Brief Genetics Report

No Evidence of Association of *ENPP1* Variants With Type 2 Diabetes or Obesity in a Study of 8,089 U.K. Caucasians

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Ectoenzyme nucleotide pyrophosphate phosphodiesterase 1 (*ENPP1*) is an inhibitor of insulin-induced activation of the insulin receptor. There is strong evidence from several previous studies that a common coding variant of *ENPP1* (K121Q) and a three-marker haplotype (Q121, IVS20delT-11, and G+1044TGA) are associated with type 2 diabetes and obesity. We examined the impact of *ENPP1* variation on type 2 diabetes and obesity in a large U.K. genetic association study. We genotyped the three previously associated polymorphisms in 2,363 type 2 diabetic case and 4,045 control subjects, as well as 1,681 subjects from 529 type 2 diabetic families. We used the same subjects for morbid and moderate obesity association studies. For type 2 diabetes, moderate and morbid obesity, and for both the Q121 and three-marker haplotype, our results exclude with >95% confidence the effect sizes from previous studies (Q121 allele: odds ratio 1.02 [95% CI 0.93–1.12], $P = 0.61$; 1.00 [0.85–1.18], $P = 0.99$; and 0.92 [0.70–1.20], $P = 0.41$; three-marker haplotype: 1.10 [0.96–1.26], $P = 0.17$; 0.97 [0.77–1.23], $P = 0.81$; and 0.86 [0.57–1.30], $P = 0.46$ for type 2 diabetes, moderate, and morbid obesity, respectively). A K121Q type 2 diabetes meta-analysis of all previously published studies remained significant after the inclusion of this study (1.25 [1.10–1.43], $P = 0.0007$), although there was some evidence of publication bias. In conclusion, we find no evidence that previously associated variants of *ENPP1* are associated with type 2 diabetes or obesity in the U.K. population. *Diabetes* 55:3175–3179, 2006

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SNP, single nucleotide polymorphism.

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Ectoenzyme nucleotide pyrophosphate phosphodiesterase 1 (*ENPP1*) is an excellent type 2 diabetes candidate gene. *ENPP1* is an inhibitor of insulin-induced activation of the insulin receptor (1), and its concentration is elevated in tissues from insulin-resistant subjects (2,3). *ENPP1* may therefore have a role in the development of insulin resistance and type 2 diabetes in humans.

There is strong evidence from several previous studies that common variation of the *ENPP1* gene predisposes to type 2 diabetes. A meta-analysis of nine studies, including a total of 2,834 case and 4,425 control subjects, suggested that the Q-allele of the K121Q nonsynonymous single nucleotide polymorphism (SNP) is associated with type 2 diabetes with an odds ratio (OR) of 1.29 (95% CI 1.09–1.53, $P = 0.003$) (4). A second recent study (5) of French and Austrian subjects, including a total of 1,255 case and 1,314 control subjects, found that a common three-marker haplotype, including the Q-allele at K121Q, is associated with type 2 diabetes with an OR of 1.56 (approximate 95% CI 1.27–1.92) versus the most common haplotype ($P = 0.00002$).

Additional data from the French study suggested that the same *ENPP1* variants associated with type 2 diabetes also associate with obesity. The three-marker haplotype was associated with morbid adult obesity (BMI >40 kg/m^2) combined with childhood obesity (>97th percentile) ($n = 2,430$; OR 1.58 [95% CI 1.28–1.95], $P = 0.00001$) and moderate obesity (717 subjects with a BMI 30–40 kg/m^2; 1.37 [1.06–1.69], $P = 0.02$). *ENPP1* variation partly explained the obesity linkage signal on chromosome 6q, and there was an overtransmission of the Q121 allele to obese subjects from heterozygous parents in 184 families ($P = 0.01$). Together, there is a strong case that variation of *ENPP1* predisposes to both type 2 diabetes and obesity.

