ENPP1 Q121 Variant, Increased Pulse Pressure and Reduced Insulin Signaling, and Nitric Oxide Synthase Activity in Endothelial Cells

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Objective—Insulin resistance induces increased pulse pressure (PP), endothelial dysfunction (ED), and reduced bioavailability of endothelium-derived nitric oxide (NO). The genetic background of these 3 cardiovascular risk factors might be partly common. The *ENPP1* K121Q polymorphism is associated with insulin resistance and cardiovascular risk.

Methods and Results—We investigated whether the K121Q polymorphism is associated with increased PP in white Caucasians and with ED in vitro. In 985 individuals, (390 unrelated and 595 from 248 families), the K121Q polymorphism was associated with PP ($P=8.0\times10^{-4}$). In the families, the Q121 variant accounted for 0.08 of PP heritability ($P=9.4\times10^{-4}$). This association was formally replicated in a second sample of 475 individuals ($P=2.6\times10^{-2}$) but not in 2 smaller samples of 289 and 236 individuals ($P=0.49$ and 0.21, respectively). In the individual patients' data meta-analysis, comprising 1985 individuals, PP was associated with the Q121 variant ($P=1.2\times10^{-3}$). Human endothelial cells carrying the KQ genotype showed, as compared to KK cells, reduced insulin-mediated insulin receptor autophosphorylation ($P=0.03$), Ser^{473}-Akt phosphorylation ($P=0.03$), and NO synthase activity ($P=0.003$).

Conclusions—Our data suggest that the *ENPP1* Q121 variant is associated with increased PP in vivo and reduced insulin signaling and ED in vitro, thus indicating a possible pathogenic mechanism for the increased cardiovascular risk observed in *ENPP1* Q121 carriers. **(*Arterioscler Thromb Vasc Biol*. 2009;29:1678-1683.)**

Key Words: ENPP-1 gene ■ cardiovascular disease ■ arterial stiffness ■ endothelial dysfunction ■ insulin resistance

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Increased pulse pressure (PP), mostly a consequence of arterial stiffness, has been associated with cardiovascular events, including myocardial infarction (MI).[1–5] Increased PP is associated with endothelial dysfunction (ED) and related reduced bioavailability of endothelium-derived nitric oxide (NO).[6,7] Both increased PP and ED are, at least partly, genetically determined.[8,9] Insulin resistance is pathogenic for both abnormalities[10,11] and is under genetic control too[12]; thus, it is possible that these conditions share some common genetic background.

ENPP1 (also known as plasma cell antigen 1 [PC-1]) is a class II membrane glycoprotein known to adversely influence insulin sensitivity by binding to and inhibiting insulin receptor (IR) function.[13] A missense polymorphism, K121Q, has been described in the *ENPP1* gene.[14] As compared to the K121, the Q121 variant is a stronger inhibitor of the IR/insulin receptor substrate-1 (IRS-1)/phosphatidylinositol 3-kinase (PI3-K) activity pathway[14,15] and, in most, although not all studies, has been associated with insulin resistance[13–16] and type 2 diabetes.[17] Most importantly, in this specific context, the *ENPP1* Q121 variant modulates susceptibility to atherosclerosis and premature MI.[18,19] To get insights about the pathogenic mechanisms underlying the deleterious cardiovascular effect of the *ENPP1* Q121 variant, we investigated in humans whether it was associated with increased PP. In endothelial cells, insulin stimulates Akt phosphorylation at Ser^{473} and subsequently increases NO release, a potent

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vasodilator[10,20] and a strong in vivo regulator of arterial stiffness and PP.[6,7] Thus, we also tested whether the *ENPP1* Q121 variant exerts a direct effect on some crucial steps of insulin signaling and on NO synthase activity in cultured human umbilical vein endothelial cells (HUVECs).

Methods

In Vivo Studies

First Stage Study

Nine hundred eighty-five white Caucasians from Gargano and surrounding area (Southern-East Italy) were investigated. Of them, 390 were unrelated subjects and 595 were from 248 pedigrees,[21,22] comprising 75 nuclear families, 153 sibships, and 20 extended sibships.

