

**THE *ENPP1* K121Q POLYMORPHISM IS ASSOCIATED
WITH TYPE 2 DIABETES IN EUROPEAN POPULATIONS:
EVIDENCE FROM AN UPDATED META-ANALYSIS IN 42,042 SUBJECTS**

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ABSTRACT

Objective: Functional studies suggest that the nonsynonymous K121Q polymorphism in the ectoenzyme nucleotide pyrophosphate phosphodiesterase ENPP1 may confer susceptibility to insulin resistance; genetic evidence on its effect on type 2 diabetes, however, has been conflicting. We therefore conducted a new meta-analysis that includes novel unpublished data from the ENPP1 Consortium and recent negative findings from large association studies to address the contribution of K121Q to type 2 diabetes.

Research Design and Methods: After a systematic review of the literature, we evaluated the effect of ENPP1 K121Q on diabetes risk under three genetic models using a random-effects approach. Our primary analysis consisted of 30 studies comprising 15,801 cases and 26,241 controls. Due to considerable heterogeneity and large differences in allele frequencies across populations, we limited our meta-analysis to those of self-reported European descent and, when available, included BMI as a covariate.

Results: We found a modest increase in risk of type 2 diabetes for QQ homozygotes in white populations (combined OR 1.38, 95% CI 1.10-1.74, $P=0.005$). There was no evidence of publication bias, but we noted significant residual heterogeneity among studies ($P=0.02$). On meta-regression, 16% of the effect was accounted for by the mean BMI of controls: this association was stronger in studies in which controls were leaner, but disappeared after adjustment for mean control BMI (combined OR 0.93, 95% CI 0.75-1.15, $P=0.50$).

Conclusions: The ENPP1 Q121 variant increases risk of type 2 diabetes under a recessive model of inheritance in whites, an effect which appears to be modulated by BMI.

Insulin resistance is a major feature of type 2 diabetes (1). Although there is evidence of heritability for this trait (2), the genes responsible are largely unknown. Indeed, recent genome-wide association studies have uncovered variants mostly related to insulin secretion rather than insulin resistance (3).

Ectoenzyme nucleotide pyrophosphate phosphodiesterase 1 (ENPP1, also known as plasma cell membrane glycoprotein 1 or PC-1) belongs to a family of E-NPP enzymes that regulate pyrophosphate and nucleotide levels. ENPP1 reduces insulin signaling by direct inhibition of the insulin receptor's tyrosine kinase activity, perhaps via interaction with its α subunit (4; 5). ENPP1 expression is elevated in skin fibroblasts and adipose tissue of insulin resistant subjects with or without type 2 diabetes (6; 7), and its content in cultured fibroblasts from non-diabetic subjects is correlated with decreased insulin receptor autophosphorylation *in vitro* and insulin sensitivity *in vivo* (8). In addition, its over-expression increases insulin resistance in mammals (9; 10). Therefore, the *ENPP1* gene is considered a plausible biological candidate for insulin resistance and associated type 2 diabetes.

However, studies of selected common variants in the *ENPP1* gene have yielded inconsistent association results with type 2 diabetes. Pizzuti *et al.* first reported an association of a common missense variant in exon 4 of *ENPP1*, K121Q (rs1044498), with insulin resistance (11). Interestingly, subsequent cellular transfection studies showed that constructs containing the Q variant led to more avid insulin receptor binding and stronger inhibition of insulin signalling than those containing the K allele (5). Follow-up genetic association studies found that the minor Q allele of K121Q increases the risk of type 2 diabetes in a Dominican population (12), in South Asians living in the US and in India, and in US white