Given the large number of errors and biases that can occur in any single study (and in meta-analyses), replication of association in sufficiently powered follow-up studies is essential to confirm an observed genetic association (6,7) and allow a more precise estimate of the effect size. Therefore, we examined the impact of *ENPP1* variation on type 2 diabetes and obesity in a large U.K. Caucasian genetic association study. We genotyped the three previously associated polymorphisms of *ENPP1* in 2,363 type 2 diabetic case and 4,045 control subjects, as well as 1,681 subjects from 529 type 2 diabetic families.

TABLE 1
Clinical details of subjects in study

	Case subjects	Control subjects	Family study probands
n	2,363	4,045	529
Male (%)	58.9	50.2	58.2
Age at diagnosis (years)*	51 (44–58)	31 (28–35)	41 (36–47)
BMI (kg/m^2)	30.3 (26.9–34.3)	24.8 (22.2–27.9)	33.0 (28.9–37.4)
Treatment D/O/I (%)	11/63/26	—	20/59/21

Data are median (interquartile range) unless otherwise indicated. Only successfully genotyped subjects were included. *Age at diagnosis for case subjects or age at study for control subjects. No clinical details were available for the European Collection of Cell Cultures and the British 1958 Birth Cohort population control samples; therefore, control characteristics are for the Exeter Family Study samples only. D/O/I, diet/oral hypoglycemic agents/insulin.

RESEARCH DESIGN AND METHODS

Informed consent was obtained from all participants. Clinical characteristics of the subjects are presented in Table 1 and online appendix Table 1 (available at http://diabetes.diabetesjournals.org). Most of these subjects have been described in detail previously (8). Briefly, all type 2 diabetic subjects were unrelated U.K. Caucasians who had diabetes defined by either the World Health Organization criteria (9) or by being treated with medication for diabetes and were recruited from three sources: *1*) a young-onset collection (defined as age ≥18 and ≤45 years at diagnosis), *2*) probands from type 2 diabetic sibships from the Diabetes U.K. Warren 2 repository described previously (10,11), and *3*) a collection of subjects with diabetes diagnosed between 35 and 65 years of age.

Population control subjects were all U.K. Caucasians. Subjects were recruited from three sources: *1*) parents from a consecutive birth cohort (Exeter Family Study) with normal (<6.0 mmol/l) fasting glucose and/or normal HbA_{1c} levels (<6%) (10), *2*) a nationally recruited population of control samples of U.K. Caucasians obtained from the European Collection of Cell Cultures, and *3*) a follow-up study, which is ongoing, of all people born in the U.K. during 1 week in 1958 (National Child Development Study; http://www.cls.ioe.ac.uk). Only results for subjects of Caucasian ethnicity are reported. Case subjects and families where the proband had high GAD autoantibody levels (>99th percentile of the normal population) were excluded from the study. Known subtypes of diabetes (e.g., maturity-onset diabetes of the young) were excluded by clinical criteria and/or genetic testing.

Subjects in the family-based study were independent of those in the case-control study. Families were selected to have either an affected proband with both parents (trios; in this study 19 of 410 trio families were missing one parent) or an affected proband with one parent and at least one sibling (duos), which has been described previously (8,12,13).

Genotyping and quality control. Genotyping was performed by Kbiosciences (Herts, U.K.) using a KASPar assay system (details of the methods used can be found at http://www.kbioscience.co.uk). Genotyping accuracy, as determined from the genotype concordance between duplicate samples (10.3% of the total), was 100% (0 discrepancies of 875 informative duplicate pairs) for K121Q (rs1044498), 99.4% (5 of 857) for IVS20delT-11 (rs1799774), and 100% (0 of 798) for A>G+1044TGA (rs7754561). The genotyping success rates were 96.8% case and 95.2% control subjects for K121Q, 94.5% case and 95.1% control subjects for IVS20delT-11, and 96.1% case and 94.6% control subjects for A>G+1044TGA. There were no Mendelian inheritance errors in the family-based samples for K121Q or IVS20delT-11; there were two for A>G+1044TGA. All SNPs were in Hardy-Weinberg equilibrium in the overall case and control groups (all $P > 0.01$).