Replication Attempt

As for replication, 3 additional samples of white Caucasians were studied. Four hundred seventy-five obese (ie, BMI $\geq$30 Kg/m^2) and 289 nonobese individuals (ie, BMI $<$30 Kg/m^2) were recruited from the outpatient Obesity Clinic at the Endocrine Unit and the hospital staff of Garibaldi Hospital in Catania (Sicily, Southern Italy), respectively. Finally, 236 newly diagnosed and never-treated hypertensive patients (systolic blood pressure $\geq$140 mm Hg or diastolic $\geq$90 mm Hg) were recruited at the Catanzaro University Hospital (Calabria, Southern Italy). As per selection criteria, all subjects from these 4 samples had fasting plasma glucose $<$126 mg/dL, were free from overt cardiovascular disease (ie, by means of self report), and were not treated with medications known to interfere with either glucose or lipid metabolism and with blood pressure.

The study, approved by local ethical committees, was performed according to the Helsinki Declaration. All study individuals gave written informed consent. In all individuals clinical examination, data from standardized interview, and blood sample collection were obtained between 8:00 and 9:00 AM after an overnight fast. Seated brachial blood pressure after 10 minutes rest period and again 5 minutes later was taken with standard mercury sphygmomanometer by the same investigator in each sample. PP was calculated as the difference between the mean of the two consecutive measurements of systolic blood pressure (SBP) and the two consecutive measurements of diastolic blood pressure (DBP).

In Vitro Studies

Cell Cultures

Umbilical cords were obtained from healthy mothers delivering at the Pescara Town Hospital consecutively asked to participate in the study. Those who were willing to participate signed a written consent form. Primary HUVECs were obtained and cultured as previously described.[23] Because of our previous data showing that HUVECs carrying either the *IRS-1* R972 or the *TRIB3* R84 variant (of the *IRS-1* G972R or the *TRIB3* Q84R polymorphism, respectively) had reduced insulin-stimulated Akt activation and NO synthase activity,[24–25] HUVEC lines used in this study (at least 2 different strains of KK- and of KQ-HUVECs) were chosen among those carrying wild-type *IRS-1* G972/G972 and *TRIB3* Q84/Q84 genotypes and used for each experiment.

Insulin-Stimulated IR Phosphorylation

HUVECs were starved for 12 hours in serum-deprived medium and incubated with or without insulin (100 nmol/L). Fifty micrograms of total cell lysates were immunoprecipitaded with anti-IR β-subunit antibody (C19, Santa Cruz Biotechnology), separated by the same SDS-PAGE and transferred to nitrocellulose membrane (Amersham Pharmacia Biotech). Blots were probed with anti-PY antibody (4G10, Millipore) stripped, and reprobed with anti-IR β-subunit antibody (C19, Santa Cruz Biotechnology). Immunocomplexes were detected with the ECL Western Blotting System (Amersham Pharmacia Biotech) and quantified with a computerized densitometric system. Densities of IR β-subunit phosphorylation were divided by the amount of immunoprecipitate IR β-subunit and the ratio indicated as arbitrary units.

Table 1. Features of the 1985 Study Subjects

Sample	Gargano	Obese* Sicily	Non obese Sicily	Calabria
n	985	475	289	236
Sex, M/F	377/608	127/348	138/151	131/105
Age, y	39.4±0.4	37.2±0.54	36.7±0.73	44.7±0.35
Smokers, %	26.0	40.4	34.3	NA
BMI, kg/m^2	25.9±0.1	42.6±0.35	24.5±0.17	26.1±0.19
$HOMA_{IR}$	1.8±0.04	4.5±0.11	1.7±0.06	2.1±0.08
PG, mg/dl	90.3±0.3	97.9±0.55	86.7±0.56	89.8±0.43
IRI, μU/ml	7.8±0.1	12.0±0.4	7.8±0.26	9.6±0.36
HDL cholesterol, mg/dl	53..3±0.4	45.0±0.63	45.8±0.73	NA
Triglycerides, mg/dl	95.7±1.9	123±3.0	99.8±3.94	NA

Data are reported as means±SE. BMI indicates body mass index; $HOMA_{IR}$, homeostasis model assessment of insulin-resistance; PG, plasma glucose; IRI, immunoreactive insulin; NA, not available. *Obesity is defined as BMI $\geq$30 kg/m^2.

