The ENPP1 Q121 Variant Predicts Major Cardiovascular Events in High-Risk Individuals

Evidence for Interaction With Obesity in Diabetic Patients

Simonetta Bacci,1 Stefano Rizza,2 Sabrina Prudente,3 Belinda Spoto,4 Christine Powers,5 Antonio Facchiorusso,6 Antonio Pacilli,1 Davide Lauro,2 Alessandra Testa,4 Yuan-Yuan Zhang,5,7 Giuseppe Di Stolfo,5 Francesca Mallamaci,4 Giovanni Tripepi,4 Rui Xu,5,7 Davide Mangiacotti,8 Filippo Aucella,9 Renato Lauro,2 Ernest V. Gervino,7,10 Thomas H. Hauser,7,10 Massimiliano Copetti,11 Salvatore De Cosmo,1 Fabio Pellegrini,11,12 Carmine Zoccali,4 Massimo Federici,2 Alessandro Doria,5,7 and Vincenzo Trischitta8,13

OBJECTIVE—Insulin resistance (IR) and cardiovascular disease may share a common genetic background. We investigated the role of IR-associated ENPP1 K121Q polymorphism (rs1044498) on cardiovascular disease in high-risk individuals.

RESEARCH DESIGN AND METHODS—A prospective study (average follow-up, 37 months) was conducted for major cardiovascular events (myocardial infarction [MI], stroke, cardiovascular death) from the Gargano Heart Study (GHS; n = 330 with type 2 diabetes and coronary artery disease), the Tor Vergata Atherosclerosis Study (TVAS; n = 141 who had MI), and the Cardiovascular Risk Extended Evaluation in Dialysis (CREED) database (n = 206 with end-stage renal disease). Age at MI was investigated in cross-sectional studies of 339 type 2 diabetic patients (n = 169 from Italy, n = 170 from the U.S.).

RESULTS—Incidence of cardiovascular events per 100 person-years was 4.2 in GHS, 10.8 in TVAS, and 11.7 in CREED. Hazard ratios (HRs) for KQ+QQ versus individuals carrying the K121/K121 genotype (KK) individuals were 1.47 (95% CI 0.80–2.70) in GHS, 2.31 (95% CI 1.22–4.34) in TVAS, and 1.36 (95% CI 0.88–2.10) in CREED, and 1.56 (95% CI 1.15–2.12) in the three cohorts combined. In the 395 diabetic patients, the Q121 variant predicted cardiovascular events among obese but not among nonobese individuals (HR 5.94 vs. 0.62, P = 0.003 for interaction). A similar synergism was observed in cross-sectional studies, with age at MI being 3 years younger in Q121 carriers than in KK homozygotes among obese but not among nonobese patients (P = 0.035 for interaction).

CONCLUSIONS—The ENPP1 K121Q polymorphism is an independent predictor of major cardiovascular events in high-risk individuals. In type 2 diabetes, this effect is exacerbated by obesity. Future larger studies are needed to confirm our finding.

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Morbidity and mortality due to cardiovascular disease (CVD) are highly prevalent (1), mostly because of the epidemics of obesity and type 2 diabetes (2–4). Environmental and genetic factors both contribute to CVD. The genes that are involved are mostly unknown (5), and only few clues have been provided about their identities by recent genome-wide association studies (6–10). Insulin resistance and related abnormalities are among the factors that have been implicated in the etiology of CVD (11–17). Because insulin resistance is also under genetic control, the two conditions may share a common genetic background (18–20), with genes that contribute to impaired insulin sensitivity being prime candidates for a predisposing effect on CVD.

The ectoenzyme nucleotide pyrophosphate phosphodiesterase (ENPP1) inhibits insulin receptor signaling (21). A nonsynonymous polymorphism (K121Q, rs1044498) of the ENPP1 gene has been described (22), with the Q121 variant determining a gain of function resulting in an increased ability to inhibit insulin receptor signaling (22–24). This variant has been associated with insulin resistance in most (22,25–27) but not all (28) large studies. In agreement with the hypothesis of a common genetic “soil” between insulin resistance and CVD, the Q121 variant has been also associated with atherosclerosis-related phenotypes in European (24,29,30) but not in Brazilian (31) or in Chinese (32) samples. Most of these associations have been mainly observed among heavier individuals (25–27,30,33), thereby suggesting a gene-by-adiposity interaction.