Statistical analysis. ORs and *P* values were determined for our case-control analyses using χ^2 tests. There was no evidence of heterogeneity between case cohorts or between control cohorts (all $P > 0.05$); therefore, we pooled all our case and control subjects for association analysis. We used cocaphase (14) for case-control haplotype analyses. For consistency with the Meyre et al. (5) study, ORs and *P* values are presented for each haplotype against the most common haplotype. We also analyzed each haplotype against all others, and the results were similar (data not shown). The 95% CIs around haplotypes were determined by using the inferred haplotype counts from the cocaphase output for our study and around the published counts for the Meyre et al. (5) study. To analyze our family-based data, we used the Family-Based Association Test (15). We combined ORs from the case-control and family-based subjects using Mantel-Haenszel meta-analysis. For the meta-analysis, we used the Mantel-Haenszel method implemented in Stats Direct (version 2.4.6; Sale, Chesire, U.K.) to combine studies based on OR summary statistics provided by Bacci et al. (4) and Meyre et al. (5). We tested for heterogeneity using the Q statistic. Power calculations were performed using the Quanto software (16). We assumed a log-additive genetic model and set α to 1%. Allele frequencies were estimated from published studies. We had >99% power to detect the type 2 diabetes OR of 1.29 reported in the meta-analysis of Bacci et al. (4) for the Q121 allele and >99% power to detect the OR of 1.56 reported by Meyre et al. (5) for the three-marker haplotype. Subsets of case and control subjects were underpowered to detect previous effect sizes.

RESULTS

The individual SNP and haplotype association results are presented in Table 2. Genotype counts for each of the SNPs by individual cohort are given in online appendix Table 2. There were no individual SNP or haplotype associations of the *ENPP1* variation with type 2 diabetes either in case-control or familial-association studies (all $P > 0.05$). The combined result for the Q121 allele was an OR of 1.02 ([95% CI 0.93–1.12], $P = 0.61$), and for the previously associated haplotype (Q121, IVS20delT-11, and G+1044TGA) the OR was 1.10 ([0.96–1.26], $P = 0.17$).

We performed an OR meta-analysis of the previously published type 2 diabetes association studies for K121Q (Fig. 1). Before adding data from this study, the Q121 allele was associated with type 2 diabetes with a random-effects OR of 1.30 ([95% CI 1.14–1.47], $P = 0.00005$) (the fixed-

TABLE 2
Type 2 diabetes association results

SNP/haplotype		Case-control test				Familial association		
	Alleles (1/2)	Control allele 2 frequency	Case allele 2 frequency	Allele 2 OR (95% CI)	*P*	Observed allele 2	Expected allele 2	*P*
K121Q	A/C	0.137	0.139	1.02 (0.92–1.13)	0.75	119.0	118.3	0.93
IVS20delT-11	T/delT	0.202	0.198	0.97 (0.89–1.07)	0.58	151.0	147.3	0.65
A>G + 1044TGA	A/G	0.230	0.227	0.99 (0.90–1.08)	0.74	190.0	184.0	0.50
Q/delT/G	—	0.065	0.074	1.11 (0.96–1.29)	0.19	57.0	56.1	0.86

Single SNP association and haplotype association results. For consistency with the Meyre et al. (5) study, ORs and *P* values are presented for the individual haplotype versus the most common haplotype for the case-control analysis. The Q/delT/G haplotype is the haplotype that was previously associated with type 2 diabetes (OR 1.56 [95% CI 1.27–1.92]) (5). Full genotype counts by cohort are given in online appendix Table 2.