samples (13); it has also been associated with earlier onset of type 2 diabetes and myocardial infarction in other populations of European ancestry (14). A French and Austrian study, consisting of 1,255 cases and 1,314 controls, examined several polymorphisms in *ENPP1* and found evidence that a three-marker haplotype (comprised by K121Q as well as IVS20delT-11 [rs1799774] and A>G+1044TGA [rs7754561]) increased risk of diabetes (OR 1.56, 95% CI 1.27-1.92; $P=0.00002$); of note, this risk was more apparent among obese subjects (15). Conversely, this association has not been replicated in three subsequent large-scale association studies: one performed in 5,863 controls and 1,386 cases from Denmark ($P=0.7$) (16), one performed in 8,089 white subjects from the UK (OR 1.02, 95% CI 0.93-1.12, $P=0.61$) (17) and our own study of 8,676 Scandinavian, Polish and North-American white subjects (OR 0.94, 95% CI 0.86-1.03, $P=0.20$) (18). Partial meta-analyses reported by the first two publications (16; 17) yielded a nominal association of the Q allele with type 2 diabetes, but they continued to show significant heterogeneity, and did not include results from each other or from our own extensive dataset. Similar limitations are present in a recent review (19). Due to the above conflicting evidence, the relatively modest effects possibly conferred by the 121Q variant, the incomplete extant meta-analyses and the remaining heterogeneity, we comprehensively re-evaluated the putative association between K121Q and diabetes in samples that have sufficient statistical power to detect a weak association while adjusting for potential sources of heterogeneity, under all three genetic models. In this analysis we have included all of the negative data from the large-scale association studies reported above, as well as results from subsequent reports (20-23) and an additional unpublished dataset.

Similar meta-analyses have served to clarify the effects of the *PPARG* P12A polymorphism (24; 25) and of variants in Calpain 10 (26-28) on risk of type 2 diabetes.

METHODS

Study sources. To identify the populations to be included in this study we initially selected all of the relevant case/control studies reported in previous meta-analyses of *ENPP1* that assessed association results with type 2 diabetes (13; 14; 16; 17), the largest consisting of 7,318 cases and 13,726 controls (17). We complemented this approach with a systematic review of the PubMed database for papers that included “ENPP1” as a keyword, last accessed in August, 2007. Where genotype counts were unpublished, we obtained results from the corresponding authors. Studies missing from the current analysis, but reported elsewhere did not furnish usable data (29). We also obtained additional currently unpublished genotype data from ongoing work by collaborators of the ENPP1 Consortium. A summary of the characteristics of each study is presented in Supplementary Table 1 (online Appendix [available at <http://diabetes.diabetesjournals.org>]).

New population. Five hundred fifty-nine subjects of self-reported European ancestry with type 2 diabetes (defined according to the World Health Organization criteria) who resided in the Gargano and the surrounding central eastern region of Italy and attended the Scientific Institute “Casa Sollievo della Sofferenza” in San Giovanni Rotondo were included in the study as cases. Controls were 483 non-diabetic, unrelated white residents of the same region who were included in the study according to the following selection criteria: fasting plasma glucose lower than 6.1 mmol/l per the same World Health Organization criteria, and absence of drug treatment known to affect glucose and lipid

metabolism. The K121Q polymorphism was determined as previously described (11).

Statistical Analyses. All statistical tests were performed with the meta-analysis software package Comprehensive Meta-Analysis (version 2.2.040; Englewood, NJ) under the allelic, dominant and recessive genetic models. Because of considerable heterogeneity among studies, we employed a random-effects model to assess the effect of genotype on diabetes risk. While a fixed-effects model considers the variability between studies as purely due to chance, the random-effects model may be most appropriate for the differing, cryptic, underlying effects each study may contribute to the overall heterogeneity.

We used Cochran's χ^2 test (Q test.) (30) to evaluate heterogeneity between studies. Where we encountered a large source of heterogeneity, we addressed it in a sequential manner: first, due to the large allele frequency differences in K121Q across populations (12% Q allele in HapMap CEU, 93% Q allele in HapMap YRI), we restricted the studies to be included in the final meta-analysis to those performed in the ethnic group with the largest number of samples (self-reported European descent); and second, given the suggestions that *ENPP1* plays a role in obesity (15; 31-33) and that obesity may modulate the effect of *ENPP1* K121Q on diabetes risk (15; 21; 22; 32-34) we included BMI as a covariate in meta-regression, and adjusted the meta-analysis accordingly. Egger's test was used to address publication bias. Because this meta-analysis represents a focused exploration of previously published hypotheses, a *P* value <0.05 was considered significant.

RESULTS

An initial random-effects meta-analysis which comprised a total of 15,801 cases and 26,241 control subjects from diverse ethnic groups (including a new unpublished cohort

from the ENPP1 Consortium), under an allelic genetic model of inheritance, yielded a very modest effect for the K121Q genotype on diabetes risk (OR 1.11, 95% CI 1.04-1.19, $P=0.001$; Supplementary Fig. 1). There was considerable heterogeneity among the 30 studies (Q test $P=0.003$) and evidence of publication bias (Egger's test $P=0.02$). Because of this strong evidence of heterogeneity, we limited our analysis to the 23 studies with populations of self-reported European ancestry, so as to include the largest ethnically homogenous sample set. This subset meta-analysis, including 13,901 cases and 21,425 controls, showed a smaller effect on risk of diabetes under the allelic model (OR 1.08, 95% CI 1.01-1.15, $P=0.03$; Fig. 1) with significant residual heterogeneity (Q test $P=0.03$) and modest evidence of publication bias (Egger's test $P=0.04$; Supplementary Fig. 2).

The risk of type 2 diabetes conferred by K121Q in studies of European ancestry under the dominant genetic model did not reach nominal significance (OR 1.06, 95% CI 0.99-1.15, $P=0.10$). Conversely, there was nominal evidence of association with type 2 diabetes under a recessive model of inheritance: we found a nominally significant increase in diabetes risk for QQ homozygotes (OR 1.38, 95% CI 1.10-1.74, $P=0.005$; Fig. 2). There was still residual heterogeneity among the 20 case/control studies that had at least one QQ homozygote (Q test $P=0.02$) but no evidence of publication bias (Egger's test $P=0.24$). In order to test the stability of this result, we repeated our meta-analysis after exclusion of the first study which reported an association with diabetes (11); such exclusion did not alter the results. We also tested whether the meta-analysis was overtly influenced by a single study: exclusion of the study with the strongest effect size (that reported by Abate *et al.* in white Americans (13)) eliminated statistical significance in the allelic model (OR 1.06, 95% CI 1.00-1.12,

$P=0.07$) but not in the recessive model (OR 1.28, 95% CI 1.05-1.56, $P=0.015$); in the latter, sequential exclusion of each single study yielded ORs ranging from 1.28 to 1.46 (mean OR 1.38, 95% CI 1.365-1.404), with corresponding P values 0.015-0.001.

To address the heterogeneity remaining in this last model, we considered BMI as a covariate in meta-regression. We examined mean control BMI, mean case BMI or the difference between the two means in the studies from which that information was available. We detected no significant correlations between mean case BMI and effect size of genotype on diabetes risk ($P=0.76$; Supplementary Fig. 3). However, there was a significant correlation between mean control BMI and genetic effect ($P=0.02$), with evidence that mean control BMI contributes to 16% of the variance (Fig. 3). A similar nominal trend was noted for the correlation between diabetes risk and the difference in mean BMI between cases and controls ($P=0.054$). When we adjusted the individual OR of each study for mean control BMI, the adjusted random-effects meta-analysis failed to reveal a significant association (combined OR 0.93, 95% CI 0.75-1.15, $P=0.50$), while some heterogeneity still remained (Q test $P=0.03$).

We further explored other possible sources of heterogeneity: under the recessive model, heterogeneity disappeared (Q test $P=0.99$) but statistical significance remained after exclusion of all six outliers (two with opposing and four with consistent odds ratios) revealed by the funnel plot (OR 1.25, 95% CI 1.04-1.51, $P=0.02$). The P value for heterogeneity also became non-significant after the single exclusion of each of four cohorts (white Americans in Abate *et al.* (13), Austrians in Meyre *et al.* (15) and the GCI USA and Polish samples in Lyon *et al.* (18)), while the P value for association of the QQ genotype with type 2 diabetes remained nominally significant after each of the four

exclusions ($P=0.001-0.02$). We did not find a significant correlation between latitude within the European continent and strength of the association (data not shown).

We found no evidence of association for either of the two other polymorphisms that together with K121Q define the putative risk haplotype. No significant association was found for rs1799774 (OR 0.97, 95% CI 0.90-1.06, $P=0.51$) or for the previously associated 3' UTR variant rs7754561 (OR 0.94, 95% CI 0.86-1.02, $P=0.15$) in our analysis of 8,212 cases and 9,209 controls for rs1799774 or 8,255 cases and 9,574 controls for rs7754561 under an allelic model of inheritance.

DISCUSSION

There have been conflicting results for the putative association between the common K121Q polymorphism in *ENPP1* and type 2 diabetes. Although inconsistent replication of original published findings is not uncommon when studying complex diseases (35; 36), given the multiplicity of both positive and negative reports for this polymorphism we have tried to examine the potential factors underlying the discrepant results. The failure to reproduce an association for *ENPP1* K121Q with type 2 diabetes may be accounted for by either false positive reports of association, false negative attempts at replication or true study heterogeneity.

False positive reports of association can be due to publication bias, confounding by population stratification or initial overfitting of genotype-phenotype correlations. In regard to the former, it is noteworthy that the meta-analyses by Weedon *et al.* (17) and our own reported here do indicate some measure of publication bias; this finding highlights the continued need to support a forum where negative association results find an open venue for publication. Additionally, positive results may be confounded by underlying population substructure within association studies, especially when both allele

frequencies and rates of disease vary among ethnic groups. In this regard, the Q allele frequencies of *ENPP1* K121Q differ substantially among African Americans (78.5%), Hispanics (21.9%), and whites (13.2%) (21); thus, cryptic population ancestry differences among cases and controls may lead to spurious results and confound the true effect size conferred by the risk Q allele, unless stratification is carefully controlled for in case/control analyses (particularly of admixed populations). Thirdly, overfitting of an association result to the best of many possible multi-marker haplotypes may prove irreproducible. In this study, we have addressed publication bias, minimized the potential confounding of population stratification by examining a single ethnic group, and avoided the overfitting of phenotypes to artificially constructed haplotypes by limiting our association analyses to individual variants.

Irreproducibility may also result from the failure of subsequent reports to confirm a real finding. Such reports may be underpowered, neglect to examine the relevant genetic model or be affected by significant heterogeneity. Lack of power may stem from an overestimation of the true genetic effect caused by the phenomenon of the “winner's curse,” with follow-up studies being too small to detect the more modest actual effects. In this regard, the power calculation performed in our previous attempt at replication (18) had assumed an allelic odds ratio of ~1.3; thus, although our sample size had >99% power to detect an effect of that size, the meta-analysis conducted here reveals a more modest overall allelic effect of 1.08. More significantly, however, our previous study did not evaluate the recessive genetic model which seems to be pertinent to this variant.

Finally, heterogeneity in the manner by which a particular genetic variant increases diabetes risk may also diminish our ability to detect a true effect. Even after minimizing

the ethnic confounding discussed above, our meta-analysis contains residual heterogeneity. To address this issue, we performed a meta-regression by considering BMI as a covariate. Our data indicate that the genetic effect is greater in studies conducted with leaner controls, and that the mean BMI of controls explains a small proportion of the observed heterogeneity. After adjustment for control BMI, unexplained heterogeneity remains, but the association of the QQ genotype with diabetes is no longer significant, suggesting that it may be mediated by its effect on BMI. Indeed, if this polymorphism contributes to diabetes via BMI, studies that magnify the BMI difference between cases and controls (e.g. by lowering mean control BMI) may be more likely to yield significant associations. A similar observation has been reported for *PPARG* P12A, which has long been established as a true diabetes-associated polymorphism (24) and its effect replicated in many populations (reviewed in (37)). However, this association has been less robust in recent genome-wide scans (38; 39): the heterogeneity of these results may be partially explained by the attenuation of the protective effect of the *PPARG* A12 variant on diabetes risk seen at higher strata of BMI (40). Indeed, a recent meta-analysis and meta-regression have shown that the deleterious effect of the *PPARG* P12/P12 genotype on diabetes risk is augmented in studies where controls were leaner (25), thus suggesting this may be a common phenomenon characterizing the effect of insulin resistance genes on the risk of type 2 diabetes. Since our meta-regression of *ENPP1* K121Q is limited by the use of population BMI averages rather than individual measures, we cannot verify whether our present finding is related to the reported interaction between the K121Q polymorphism and BMI in modulating the risk of type 2 diabetes (15; 21; 22).

If *ENPP1* K121Q truly increases risk of diabetes via a recessive model and in concert

with increased BMI, it is not surprising that recent genome-wide association scans (largely conducted in samples that overlap those studied here) have failed to detect the association. For instance, in the publicly available dataset of the Diabetes Genetics Initiative (www.broad.mit.edu/diabetes), the single nucleotide polymorphism rs7767502 (which is in perfect linkage disequilibrium with K121Q in the HapMap CEU population, $r^2=1.0$) is not associated with type 2 diabetes (OR 0.86, 95% CI 0.71-1.03, $P=0.38$). In that cohort, cases and controls were matched for BMI and only allelic odds ratios are reported. In that scan, the OR of true diabetes-associated variants were all larger than the OR of 1.08 for the Q allele we have found here (39). The sample size of this meta-analysis is also much larger than that of the genome-wide association scan of Sladek *et al.*, where a borderline association of this variant with type 2 diabetes was noted (38).

In summary, we have carried out an extensive, up-to-date and unbiased meta-analysis of 30 study populations comprising 15,801 cases and 26,241 control subjects for association of any of the three previously reported *ENPP1* variants K121Q, rs1799774 and rs7754561 with type 2 diabetes. We have found nominally significant evidence for a modest increase in risk of type 2 diabetes for homozygous carriers of the Q allele in populations of European descent. Residual heterogeneity may be partially explained by an interaction between genotype and BMI. We do not find any evidence that the other two *ENPP1* polymorphisms (which, together with K121Q, define a previously reported haplotype (15)) have an individual effect on diabetes risk, a result which is consistent with a recent population study by the same investigators (23).

We caution that the modest statistical evidence attained here is orders of magnitude below that of other diabetes-associated variants found through genome-wide

association (38; 39). Nonetheless, several conclusions can be drawn from these observations. First, very large sample sizes are necessary to tease out the possible modifiers of genetic risk conferred by variants of modest effects, particularly when they exert their action via recessive models of inheritance. Second, it is essential to allow the publication of negative genetic association studies, especially when conducted in large samples. Third, the availability of individual level data would greatly facilitate a thorough evaluation of potential genetic or environmental modifiers. Fourth, when evaluating the association of *ENPP1* K121Q – or of any other polymorphism whose allele frequency varies across different populations – with type 2 diabetes, population stratification must be conclusively ruled out. And finally, a comprehensive examination of all variation around *ENPP1* is still necessary to parse the true association signal, in an effort to fully understand the role of this gene in diabetes, insulin resistance and obesity.

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FIGURE LEGENDS

Figure 1. Cumulative effect of published and unpublished studies of the K121Q single nucleotide polymorphism (rs1044498) on the risk of type 2 diabetes in 13,901 cases and 21,425 control subjects using a random effects model and an allelic model of inheritance. A subset of 23 European-descent populations was used in the analysis to limit heterogeneity. There was significant heterogeneity by Q test ($P=0.03$). Population ancestry for each study is given in parentheses and 95% confidence intervals are shown. Studies are ordered by publication date, with the summary estimate reported on the last line.

Figure 2. Random-effects meta-analysis of 20 studies of European descent, comprising 12,889 cases and 20,363 controls, for an association of the homozygous QQ genotype with type 2 diabetes (two family-based studies and one small study with no QQ homozygotes were excluded). There was residual heterogeneity in the study, determined by a Q test ($P=0.02$) with an OR of 1.38 (95% CI 1.10-1.74), ($P=0.005$), but no publication bias (Egger's test $P=0.24$). Studies are ordered by publication date, with the summary estimate reported on the last line.

Figure 3. Meta-regression of mean control BMI and K121Q genetic effect for 18 populations of European descent using a recessive model of inheritance. There was a significant correlation between mean control BMI and genetic effect ($P=0.02$), which explains 16% of the variance of results. Adjustment of the recessive model for mean control BMI made the association of the QQ genotype with type 2 diabetes disappear. Different ascertainment criteria or matching procedures may explain the differences in mean BMI among controls drawn from the same populations.

FIGURE 1

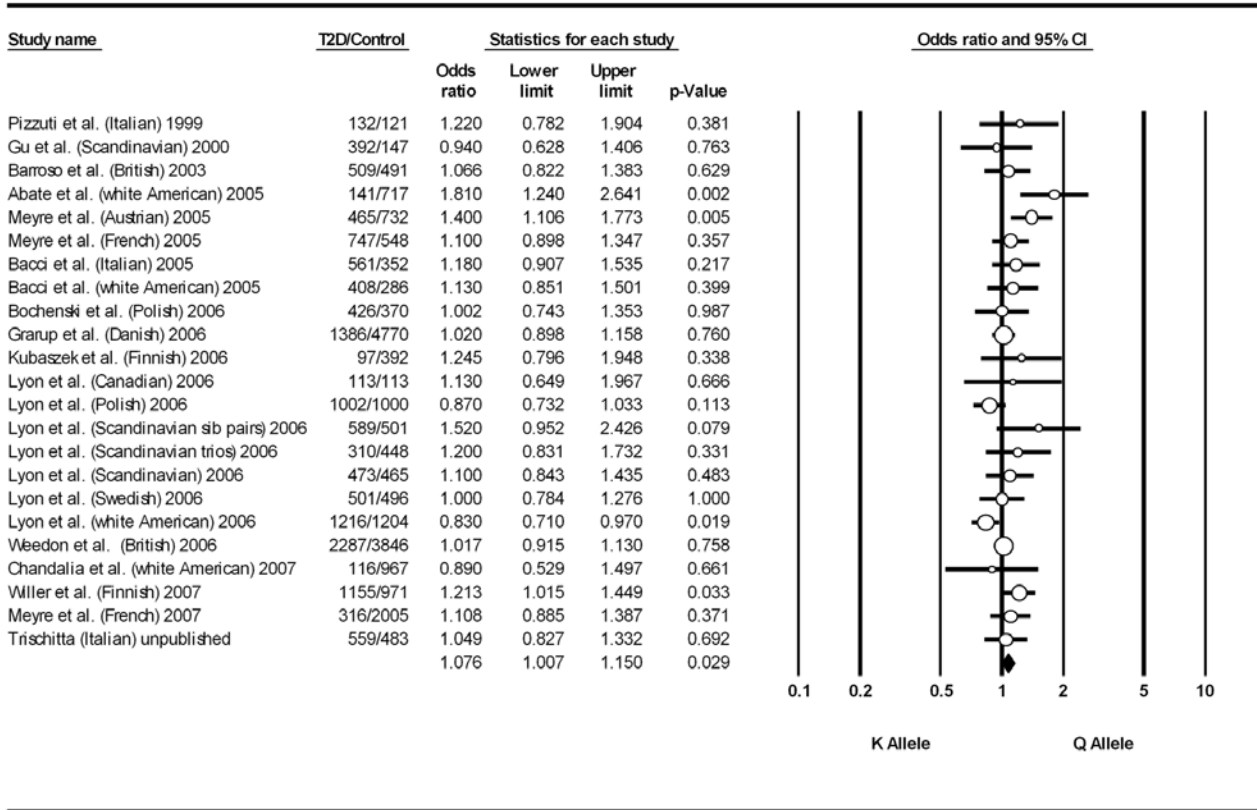


FIGURE 2