The aim of our study was to investigate the role of the ENPP1 Q121 variant and its interaction with obesity (i.e., BMI ±30 kg/m2) in accelerating major cardiovascular events in very high-risk individuals.

RESEARCH DESIGN AND METHODS

Study participants

Prospective studies. Sample 1—the Gargano Heart Study, prospective analysis. The study included 340 whites from Italy with type 2 diabetes (according to American Diabetes Association 2003 criteria) and coronary
artery disease (CAD), as indicated by previous myocardial infarction (MI) or >50% stenosis of at least one major vessel at coronary angiography, or both. These individuals were cases of the cross-sectional case-control Gargano Heart Study (GHS) (30) and were consecutively recruited at the Scientific Institute "Casa Sollievo della Sofferenza" in San Giovanni Rotondo (Gargano, center east coast of Italy), as cases of the cross-sectional case-control GHS (29). Most of these patients (n = 160) were further studied for incident major cardiovascular events in the prospective GHS (see above). Another 170 were recruited in Boston from the Beth Israel Deaconess Medical Center (BIDMC) and the Joslin Clinic (which serves as the BIDMC Diabetes Clinic) as part of an ongoing investigation on the genetics of CAD in type 2 diabetes and the ENPP1 K121Q variant and age (69 to 72 years of age). The average follow-up (mean ± SD) was 11.9 years (range, 1–37) in the GHS, and 36.3 (22.0) months in the CREED. During follow-up, 43 major cardiovascular events occurred in the GHS, 39 in the TVAS, and 94 in the CREED, resulting in respective incidence rates of 4.2, 10.8, and 11.7 per 100 person-years (Years vs. 8.1 in the TVAS, and 14.1 vs. 10.8 in the CREED (Table 2). The difference was significant in the TVAS (P = 0.025) and in a pooled analysis of the three studies (P = 0.005). No difference in the magnitude of the genetic effect was observed among studies (P = 0.32 for interaction).

In a time-to-event analysis, the HR of cardiovascular events for Q121 carriers versus KK homozygotes was 1.47 (95% CI 0.80–2.70, P = 0.21) in the GHS, 2.31 (95% CI 1.2–4.34, P = 0.01) in the TVAS, and 1.36 (95% CI 0.88–2.10, P = 0.16) in the CREED (Fig. 1A, B, and C, respectively; P = 0.32 for gene-by-sample interaction). In a pooled analysis (i.e., individual patient data meta-analysis) of the three studies, which included 737 subjects with 176 events, the HR for Q121 carriers versus KK homozygotes was 1.56 (95% CI 1.15–2.12, P = 0.004; Fig. 1D). Age at study entry (HR 1.04 [95% CI 1.03–1.06], P < 0.0001), diabetes (2.23 [95% CI 1.50–3.31], P < 0.0001), and smoking status (1.71 [95% CI 1.24–2.36], P = 0.001) were additional predictors of incident events. BMI (HR 1.03 [95% CI 0.99–1.06], P = 0.08), hypertension (1.50 [95% CI 0.99–2.27], P = 0.054), and sex
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Interaction between Q121 variant and obesity in predicting major cardiovascular events. Given the previous evidence for an ENPP1-by-obesity interaction in the modulation of traits related to insulin resistance (25–27,29,30,39–41), we investigated this hypothesis in our study. In the GHS, which entirely consisted of patients with type 2 diabetes, we indeed observed a significant interaction between the Q121 variant and obesity. An association between the variant and risk of events was present among the 159 subjects who had a BMI ≥30 kg/m² (HR 3.56 [95% CI 1.21–10.5], $P = 0.02$), but not among the 171 individuals who had a BMI <30 kg/m² (0.91 [95% CI 0.40–2.06], $P = 0.82$; $P = 0.039$ for interaction).