Insulin Stimulation of Akt Phosphorylation and NO Synthase Activity

HUVECs were starved for 2 hours in serum-deprived medium and incubated with or without insulin (100 nmol/L). Because in our hands phospho-Akt levels in HUVECs are comparable after 2, 5, and 10 minutes insulin stimulation (data not shown), the longest incubation (ie, 10 minutes) was chosen. Equal amounts of total cell lysate proteins were resolved by SDS-PAGE, transferred to nitrocellulose membrane, immunoblotted with anti-Akt, anti-Ser^{473}-Akt, and β-actin primary antibodies, and incubated with peroxidase-conjugated secondary antibodies. Proteins were detected using enhanced chemiluminescence and quantified with a computerized densitometric system. Densities of Akt phosphorylation were divided by those of total Akt content and the ratio indicated as arbitrary units.

NO synthase activity was determined in duplicate for each cell strain by measuring the conversion of L-(^{3}H)-arginine into L-(^{3}H)-citrulline as previously described.[26] In selected experiments, L-NAME (1 mmol/L) was added 40 minutes before L-(^{3}H)-arginine.[23] Data of NO synthase activity were normalized to cell protein content.

Genotyping

Genomic DNA from both blood samples and umbilical cord arteries and genotyping were obtained as previously described.[18]

Statistical Methods

General features of study subjects are reported in Table 1 as means±SE. A χ^2 test was used to assess whether genotype distribution was in Hardy–Weinberg Equilibrium (HWE). Association between PP, SBP, or DBP values and K121Q polymorphism in 3 samples (obese and not obese groups from Sicily, and hypertensive patients from Calabria) was assessed with a multivariate linear regression model. As for the Gargano sample, to account for within-family correlation in PP, SBP, and DBP values, a multivariate linear mixed model with an unstructured correlation matrix was used.[27,28] All 4 samples were adjusted for age, sex, smoking habit (except for the Calabria sample, where the latter variable was not available). PP (as well SBP and DBP) values according to *ENPP1* K121Q genotype are expressed as estimated means±SE (Table 2). Furthermore, when a significant association between K121Q polymorphism and PP was observed, analyses were adjusted also for $HOMA_{IR}$. Significance was assessed on the log transformed data because of the skewed distributions of PP, SBP, and DBP values; *ENPP1* genotype was tested in the regression models according to additive genetic model. Last, to provide a combined analysis, an individual patients' data meta-analysis using a linear mixed model[29–31] was performed on the 4 samples. Linear mixed models allowed to take into account potential between-sample heterogeneity (tested as a geno-

Table 2. Pulse Pressure (PP), Systolic Blood Pressure (SBP), and Diastolic Blood Pressure (DBP) of the 1985 Subjects According to Sample Location and *ENPP1* K121Q Genotype

	K121K	K121Q	Q121Q	*P**
SGR				
n	716	248	21	
PP, mm Hg	38.1±0.4	40.8±0.6	39.8±2.0	8.0×10^{-4}
SBP, mm Hg	114.8±0.5	117.0±0.8	117.1±2.8	1.1×10^{-2}
DBP, mm Hg	76.7±0.4	76.2±0.6	78.3±1.9	0.84
Obese Sicily				
n	326	137	12	
PP, mm Hg	43.8±0.4	44.6±0.7	49.3±2.3	2.6×10^{-2}
SBP, mm Hg	123.9±0.6	124.7±1.0	130.5±3.4	0.14
DBP, mm Hg	80.1±0.5	80.1±0.7	81.2±2.4	0.95
Non obese Sicily				
n	202	78	9	
PP, mm Hg	41.7±0.6	40.1±0.9	44.2±2.7	0.49
SBP, mm Hg	115.8±0.8	115.0±1.2	117.8±3.6	0.78
DBP, mm Hg	74.1±0.6	74.8±1.0	73.5±2.9	0.74
Calabria				
n	167	64	5	
PP, mm Hg	57.8±1.0	58.0±1.7	72.9±5.9	0.21
SBP, mm Hg	150.4±1.5	149.5±2.4	169.3±8.5	0.45
DBP, mm Hg	92.5±0.8	91.4±1.3	96.4±4.8	0.91
IPD meta–analysis				
n	1411	527	47	
PP, mm Hg	45.1±0.3	46.3±0.4	49.5±1.4	1.2×10^{-3}
SBP, mm Hg	125.7±0.4	126.7±0.6	131.0±1.9	1.7×10^{-2}
DBP, mm Hg	80.6±0.3	80.3±0.4	82.1±1.4	1.0

Data are reported as adjusted means±SE. *P values refer to the additive model after adjustment for age, sex, and smoking habit (except for the Calabria sample, where smoking was not available), assessed with linear (mixed) models. IPD indicates individual patients' data.

type-by-sample interaction),[30] and to simultaneously address within-family correlation[31] in 1 of the samples (ie, data from Gargano). The Sequential Oligenic Linkage Analysis Routines (SOLAR) software package[32] was used to estimate the heritability of PP and the contribution of K121Q polymorphism on it, as previously described.[21,22] All analyses were assessed after adjusting for age, age^2, sex, and smoking habit. For in vitro experiments, results are expressed as mean±SD of at least 3 different experiments, and the differences were assessed by Student *t* test. All statistical analyses were performed using SPSS Statistical Package (Version 13) and SAS Release 9.1 (SAS Institute). probability values <0.05 were considered statistically significant.

Results

In Vivo Studies

Clinical features of all study subjects are shown in Table 1. Genotype distribution was in HWE in each sample.

First Stage Study

In the first stage study (ie, the sample from Gargano) of 985 nondiabetic individuals, 716 carried the K121/K121 (ie, KK), 248 the K121/Q121 (ie, KQ), and 21 the Q121/Q121 (ie, QQ) genotype. In the multivariate linear mixed model the *ENPP1* K121Q polymorphism was significantly associated ($P=8.0\times10^{-4}$) with PP and to a lower extent SBP but not with DBP (Table 2). Of note, the association between Q121 variant and PP was still significant when adjusting also for $HOMA_{IR}$ ($P=6.0\times10^{-4}$). In the 248 families (n=595 individuals), variance component analysis showed that PP variability was partly explained by genetic factors (ie, heritability=0.29±0.08, adjusted $P=1.0\times10^{-4}$). Of note, the K121Q polymorphism, which was significantly associated with PP also in this subset (adjusted $P=7.3\times10^{-4}$), accounted for approximately one fourth of this heritability (ie, 0.08, adjusted $P=9.2\times10^{-4}$). Also SBP and DBP were heritable (0.46±0.08, adjusted $P=3.0\times10^{-7}$ and 0.31±0.09, adjusted $P=3.1\times10^{-5}$, respectively). However, the K121Q polymorphism accounted for only a small proportion of genetic variability of SBP (ie, 0.03, adjusted $P=2.3\times10^{-2}$) and not at all for that of DBP (ie, <0.001, adjusted $P=0.1$).

Replication Attempt

As for replication, 3 additional samples of white Caucasians (ie, 2 from Sicily and 1 from Calabria) were studied.

Samples From Sicily

Of the 475 obese unrelated individuals, 326 carried the KK, 137 the KQ, and 12 the QQ genotype. In the multivariate linear model the K121Q polymorphism was significantly ($P=2.6\times10^{-2}$) associated with PP (Table 2). No significant associations were observed with SBP and DBP (Table 2). The association between Q121 variant and PP remained significant after adjusting also for $HOMA_{IR}$ ($P=2.1\times10^{-2}$). Because a small proportion (22.3%) of untreated hypertensive patients was observed in this sample, data were analyzed after adjusting also for hypertensive status and showed that the association between the Q121 variant and PP was still significant ($P=4.0\times10^{-2}$).

Of the 289 non obese unrelated individuals, 202 carried the KK, 78 the KQ, and 9 the QQ genotype. No significant association was observed between PP values and the *ENPP1* genotypes (Table 2).

Sample of Hypertensive Patients From Calabria

Of these 236 unrelated patients, 167 carried the KK, 64 the KQ, and 5 the QQ genotype. PP values were not significantly different across genotype groups (Table 2).

Combined Analysis

Individual Patients' Data Meta-Analysis

The pooled dataset of the 4 samples was adjusted for age, gender, smoking habit, and sample location. The genotype-by-sample interaction did not show between-samples heterogeneity in the K121Q polymorphism effect on PP ($P=0.16$). Therefore, a fixed effects genotype individual patients' meta-analysis was performed, where the use of a linear mixed model was still necessary to account for the within-family correlation of 1 of the samples (ie, the Gargano sample). The K121Q polymorphism was associated with PP ($P=1.2\times10^{-3}$, $\beta=1.53$) and SBP ($P=1.7\times10^{-2}$, $\beta=1.50$) but not with DBP ($P=1.0$; Table 2). The association with PP did not change after adjusting also for $HOMA_{IR}$ ($P=1.4\times10^{-3}$). No significant gene × obesity (ie, BMI ≥30 Kg/m^2) interaction was observed in modulating PP (adjusted $P=0.93$). Neither in this pooled analysis ($P=0.67$) nor in any sample singly considered (data not shown) was a genotype-by-

