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LETTER

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Reply to comment on: Bion-Laubert 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

To the Editor: Some concerns have been raised that the analytical methods we used for genotyping may be responsible for the striking differences observed in the distribution of the genotypes of the *PAX4* A1168C SNP, which deviated from Hardy–Weinberg equilibrium (HWE). In our certified laboratory (ISO/IEC 17025, accreditation STS286) both genotyping methods used (RFLP and allelic discrimination) were validated as described below.

Pre-analytical phase To check the influence of sample preparation (DNA extraction), transport and storage on the results, DNA extractions were performed on ten EDTA blood samples. DNA extraction was performed immediately after collection, 48 h after storage at room temperature in the dark (mimicking the average shipment time), and from blood spots on filter paper. All extractions were frozen, kept at -20°C for 7 days and then thawed before

analysis. The DNA extractions were performed as described previously [1].

Analytical phase (1) Precision. To check reproducibility, we performed ten analyses of the ten EDTA blood samples on the same day. We also assessed day-to-day precision by performing a single analysis of each of the ten samples on five different days. Laboratory precision was checked by a single analysis of each of the ten samples by two different operators. (2) Accuracy. Five DNA samples, received from an associated laboratory, with known genotype (unknown to us) were each assayed on the same day. (3) Re-finding. Although the results are qualitative, in order to exclude artefacts due to DNA concentration, ten samples were diluted 1:5, 1:10 and 1:100 and analysed on the same day. Linearity and detection limits were not tested in this qualitative assay.

Our acceptance criterion was set at <5% discrepancy. In case of a discrepancy either within one of the methods or between methods the discordant samples were analysed by sequencing of PCR-amplified exonic fragments of exon 9. As a reference, three DNAs representing the three genotypes (A/A, A/C and C/C) were used in every series. The reference sequences were CH236947.1 and AF043978. The discrepancy between the results of RFLP and sequencing was 3%, whereas that between allelic discrimination and sequencing was 2%.

All the case–control study samples were analysed by both methods. As an internal control the same three samples with genotype confirmed by sequencing for the three variants (A/A, A/C and C/C) plus a blank (no DNA) were used in every series. To check the validity of the method, we re-analysed the same ten samples per population every fourth series (every $30 \times 4 = 120$ analyses for RFLP, and $83 \times 4 = 332$ analyses for allelic discrimination). Since the batches of reagents are sufficient for 200 and 600 RFLP and allelic discrimination reactions, respectively, this procedure

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evaluates the influence of batch differences on the results. The discrepancy rate was comparable to that found in the validation series (2.9% for RFLP and 2.1% for allelic discrimination).

Post-analytical phase (interpretation) To decrease the possibility of biased interpretation of the results, we used two approaches. Firstly, all samples were analysed in a blind fashion—the samples were coded by numbers only. The code was revealed after completion of the analysis of the entire population (Swiss or German). For the German population, operator and code-breaker were two different individuals. Secondly, to minimise congruency errors (correspondence case-result) due to data transfer errors (e.g. errors in the ordering of data in the Excel tables), the two populations were analysed separately and the results were listed in separate tables. The chance that such errors occur repeatedly and still generate the same trend in distribution should be very low.

We realised that although we found an association between *PAX4* variants and type 1 diabetes, the data are not in HWE. Of particular concern were the ‘excess heterozygotes’ in the control group of both populations. Since deviation from HWE in this case is most likely caused by genotyping errors, we decided to address this issue using a third genotyping method, namely, sequencing (in addition to RFLP and allele discrimination, as described above and in [1]) to confirm our results. Sequencing produced similar results, suggesting that the genotyping was correct. The most probable explanation for this phenomenon is that the polymorphism represents a heterozygote advantage, one of the known causes of deviation from HWE (together with outbreeding, mutations, gene migration, genetic drift and non-random mating). This is not an isolated phenomenon as it has been previously described and discussed [2–6]. That the A/C genotype might confer a functional advantage is also confirmed by our expression studies. Although rather unlikely, there is always the possibility that a cryptic population stratification (a systematic difference in ancestry rather than association of genes with disease) might influence our case–control data and that the correlation between type 1 diabetes and *PAX4* observed in our populations, although representative of the average populations of these countries, might not be reproduced in other groups. Similarly, *PAX4* variants have been linked to type 2 diabetes in some ethnic groups but not in others [7, 8]. Thus, as underlined by A. Paterson, it is important to investigate other populations [9]. In this light, the data by Maier et al. [10], which are based on family studies, are very interesting. We are therefore currently studying other pop-

ulations (Italian patients and control subjects) to find out whether the ‘Swiss-German’ variant correlation will be confirmed.

Although diabetes remains a multifactorial disease and can by no means be ascribed to a variation in a single gene, the role of *PAX4* in the regeneration of beta cells is supported by our work [11] and indicates a link, at least in certain populations, between *PAX4* defects and defects in the regeneration of beta cells. Further studies, such as those outlined by M. Tiedge in his commentary on our study [12], are necessary and are currently underway to identify other components of this pathophysiological network.

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