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Joint effect of insulin signalling genes on cardiovascular events and on whole body and endothelial insulin resistance

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VT had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Abstract

Objective—Insulin resistance (IR) and cardiovascular disease (CVD) share a common soil. We investigated the combined role of single nucleotide polymorphisms (SNPs) affecting insulin signaling (*ENPP1* K121Q, rs1044498; *IRS1* G972R, rs1801278; *TRIB3* Q84R, rs2295490) on CVD, age at myocardial infarction (MI), *in vivo* insulin sensitivity and *in vitro* insulin-stimulated nitric oxide synthase (NOS) activity.

Design and Setting—1. We first studied, incident cardiovascular events (a composite endpoint comprising myocardial infarction -MI-, stroke and cardiovascular death) in 733 patients (2,186 person-years, 175 events). 2. In a replication attempt, age at MI was tested in 331 individuals. 3. OGTT-derived insulin sensitivity index (ISI) was assessed in 829 individuals with fasting glucose < 126 mg/dl. 4. NOS activity was measured in 40 strains of human vein endothelial cells (HUVECs).

Results—1. Risk variants jointly predicted cardiovascular events (HR=1.181; p=0.0009) and, when added to clinical risk factors, significantly improved survival C-statistics; they also allowed a significantly correct reclassification (by net reclassification index) in the whole sample (135/733 individuals) and, even more, in obese patients (116/204 individuals). 2. Risk variants were jointly associated with age at MI (p=0.006). 3. A significant association was also observed with ISI (p=0.02). 4. Finally, risk variants were jointly associated with insulin-stimulated NOS activity in HUVECs (p=0.009).

Conclusions—Insulin signaling genes variants jointly affect cardiovascular disease, very likely by promoting whole body and endothelium-specific insulin resistance. Further studies are needed to address whether their genotyping help identify very high-risk patients who need specific and/or more aggressive preventive strategies.

Keywords

genetic susceptibility; non synonymous polymorphism; insulin sensitivity; insulin dependent endothelial function

INTRODUCTION

Cardiovascular disease (CVD) is highly prevalent (1) and imposes a tremendous burden to health care systems as well as to patients and their families. Genetic and environmental factors both contribute to CVD. Genes predisposing to CVD are mostly unknown (2) with only few clues about their identities being provided by recent genome-wide association studies (GWAS) (3–9). Insulin resistance, and the related cluster of abnormalities, is pathogenic for CVD (10–11). Since also insulin resistance is under genetic control (12–13), genes that contribute to impaired insulin sensitivity may also be involved in shaping cardiovascular risk (13).

Several variants harbored by genes involved in the modulation of insulin action have been so far described. Among these, there are three single nucleotide polymorphism (SNPs) of

insulin signalling regulating genes, namely rs1044498 (i.e. *ENPP1* K121Q), rs1801278 (i.e. *IRS1* G972R) and rs2295490 (*TRIB3* Q84R). These are the only non synonymous SNPs whose ability to affect the insulin signaling pathway has been thoroughly characterized *in vitro* not simply in transfected cell lines but also in human cells naturally carrying them (14–21). In addition, several (22–26), although not all (27), *in vivo* studies have reported that each of these SNPs is associated with human insulin resistance. Finally, in human endothelial cells these SNPs have been reported to inhibit insulin-stimulated nitric oxide (NO) production (16, 18, 20), a potent vasodilator whose deficiency is an early step in the development of atherosclerosis (28). Of note in this specific context, *ENPP1* K121Q has been also reported to predict major cardiovascular events (29). So, these three SNPs represent a unique model perfectly suited to answer the question of whether a progressive impairment of the insulin signaling pathways, as a consequence of their combined effect, plays a role in modulating the risk insulin resistance-related alterations.

Accordingly, our present aim was to investigate whether these three functional SNPs (with risk variants being *ENPP1* Q121, *IRS1* R972 and *TRIB3* R84, respectively) exert a combined effect on major cardiovascular events in high-risk individuals and whether in these patients such genetic information improves the ability to predict CVD. In addition, in order to unravel the biology underlying the joint associated effect of the 3 risk variants on CVD we in fact observed, their combined roles on insulin action both *in vivo* (i.e. on glucose disposal) and *in vitro* (i.e. on NO production by cultured human endothelial cells) was also investigated.

METHODS

Study samples and designs

Salient features of study subjects and designs regarding the prospective analysis on incident cardiovascular events, the cross-sectional association with age at myocardial infarction (MI) and the cohort study on insulin sensitivity are described into details in the online supplemental methods (i.e. “Study participants and design” section). Methods for performing studies on NO synthase (NOS) activity in human vein endothelial cells (HUVECs) are described in the same section of online supplemental methods.

All four study protocols were approved by the local institutional review boards and performed according to the Helsinki Declaration. Written informed consent was obtained from each study participant.

Genotyping

DNA was extracted from whole blood or HUVECs by standard methods. Genotyping of the *ENPP1* K121Q (rs1044498) *IRS1* G972R (rs1801278) and *TRIB3* Q84R (rs2295490) polymorphisms was performed by TaqMan allele discrimination (assays C_1207994_20, C_2384392_20 and C_16190162_10 respectively, Applied Biosystems, Foster City, CA) on the HT7900 platform (Applied Biosystems). Genotyping quality was assessed by including in each 96 wells plate positive controls with known genotypes (i.e. homozygous for the major allele, heterozygous or homozygous for the minor allele, previously evaluated for each SNP of interest by direct Sanger sequencing, each in duplicates). The agreement rate was >99% for each SNP assay. Genotype distribution was in Hardy-Weinberg equilibrium (HWE) in all study samples.

Statistical Analysis

Patients' characteristics are reported as mean and standard deviation (SD) for continuous variables and frequencies and percentages for categorical ones.

Deviations from HWE of each single SNP were investigated by exact χ^2 test.

The association between each single SNP and any given phenotype of interest was assessed according to the additive model of inheritance.

The association between the three SNPs considered in a combined fashion and any given phenotype of interest, was assessed by creating a weighted genotype risk score (GRS); this was obtained by summing the risk alleles from each SNP weighted by their estimated effect sizes, using regression coefficient as appropriated, on each outcomes of interest from current data. To facilitate results interpretation, each individual's GRS was divided by the maximum observed GRS and multiplied by 6 (i.e. the maximal theoretical number of risk alleles). Each GRS weights were estimated following a bootstrap approach with 10,000 re-samplings with replacement in order to reduce the risk of too optimistic discoveries.

In prospective studies, time-to-event analysis was conducted by means of Cox proportional hazards regression models and reported as hazard ratios (HR) along with their 95% confidence interval (CI). Time variable was defined as the time between enrolment date and the date of any of the three major cardiovascular events comprised in our composite end-point. For subjects who did not experience any cardiovascular event, time variable was defined as the time between the enrolment date and the date of the last available clinical follow-up. The assumption of proportionality of the hazards was tested by using scaled Schoenfeld residuals.

At the time of study design, we calculated that our prospective study on incident cardiovascular events had 80% power at a 0.05 significance level (type I error - α) to detect a HR=1.16, for each CV- GRS unit in the whole sample (n=733).

Details on addressing predicted risk probabilities, models' discrimination, reclassification improvement are given in the online supplemental methods (i.e. "Statistical analysis" section).

In cross-sectional studies, multivariate logistic regression analysis was used to test for association with dichotomous outcomes and results were reported as odds ratios with 95% confidence intervals. One-way analysis of variance (ANOVA) or covariance (ANCOVA; i.e. in multivariate models) was used to test for association with continuous traits.

Details about analyses on pooled samples also are given in the on line supplemental methods (i.e. "Statistical analysis" section).

Data on insulin-stimulated NOS activity in HUVECs were analyzed as percentage increase of insulin-stimulated NOS activity over basal, unstimulated value for each cell line.

Normal distribution was checked using Kolmogorov-Smirnov and Shapiro-Wilk tests as well as by graphical inspection of nonparametric kernel density estimation and Q-Q plot. Log-transformation was used for suspicious non normal distributed variables.

A p-value smaller than 0.05 was considered as significant. All analyses were performed using SAS Release 9.1 (SAS Institute, Cary, NC).

RESULTS

Prospective study on incident cardiovascular events

Characteristics of cohort members at baseline—The three study samples comprise, as by inclusion criteria, subjects who were at high risk of major cardiovascular events: the

Gargano Heart Study (GHS) – prospective design, a cohort of patients with T2DM and a previously diagnosed coronary artery disease; the Tor Vergata Atherosclerosis Study (TVAS), a cohort of subjects from the general population who had experienced a MI; and the Cardiovascular Risk Extended Evaluation in Dialysis (CREED) database, a cohort of individuals with ESRD who were on dialysis. Clinical characteristics of 733 study subjects at study entry are shown in appendix Table 1.

Prediction of major cardiovascular events—The average (mean + SD) follow-up time was 37.3 ± 19.6 months (range 1–91) in the GHS, 31.2 ± 10.8 (1–37) in the TVAS and 36.2 ± 22.0 (0–69) in the CREED. Number of each major cardiovascular events occurred during follow-up and overall incidence rates of study end point in the three studies either singly considered or pooled together are shown in the appendix Table 2. Estimated risk probabilities according to a clinical model comprising age, sex, smoking habit, diabetes and hypertension are shown in appendix Figure 1. Such clinical model showed a predicting ability of major cardiovascular events of approximately 68%, as indicated by a survival C-statistic of 0.678 (95% CI=0.633–0.723, $p=4 \times 10^{-15}$).

Number of events across genotypes of each SNP are shown in appendix Table 3, first panel.

HR (95%CI) for cardiovascular events given by each SNP in each sample as well as in the combined analysis of all 733 individuals are shown in Table 1, panel A. Of these, 311 individuals had no risk variants, while 299, 98, 21 and 4 individuals had 1, 2, 3 and 4 risk variants, respectively. Distribution of the weighted cardiovascular genotype score (CV-GRS) in the pooled sample is shown in appendix Figure 2, panel A. A significant association was observed between CV-GRS and incident events (HR=1.181, 95% CI=1.070–1.302, $p=0.0009$) (Table 1, panel A). Un-weighted genetic score (i.e. simply derived by summing the number of risk alleles) gave similar results (HR=1.260, 95% CI=1.074–1.477, $p=0.004$). Age at study entry (HR=1.039, 95% CI=1.025–1.053, $p=2 \times 10^{-07}$), smoking habit (HR=1.623, 95% CI=1.179–2.235, $p=0.003$), diabetes (HR=2.228, 95% CI=1.490–3.332, $p=0.0001$) and hypertension (HR=1.569, 95% CI=1.008–2.440, $p=0.046$) were additional predictors of incident events. Sex (HR=1.323, 95% CI=0.953–1.835, $p=0.094$) also tended to be associated with increased rate of events, although not reaching nominal statistical significance. The increased risk of events associated with CV-GRS remained significant after adjusting for all these variables (HR=1.128, 95% CI=1.021–1.246, $p=0.017$) (Table 2). In this multivariate model also age, smoking status and diabetes (but not sex) were independent predictors (Table 2). Hypertension tended to remain associated too; however, probably because of insufficient statistical power, this association was no longer significant. Of note, the CV-GRS was not associated with baseline values of any of these covariates as well as baseline BMI (data not shown).

Given previous evidences suggesting that some of these SNP are preferentially associated with insulin resistance and related traits among obese individuals (13), we stratified patients according to individual BMI being < or ≥ 30 Kg/m² (i.e. non-obese or obese). The association between CV-GRS and cardiovascular events in the adjusted model was statistically significant among the 204 obese patients (38 events, adjusted HR=1.285, 95% CI=1.034–1.597, $p=0.024$) while not significant and barely observable among the 508 non obese patients (131 events, adjusted HR=1.073, 95% CI=0.949–1.212, $p=0.262$) (p for CV-GRS-by-obesity interaction=0.15).

Survival C-statistic showed by CV-GRS was 0.634 (0.589–0.678; $p=2 \times 10^{-9}$) and 0.756 (0.677–0.835; $p=9 \times 10^{-11}$) in the whole sample and in the obese subgroup, respectively. The addition of CV-GRS to survival C-statistic obtained with the clinical model produced a significant improvement (from 0.678 to 0.688, $p=0.02$) with both models resulting

adequately calibrated (HL p-values being 0.13 and 0.14, respectively). Such improvement in risk discrimination was much more evident in the subgroup of obese individuals with survival C-statistic increasing from 0.759 to 0.788, $p=0.008$). Also in this case, both models turned out to be calibrated (HL p-values being 0.87 and 0.44, respectively). In 4-year risk prediction, using of NRIc indicated that the addition of the CV-GRS to the clinical model allowed a net correct reclassification (i.e. number of patients correctly reclassified minus number of patients wrongly reclassified) of 135 out of 733 individuals ($p=0.036$; 12.9% and 5.6% in those with and without incident events, respectively) in the whole sample and 116 out of 204 individuals ($p=0.003$; 32.8% and 24.2% in those with and without incident events, respectively) in the obese subgroup.

Cross-sectional study on age at MI

To seek replication of the joint associated effect of the three SNPs on cardiovascular events, we analyzed their association with 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 study subjects are summarized in appendix Table 4.

Age at MI across genotypes of each SNP is shown in appendix Table 3, second panel.

Effect sizes and p value of the associations with age at MI of each SNP in each sample as well as in the pooled analysis of all 331 individuals are reported in Table 1, panel B. Of these, 134 individuals had no risk variants, while 144, 45 and 8 individuals had 1, 2 and 3 risk variants, respectively. Distribution of the weighted myocardial infarction genotype risk score (MI-GRS) in the pooled sample is shown in appendix Figure 2, panel B.

When the joint associated effect of the three SNPs was investigated, a significant inverse relationship was observed between MI-GRS and age at MI (Table 1 panel B; $p=0.006$). A similar association was observed after adjusting for sex, smoking habit and hypertension (neither age - due to its collinearity with age at MI - nor diabetes - because all study participants were diabetic - were used as covariates) ($p=0.001$). The association between MI-GRS and age at MI was statistically significant among the 174 obese ($p=0.003$) but not among the 140 non obese ($p=0.07$) patients (p for MI-GRS-by-obesity interaction= 0.60). In the whole sample, un-weighted genetic score gave similar results ($p=0.016$). It is of note that also the genetic score we derived from prospective studies (i.e. CV-GRS) was associated with age at MI, with effect size being -0.84 yr for one score unit ($p=0.027$).

Study on insulin sensitivity

Clinical characteristics of the 829 study individuals whose OGTT-derived insulin sensitivity index (ISI) was investigated are shown in appendix Table 5.

ISI values across genotypes of each SNP is shown in appendix Table 3, third panel. