% CI 0.97–1.15, $p=0.18$). Case-control data were stratified by broad geographical region within the UK to reduce to a minimum any confounding due to variation in allele frequencies across the country [6].

To further reduce the possibility of genotyping error, we assessed the *PAX4* variant using a second independent technology (MegAllele genotyping reagents [ParAllele, San Diego, CA, USA] and GeneChip Tag arrays [Affymetrix, Santa Clara, CA, USA] using ParAllele's molecular inversion probes) [7] in 174 type 1 diabetes cases and 305 control subjects (unpublished observations). The concordance between the genotyping data obtained from the MegAllele and TaqMan methods were 97.7% in control subjects (seven discrepancies) and 99.43% in cases (one discrepancy).

We note that the frequency of the CC genotype in diabetic offspring and cases (both 62%; Table 1) is considerably lower in our samples than that reported by Biason-Lauber et al. (77% in Swiss cases and 72% in German cases). In our studies the frequency of the CC genotype in parents and control subjects was calculated to be 64 and 60%, respectively (Table 1); these values are considerably higher than those reported by Biason-Lauber et al. (31% in Swiss control subjects and 34% in German control subjects).

Clearly, there are differences between the collections reported in this study and those reported by Biason-Lauber et al. [1], such as sample size (in total, our case-control collection alone consisted of greater than eight times more type 1 diabetes cases and three times more control subjects); age (only schoolchildren were genotyped in [1]); populations (Swiss and German in [1] vs samples from the UK, USA, Norway and Romania in our study); and the availability of phenotypic parameters (e.g. anti-islet cell antibody positivity). However, given that our cases, control subjects and family sample all support HWE for this *PAX4* SNP, with no evidence of association with type 1 diabetes, we suspect the presence of a genotyping problem in the study of Biason-Lauber and colleagues [1] and conclude that the *PAX4* variant (1168C>A, Pro321His, rs712701) is unlikely to have a major effect in type 1 diabetes.

Table 1 Numbers of parent-child trios/subjects, minor allele frequencies and genotype frequencies of the 1168C>A/Pro321His/rs712701 variant in our study populations

Families	Parent-child trios with genotypes	MAF (%)	Genotype frequency (%) in parents and offspring						HWE p value		P_{TDT}	P_{GTRR}	T	NT	% T		
			Parents	Offspring		CC	AC		AA	Parents						Offspring	
				Parents	Offspring		Parents	Offspring									
UK	704	19.68	20.64	64.52	62.79	31.59	33.13	3.89	6.08	0.98	0.75	0.77	0.56	202	208	49.27	
USA	507	20.02	21.59	63.64	60.23	32.69	36.36	3.68	3.41	0.64	0.09	0.32	0.35	154	172	47.24	
Norway	160	19.02	19.54	66.57	66.31	28.82	28.30	4.61	5.39	0.06	0.05	0.11	0.15	51	36	58.62	
Romania	187	21.70	22.20	61.24	58.95	34.11	19.55	4.65	3.40	0.95	0.11	0.47	0.75	58	66	46.77	
Total	1,558	20.03	20.94	64.03	62.16	31.87	33.81	4.10	4.04	0.81	0.34	0.58	0.19	465	482	49.10	
Case-control	Subjects with genotypes	MAF (%)	Genotype frequency (%)			HWE p value		p value									
			CC	AC	AA												
Control subjects	3,418	22.50	60.36	34.35	5.30	0.41											
	3,199	21.60	62.24	32.32	5.44	0.01											

MAF minor allele frequency, P_{TDT} significance value obtained by transmission/disequilibrium test, P_{GTRR} significance value obtained for genotype relative risk analysis using a χ^2 test in a case-pseudo control framework, T number of transmissions of major allele, NT number of non-transmissions of major allele, $%T$, percentage transmission of major allele

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