No evidence of gene-by-obesity interaction was instead observed in the TVAS ($P = 0.53$) and the CREED ($P = 0.41$). Because approximately 85% of these two cohorts consisted of nondiabetic individuals, we hypothesized that the genotype-by-obesity interaction might be specific to diabetes. Indeed, a pattern consistent with such an effect was also observed in these two studies when the analysis was restricted to individuals with diabetes, even though the small sample size prevented statistical significance (data not shown).

Thus, we further investigated the Q121-by-obesity interaction in a pooled analysis of the three studies after stratification by diabetes status. In the diabetic stratum (n = 395), the Q121 variant was associated with an increased risk of incident events among the 177 obese (Fig. 2A) but not among the 218 nonobese (Fig. 2B) individuals, with adjusted HRs of 5.94 (95% CI 1.88–18.78, $P = 0.002$) vs. 0.62 (95% CI 0.32–1.24, $P = 0.18$). The interaction between the Q121 variant and obesity was significant ($P = 0.003$). By contrast, no evidence of interaction was observed in the nonobese diabetic stratum (n = 344, $P = 0.26$), with adjusted HRs of 0.82 (95% CI 0.22–3.11, $P = 0.77$) in the 39 obese individuals and 1.85 (95% CI 1.18–2.90, $P = 0.008$) in the 305 nonobese subjects.

Among obese diabetic individuals, the addition of the K121Q genotype to the multivariable model produced a slight improvement from 0.802 to 0.831 in risk discrimination if this was assessed by the survival c-index (0.91, $P = 0.003$). By contrast, no evidence of interaction was observed in the nondiabetic stratum (n = 344, $P = 0.26$), with adjusted HRs of 0.82 (95% CI 0.22–3.11, $P = 0.77$) in the 39 obese individuals and 1.85 (95% CI 1.18–2.90, $P = 0.008$) in the 305 nonobese subjects.

The addition of the K121Q genotype did not improve the risk discrimination provided by the predictive model that included age, sex, BMI, smoking status, hypertension and diabetes, as indicated by the survival c-index, which went from 0.704 to 0.713 (P = 0.94), or by the IDI, with 0.42% improvement (P = 0.16).

### Table 1
Clinical features of very high-risk individuals in the three prospective studies

| Variables | Whole sample | KK | Q121 |
|-----------|--------------|----|------|
| GHS | n = 330 | n = 222 | n = 108 |
| Male | 68.8 | 68.0 | 70.4 |
| Age (years) | 64 (8) | 64 (8) | 65 (7) |
| Smokers | 47.2 | 49.8 | 41.8 |
| BMI (kg/m²) | 30.2 (4.8) | 30.1 (4.6) | 30.5 (5.2) |
| Hypertension | 77.8 | 78.0 | 77.4 |
| Diabetes | 100 | 100 | 100 |
| TVAS | n = 141 | n = 102 | n = 39 |
| Male | 83.7 | 84.2 | 82.1 |
| Age (years) | 62 (10) | 62 (10) | 63 (9) |
| Smokers | 75.2 | 73.3 | 82.0 |
| BMI (kg/m²) | 28.0 (3.7) | 28.4 (3.8) | 27.0 (3.4) |
| Hypertension | 98.6 | 98.0 | 100.0 |
| Diabetes | 15.6 | 16.8 | 12.8 |
| CREED | n = 266 | n = 188 | n = 78 |
| Male | 56.0 | 52.1 | 65.4 |
| Age (years) | 61 (15) | 60 (16) | 64 (14) |
| Smokers | 40.0 | 38.3 | 43.6 |
| BMI (kg/m²) | 24.9 (4.4) | 24.9 (4.6) | 24.8 (4.0) |
| Hypertension | 72.9 | 73.9 | 70.5 |
| Diabetes | 16.2 | 16.5 | 15.4 |

Data are expressed as absolute numbers, percentage, or mean (SD).

(1.30 [95% CI 0.95–1.79], $P = 0.10$) also tended to be associated with increased rate of events, although these did not reach statistical significance. The increased risk of events associated with the Q121 variant remained significant (HR 1.55 [95% CI 1.14–2.12], $P = 0.005$) after adjusting for BMI and diabetes, both of which had been reported to be associated with the Q121 variant (38–40), as well as after further adjustment for age, sex, hypertension, and smoking status (HR 1.45 [95% CI 1.05–2.00], $P = 0.022$).