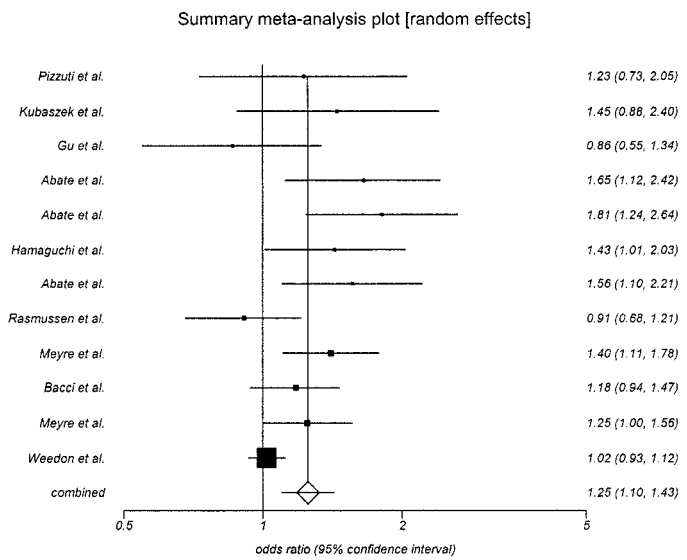

FIG. 1. Meta-analysis of all studies for the Q121 allele at K121Q. The studies are presented in order of Mantel-Haenszel weight. There is significant evidence of heterogeneity from a Q test ($P = 0.003$), so a random-effects meta-analysis was performed. There was evidence of publication bias ($P = 0.03$ from Eggers test [17]), with smaller studies having larger ORs than bigger studies. The fixed-effects OR was 1.14 ([1.07–1.22], $P = 0.00007$). References for the individual studies can be found in Bacci et al. (4) and Meyre et al. (5).

effects OR was 1.28 [1.17–1.41], $P = 2 \times 10^{-7}$). There was no significant heterogeneity (Q test $P = 0.09$) and no evidence of publication bias (Eggers test $P = 0.60$). After adding our study to the meta-analysis, the random-effects OR was 1.25 ([1.10–1.43], $P = 0.0007$) (fixed-effects OR 1.14 [1.07–1.22], $P = 0.00007$). There was strong evidence for heterogeneity (Q test $P = 0.003$) and publication bias (Eggers test $P = 0.03$) (17).

We went on to assess *ENPP1* variation association with obesity in the same cohorts. This was important as the French and Austrian control subjects used by Meyre et al. (5) in the type 2 diabetes case-control study were selected to be nonobese and had mean BMIs of 22 and 26 kg/m^2, respectively; thus, the type 2 diabetes association seen could be driven by an association with obesity. We compared allele and haplotype frequencies of 1,115 normal-weight adults (BMI <25 kg/m^2, 352 with type 2 diabetes, 763 without diabetes) with *1*) 1,500 moderately obese adults (BMI 30–40 kg/m^2, 1,297 with type 2 diabetes, 203 without diabetes) and *2*) 303 morbidly obese adults (BMI >40 kg/m^2, 292 with type 2 diabetes, 11 without diabetes). This study had 89 and 72% power (at $P < 0.05$) to detect the previous association seen in moderate obesity and morbid obesity, respectively (5). We found no evidence that the Q121 allele or the three-marker haplotype are associated with moderate or morbid obesity (Table 3).

TABLE 3
Obesity association results: single SNP and haplotype association results for moderate and morbid obesity

SNP/haplotype	Alleles (1/2)	Moderate obesity case-control				Morbid obesity case-control			
		Control allele 2 frequency	Case allele 2 frequency	Allele 2 OR (95% CI)	*P*	Control allele 2 frequency	Case allele 2 frequency	Allele 2 OR (95% CI)	*P*
K121Q	A/C	0.135	0.135	1.00 (0.85–1.18)	0.99	0.135	0.125	0.92 (0.70–1.20)	0.41
IVS20delT-11	T/delT	0.205	0.197	0.95 (0.83–1.10)	0.48	0.205	0.173	0.83 (0.65–1.05)	0.12
A>G + 1044TGA	A/G	0.225	0.230	1.03 (0.90–1.18)	0.64	0.225	0.224	1.00 (0.80–1.24)	0.97
Q/delT/G	—	0.066	0.064	0.97 (0.77–1.23)	0.81	0.066	0.058	0.86 (0.57–1.30)	0.46