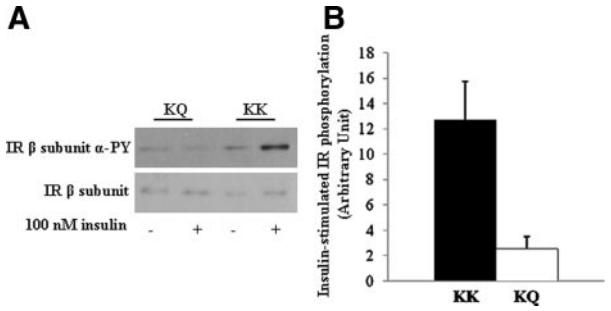

Figure 1. Impact of *ENPP1* K121Q variant on insulin-stimulated IR phosphorylation. A, KQ- and KK-HUVECs were either stimulated (lanes 2 and 4) or not (lanes 1 and 3) with 100 nmol/L for 10 minutes at 37°C. After solubilization, stimulated and unstimulated cell lysates were immunoprecipitated with anti-IR β-subunit antibody and loaded into the same gel. IR β-subunit autophosphorylation was assessed with anti-PY antibody. The blot was then stripped and reprobed with anti-IR β-subunit. A representative experiment is shown. B, Band density of IR β-subunit phosphorylation was expressed as a ratio of corresponding density for immunoprecipitate IR β-subunit. The obtained value was then divided by that obtained in unstimulated cells and then expressed as arbitrary units. Data shown are mean±SD of 3 experiments. *P=0.03 vs KK-HUVECs.

gender interaction observed, thus making unlikely the possibility of a gender-specific effect of the K121Q polymorphism on PP.

In Vitro Studies

Impact of ENPP1 *K121Q Variant on Insulin-Stimulated IR Phosphorylation*

Insulin-stimulated IR β-subunit phosphorylation was lower in KQ- (ie, 2.5-, 4.0-, and 1.1-fold stimulation over basal unstimulated phosphorylation level) than KK-HUVECs (14.1-, 17.2-, and 7.0-fold stimulation). A representative experiment is shown in Figure 1A. Mean data are shown in Figure 1B.

Impact of ENPP1 *K121Q Variant on Insulin-Stimulated Akt Phosphorylation*

Insulin-induced Akt phosphorylation at Ser^{473} was impaired in KQ- (ie, virtually no stimulation over basal unstimulated phosphorylation level) as compared to KK-HUVECs (ie, 1.49-, 1.50-, and 1.48-fold stimulation over basal unstimulated levels). Mean data are shown in Figure 2. In contrast, no significant differences were observed in Akt and β-actin protein expression (Figure 2).

Impact of ENPP1 *K121Q Variant on Insulin-Stimulated NO Synthase Activity*

To assess the impact of *ENPP1* K121Q variant on NO synthase activity, insulin-stimulated conversion of L-[^{3}H]arginine into L-[^{3}H]citrulline was measured. Basal NO synthase activity was not different between KK- and KQ-HUVECs (Figure 3). In contrast, after 100 nmol/L insulin stimulation, virtually no changes in NO synthase activity was observed in KQ-HUVECs, whereas a clear increase (ie, 1.88-, 1.77-, and 1.83-fold stimulation) was observed in KK-HUVECs. Mean data are shown in Figure 3. Preincubation with 1 mmol/L L-NAME (ie, an NO synthase inhibitor), abolished insulin-stimulated NO synthase activity in KK-HUVECs and had no effect on KQ-HUVECs (Figure 3).

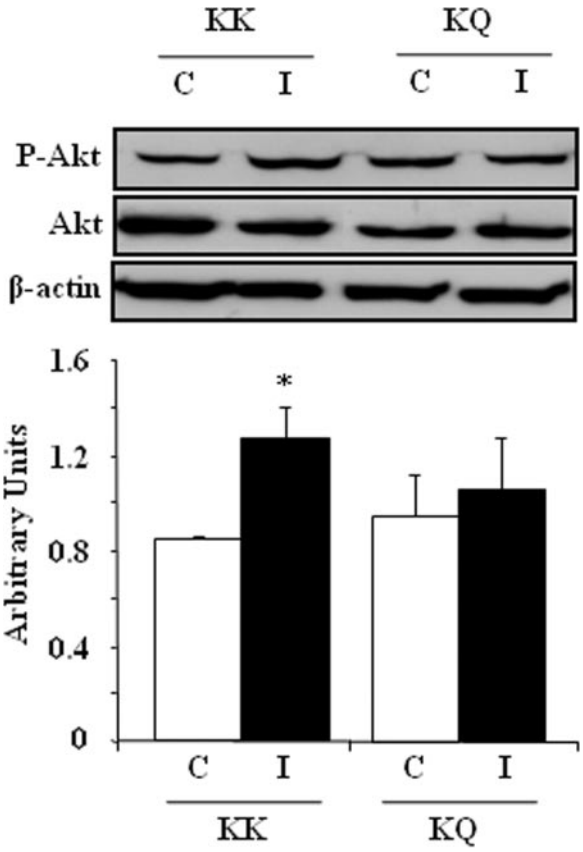

Figure 2. Impact of *ENPP1* Q121 variant on insulin-stimulated Akt phosphorylation. Upper panel, Ser^{473}, total Akt, and β-actin levels were evaluated in 100 nmol/L insulin stimulated KK and KQ HUVECs. A representative experiment is shown. Lower panel, Each bar represents the mean±SD of 3 independent experiments. Data of Akt phosphorylation were normalized against total Akt and expressed as arbitrary units. C indicates control cells; I, insulin-stimulated cells. *P=0.03 vs KK control cells.

Discussion

Our present study, performed in 4 samples of white Caucasians from Southern Italy comprising a total of 1985 individuals, suggests that the *ENPP1* Q121 variant is independently associated with increased PP, a useful clinical marker of arterial stiffness. When looking at the individual samples analyzed, statistically significant association was observed in the first stage study and in 1 of the 3 samples analyzed in the replication attempt, thus providing evidence of formal replication of the observed association. As an additional novelty of the present study, data from families indicated that the Q121 variant plays a role in PP heritability. Because the first sample mostly comprised healthy individuals of young-middle average age, the effect of the Q121 variant on PP might represent a very early alteration in the multi-step proatherogenic process. At variance with what was observed in the modulation of other phenotypes including insulin sensitivity,[33–35] type 2 diabetes,[36,37] and atherosclerosis,[18] no significant gene-by-obesity interaction was observed in modulating PP.

The Q121 variant was associated with SBP in 1 sample; however, no replication was observed in the other samples. In the pooled analysis, the Q121 variant showed a weaker association with SBP, as compared to that with PP. Similarly, data

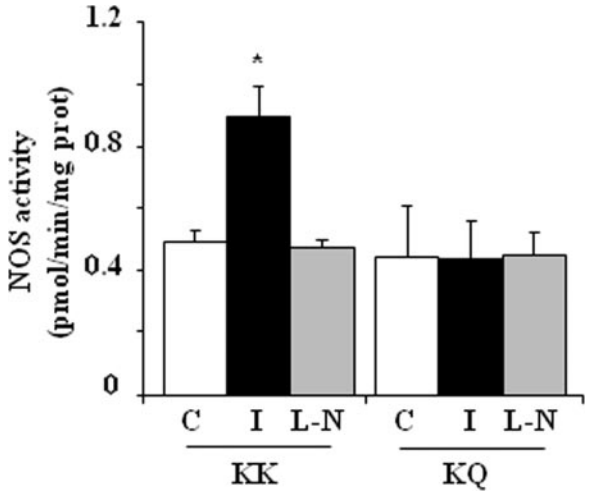

Figure 3. Impact of *ENPP1* Q121 variant on NO synthase activity. Conversion of L-[^{3}H]arginine into L-[^{3}H]citrulline in KK and KQ HUVECs. C indicates control cells; I, insulin-stimulated cells; L-N, 1 mmol/L L-NAME, added 40 minutes before L-[^{3}H]-arginine in 100 nmol/L insulin-stimulated cells. Each bar represents the mean±SD of 3 independent experiments with data normalized to cell protein content. *P=0.003 vs KK control cells.

from families indicated that the Q121 variant explained a much smaller proportion of SBP heritability as compared to that of PP. Some associations of the Q121 variant with incidence of hypertension[38] and increased SBP[39] have been reported in previous studies; unfortunately no measurement of PP were available in these studies,[38,39] thus making it impossible to compare previous data with our present findings. It is of note that in the Diabetes Genetics Initiative (www.broad.mit.edu/diabetes) database, SNP rs7767502, which is a perfect proxy of the K121Q polymorphism (r^2=1), is not associated with SBP, DBP, and hypertension (P=0.37, P=0.78, and P=0.07; of note, in all 3 cases, the tendency of association was toward the opposite direction than that observed in our study). Taken altogether, these data make uncertain the association with SBP and render unlikely that the association between the Q121 variant with PP is mediated by a genetic effect on SBP.

As a limitation of our study, we acknowledge that PP is only a surrogate of endothelial function or arterial stiffness. Therefore, a better and more direct phenotyping of 1 of these 2 parameters (or both) would have improved the quality of our study. However, this could have been performed only in small subgroups, thus probably lacking the statistical power to reach robust probability values of significance and leaving uncertain the interpretation of the results obtained. This finding have allowed us to pool and analyze together data from the 4 different populations, thus increasing statistical power.

Insulin resistance is a major contributor to atherosclerosis by altering both metabolic and vascular homeostasis, including endothelial function. The Q121 variant has been reported to confer an increased risk of insulin resistance in most, although not all, studies.[13–16] Thus, the association between this variant and PP may be mediated by insulin resistance–related mechanisms. Our in vitro data obtained in HUVECs, a model uniquely suited to determine a direct effect in human endothelium, help get deeper insights about the molecular mechanism of this association. In fact, cells carrying the Q121 variant have an impaired response to insulin stimulation in terms of IR autophosphorylation, Akt phosphorylation, and NO synthase activity. Although we cannot exclude that other yet unknown genetic variants able to affect insulin-induced NO synthase activity have played a role in the results we obtained, if by chance they have been carried by KQ- but not KK-HUVECs, these data are in line with the notion that, as compared to the K121, the Q121 variant is a stronger inhibitor of upstream insulin signaling at the level of IR/IRS-1/PI3-Kinase activity pathway.[14,15] Because reduced NO bioavailability at the endothelium may increase arterial stiffness and PP,[6,7] it may be hypothesized that the effect of *ENPP1* Q121 on PP is mediated, at least partly, by a direct detrimental effect on insulin-dependent endothelial function. A direct effect on the arterial wall is also suggested by the observation that, the association between the Q121 variant and high PP values was independent from $HOMA_{IR}$, a surrogate of "systemic" insulin resistance. A deleterious role on insulin signaling and NO synthase activity has been reported also in HUVECs naturally carrying other genetic determinants of human insulin resistance,[24,25] thus suggesting that this is a generalized phenomenon related to "insulin resistance genes."

It is quite well established that PP and arterial stiffness are strong predictors of premature cardiovascular events,[2] and of early coronary disease.[3] Thus, our present data suggest a possible pathogenic pathway contributing to the reported increased risk of early MI carried by Q121 carriers.[18,19]

False-positive results are not uncommon in association studies. In our case, the risk of population stratification was minimized by reporting the association in 2 genetically homogenous samples (both being recruited in restricted regions of Southern Italy) and by including families in the sample from Gargano. The lack of nominal association observed in the 2 additional samples is likely to be a consequence of low statistical power because of small sample size. As a matter of fact the power to detect an effect similar to that observed in the first sample from Gargano was ≤50% both in the sample comprising non obese individuals from Sicily and in that from Calabria. In the case of the sample from Calabria, also the intrinsic nature of the study (comprising only hypertensive patients in whom the range of PP distribution is reduced because limited to the high end of the PP values spectrum) might have reduced the chance to detect significant difference across genotype groups.

Thus, although the need of further replications in larger cohorts remains an essential next step to achieve even more robust, possibly genome-wide, P level of significance, the risk of a false-positive finding seems to be modest.

In conclusion, our data suggest that the *ENPP1* Q121 variant is associated with PP in white Caucasians from Southern Italy and with reduced insulin signaling and insulin-mediated NO synthase activity in human endothelium.

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Disclosures

None.

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