### Table 2
Incidence of major cardiovascular events in GHS, TVAS, and CREEDS

|              | N   | Person-years | Nonfatal MI | Nonfatal stroke | CV death | Total events | Incidence rate* | P     |
|--------------|-----|--------------|-------------|----------------|----------|--------------|----------------|-------|
| **GHS**      |     |              |             |                |          |              |                |       |
| All          | 330 | 1,027        | 3           | 8              | 32       | 43           | 4.2            | 0.22  |
| KK           | 222 | 694          | 1           | 3              | 21       | 25           | 3.6            |       |
| Q121         | 108 | 333          | 2           | 5              | 11       | 18           | 5.4            |       |
| **TVAS**     |     |              |             |                |          |              |                |       |
| All          | 141 | 361          | 20          | 13             | 6        | 39           | 10.8           |       |
| KK           | 102 | 272          | 11          | 9              | 2        | 22           | 8.1            |       |
| Q121         | 39  | 89           | 9           | 4              | 4        | 17           | 19.2           | 0.025 |
| **CREED**    |     |              |             |                |          |              |                |       |
| All          | 266 | 804          | 1           | 10             | 83       | 94           | 11.7           |       |
| KK           | 188 | 592          | 1           | 8              | 55       | 64           | 10.8           |       |
| Q121         | 78  | 212          | 0           | 2              | 28       | 30           | 14.1           | 0.225 |
| **GHS+TVAS-CREED** | | 737 | 2,192 | 24 | 31 | 121 | 176 | 8.1 | 0.005 |
| KK           | 512 | 1,558 | 13 | 20 | 78 | 111 | 7.0 |       |
| Q121         | 225 | 634 | 11 | 11 | 43 | 65 | 10.9 |       |

*Per 100 person-years. CV, cardiovascular.
Interaction between Q121 variant and obesity on age at MI in cross-sectional studies. To seek replication of the gene-by-obesity interaction observed in patients with diabetes, we analyzed the association between the Q121 variant and age at MI in two cross-sectional samples of individuals with type 2 diabetes who had had a previous MI. One sample was from the Gargano area in Italy, the other was from Boston. Salient clinical features of the

FIG. 1. Kaplan-Meier survival curves are shown for major cardiovascular events in GHS (A), TVAS (B), and CREED (C). D: Estimates generated by Cox regression in the pooled analysis are shown.

FIG. 2. Survival curves for major cardiovascular events in obese (A) and nonobese (B) patients with type 2 diabetes. Curves are estimates generated by Cox regression in the pooled analysis of the three prospective studies.
study subjects are summarized in Table 3. Because no significant genotype-by-sample interaction was observed in the association with age at MI (P = 0.11), pooled analyses were performed by adjusting for “study sample.” To make the analysis comparable to that of prospective studies, sex, smoking status, hypertension, and BMI, but not age (due to its collinearity with age at MI—and diabetes—because all study participants were diabetic) were included as covariates. Among obese subjects, 64 Q121 carriers had had the MI almost 3 years earlier than the 124 KK homozygotes, at 54.5 (9.6) vs. 57.2 (8.9) years of age (P = 0.035). In contrast, no significant difference in age at MI was observed among nonobese subjects: 59.2 (10.5) in 41 Q121 carriers versus 57.2 (10.4) in 110 KK homozygotes (P = 0.16; P = 0.025 value for Q121-by-obesity interaction).

Virtually identical results were obtained when duration of diabetes was also added to the multivariate model, with Q121 carriers having had an MI at a significantly younger age than KK individuals among obese (P = 0.039) but not among nonobese (P = 0.20) individuals (P = 0.032 for interaction). In addition, when BMI was considered as a continuous trait, it was inversely related to the age at MI among Q121 carriers (β = −0.44 [95% CI −0.75 to −0.12], P = 0.008; Fig. 3A), but not among KK individuals (β = −0.17 [95% CI −0.41 to 0.07], P = 0.16; P = 0.069; for Q121-by-BMI interaction; Fig. 3B).

TABLE 3
Clinical features of patients with type 2 diabetes who survived an MI in the two cross-sectional studies

|                         | Whole sample | KK     | Q121   |
|-------------------------|--------------|--------|--------|
| **Gender**              |              |        |        |
| Male (%)                | n = 169      | n = 112| n = 57 |
| Smokers (%)             | 70.4         | 72.3   | 66.7   |
| Age (years)             | 64 (9)       | 64 (9) | 64 (8) |
| BMI (kg/m²)             | 30.7 (4.8)   | 30.6 (4.6)| 30.9 (5.3)|
| Hypertension (%)        | 86.9         | 88.3   | 82.1   |
| Duration of diabetes (years) | 14.2 (9.5) | 14.7 (9.9)| 13.3 (8.5)|
| HbA1c (%)               | 8.6 (2.0)    | 8.6 (1.8)| 8.5 (2.2)|
| Treatment of hyperglycemia |            |        |        |
| Diet alone (%)          | 7.2          | 5.4    | 10.9   |
| Oral hypoglycemic agents (%) |        |        |        |
| Insulin ± oral hypoglycemic agents (%) |        |        |        |
|                         | 52.4         | 55.9   | 45.5   |
| **Boston**              |              |        |        |
| Male (%)                | n = 170      | n = 122| n = 48 |
| Smokers (%)             | 72.4         | 73.3   | 70.8   |
| Age (years)             | 64.6 (7.9)   | 64.7 (7.8)| 64.3 (8.1)|
| BMI (kg/m²)             | 69.3         | 66.9   | 75.6   |
| Hypertension (%)        | 31.8 (5.9)   | 31.3 (5.6)| 33.1 (6.4)|
| Duration of diabetes (years) | 12.5 (9.3) | 11.8 (9.4)| 14.1 (9.1)|
| HbA1c (%)               | 7.4 (1.4)    | 7.5 (1.5)| 7.3 (1.3)|
| Treatment of hyperglycemia |            |        |        |
| Diet alone (%)          | 5.3          | 4.1    | 8.3    |
| Oral hypoglycemic agents (%) |        |        |        |
| Insulin ± oral hypoglycemic agents (%) |        |        |        |
|                         | 42.9         | 47.5   | 31.3   |

Data are expressed as number, percentage, or mean (SD).

DISCUSSION
Our results indicate that the ENPP1 K121Q polymorphism predicts acceleration of major cardiovascular events in very high-risk patients. The increased risk conferred by the Q121 variant is independent of that of age, sex, BMI, diabetes, and cigarette smoking. Our findings are in agreement with a previous cross-sectional study of 445 MI survivors from Central Europe (29). By contrast, case-control genome-wide association studies reported that a single nucleotide polymorphism (SNP, rs7767502), which is in perfect linkage disequilibrium with the ENPP1 K121Q polymorphism, was not associated with CAD (6–10). Several differences between our study and the genome-wide association studies, such as the prospective versus cross-sectional designs, the different end points under investigation, and the different baseline cardiovascular risk, with only the patients enrolled in our study being very high-risk as per selection criteria, might be responsible for this apparent discordance.

An additional important result of our study is that the effect of the Q121 variant was modulated by obesity in diabetic patients among whom the risk of incident events was five times higher in Q121 than in KK genotype carriers. Although not the aim of our study, one can infer that obese individuals (Fig. 2A) as a whole tend to have a lower risk of future cardiovascular events than nonobese patients (Fig. 2B; adjusted HR 0.68 [95% CI 0.41–1.24], P = 0.13). This paradoxical protective effect of obesity resembles that observed in patients with CAD (41), ESRD (42), heart failure (43), and older age (44), all conditions heavily overrepresented in our samples. In this context, the Q121 variant seems to eliminate the paradoxical protective effect of obesity.

An important finding was that the Q121 variant-by-obesity interaction observed in the prospective study was replicated in a cross-sectional study on age at MI in diabetic patients. Information on the K121Q genotypes tended to improve risk prediction in these patients when the improvement was measured by the IDI, the approach that is currently favored to evaluate predictive ability increase conferred by a new marker when added to a well-performing model (39). Thus, pending further validation in larger studies, one can hypothesize clinical implementation of the Q121 variant as a marker of early cardiovascular events among obese diabetic patients. Given the increasing incidence worldwide of both obesity and diabetes (2–4) and the poor ability to stratify cardiovascular risk among diabetic patients, a large sector of society would likely benefit in the future from the availability of such a test.

The synergistic effect of the genetic marker and obesity in the modulation of cardiovascular risk resembles results repeatedly reported in the risk modulation of insulin resistance and related traits (25–27,30,33,45–49). Placed in a broader perspective, this is an excellent example of genetic heterogeneity (i.e., different genetic effects being at play in different population subgroups) and clearly illustrates how accounting for such heterogeneity may be critical to dissect the genetic architecture of multifactorial diseases.

Understanding the mechanisms through which the Q121 variant is associated with CVD is beyond the scope of this study. However, one can speculate that the Q121 variant exacerbates cardiovascular risk by inducing systemic insulin resistance (22,25–27) and proatherogenic phenotypes...
(24,29,30,33). It may also act by way of a direct detrimental effect on insulin-dependent endothelial function, as suggested by the observation that human endothelial cells carrying the Q121 variant show impaired insulin receptor signaling and, most importantly, reduced release of nitric oxide (24), a potent vasodilator whose deficiency is an established early step in the pathway development of atherosclerosis (50).

One can hypothesize that the interaction between the Q121 variant and obesity is sustained by the different sites of action on the insulin-signaling pathway. Although ENPP1 acts at the insulin receptor level (21,23), obesity acts by different mechanisms, mostly at a postreceptor level (51). It is, therefore, possible that postreceptor insulin-signaling abnormalities are necessary for the Q121 variant to be fully effective in inducing insulin resistance and, eventually, related clinical outcomes.

The three cohorts of very high-risk individuals that we studied were quite different from each other: one comprised only patients with type 2 diabetes and CAD, another included patients with a previous MI who did not have frank type 2 diabetes, and the third included only patients with ESRDs. Despite such apparent phenotypic heterogeneity, the effect of the Q121 variant was not heterogeneous across the three studies. Not only did this allow us to analyze the three cohorts together, increasing statistical power, but it also suggests that our findings may be generalizable to all high-risk patients, irrespective of their background clinical characteristics. Whether the predictive role of the Q121 variant extends to situations characterized by a more moderate cardiovascular risk remains to be determined.

We acknowledge that, mainly because of the relatively small size of our samples, the significance level of our findings is still compatible with a false-positive result. However, this seems unlikely given that the association between Q121 was not heterogeneous across the three cohorts and, importantly, was further confirmed in cross-sectional studies as far as the interaction with obesity in diabetic patients is concerned. We also acknowledge that due to the relatively small sample size of the studies that we analyzed, we cannot exclude that the gene-by-obesity interaction that we observed among diabetic patients also occurs among nondiabetic individuals, as is the case for the modulation of insulin resistance (25–27). Therefore, our findings need further replication in larger samples before they can be considered as established. Finally, because this study was entirely performed in individuals of European ancestry, we do not know whether our findings can be extended to populations of different race.

In conclusion, pending confirmation in further larger studies, the Q121 variant has the potential to become a clinical tool for identifying those very high-risk patients who are especially prone to major cardiovascular events and need, therefore, to be targeted with specific and even more aggressive preventive strategies.

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S.B. designed the study; acquired, analyzed, and interpreted the data; and wrote the manuscript. S.R. and S.P. acquired, analyzed, and interpreted the data and reviewed and edited the manuscript. B.S., C.P., A.F., A.P., D.L., A.T., Y.-Y.Z., and G.D.S. acquired data and reviewed the manuscript. F.M. reviewed the manuscript. G.T., R.X., D.M., F.A., R.L., E.V.G., and T.H.H. acquired data and reviewed the manuscript. S.D.C. acquired and interpreted the data and reviewed the manuscript. F.P.
acquired and analyzed the data and reviewed and edited the manuscript. C.Z. and M.F. acquired data and reviewed and edited the manuscript. A.D. and V.T. designed the study, analyzed and interpreted the data, and wrote the manuscript.

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