DISCUSSION

In a study of 8,089 U.K. Caucasian subjects, we have failed to replicate the association of variation of *ENPP1* with type 2 diabetes and obesity. The accompanying study by Lyon et al. (18) involving 11,814 subjects similarly did not find any evidence of association. These results do not support the previous strong evidence for association of the Q121 allele with type 2 diabetes from a meta-analysis of nine small to moderately sized studies (7,259 total subjects) (OR 1.29 [95% CI 1.09–1.53]) (4) or the association of a common haplotype containing the Q121 allele (2,569

total subjects) with type 2 diabetes (1.56 [1.27–1.92]) (5). It is therefore important to consider potential reasons why these two large studies failed to replicate the previous studies.

Although our study had >99% power to replicate the previously observed size of association, the previous studies may have overestimated the effect size of the *ENPP1* variants. The upper 95% CI for our studies of an OR of 1.12 for the Q121 allele and ~1.26 for the associated haplotype were both lower than the previously described associations (4,5); thus, our data are not consistent with the previously reported effect size.

Our study may have underestimated the effect size, as our control subjects are not age matched to cases and glycemia has not been measured in 61% of the control subjects. However, the prevalence of type 2 diabetes in Caucasian U.K. subjects is <5% (and considerably less than this at 51 years of age, which is the mean age of diagnosis of case subjects in this study), so this would have resulted in only a slight reduction in any effect seen. These case and control subjects have previously provided significant evidence for two type 2 diabetes risk variants, P12A of *PPARG* and E23K of *KCNJ11*, the only two diabetes variants that reach genome-wide significance on meta-analysis (19,20).

Our data do not provide any evidence that *ENPP1* variation is associated with obesity despite good power to detect the same effects seen with moderate and morbid obesity reported by Meyre et al. (5). The upper 95% CIs from our data are not consistent with the effect sizes estimated from previous studies. However, there are limitations in using this dataset to examine obesity, as it was primarily established to detect type 2 diabetes genes, and type 2 diabetes or its treatment may alter BMI; the optimal study design would be to use a nondiabetic population.

Population stratification might contribute to the previous association or prevent an association being seen in our cohort. Two of the three SNPs in the haplotype, including K121Q, have allele frequencies that vary greatly across different racial groups. For example, the Q121 allele frequency is ~0.14 in U.K. Caucasians but ~0.80 in Africans. This may have made the results with these polymorphisms more susceptible to confounding by population admixture. Our results did not change when we analyzed our data stratified by 12 geographical regions of the U.K. (data not shown). We also note that the lactase polymorphism rs4988235, which varies twofold in frequency across the U.K. (21), is not associated with type 2 diabetes in our population (OR 0.97 [0.90–1.06], $P = 0.52$), suggesting that population stratification is unlikely in our study (M.N.W., T.M.F., A.T.H., unpublished data).

The asymmetry of the funnel plot from our meta-analysis (Eggers test $P = 0.03$), with smaller studies reporting larger ORs than bigger studies, suggests some form of small-study bias. This may represent publication bias or it may be due to other biases such as poorer methodological design for the smaller studies (17). It may also be due to differences in subject selection criteria between our study and the previous studies, as our subjects are generally younger at diabetes onset and are enriched for a family history of type 2 diabetes.

ENPP1 may yet prove to be a type 2 diabetes or obesity gene. However, the currently conflicting results provide some lessons. They suggest that association studies need to continue until the same allele of the same variant is associated with the same phenotype at genome-wide levels of significance (approximate $P < 1 \times 10^{-7}$) before declaring it a diabetes gene. It also illustrates the importance of using very large studies as replication samples in genetic association studies. In conclusion, we have found no evidence of association of previously associated *ENPP1* variants with type 2 diabetes or obesity in the U.K. Caucasian population.

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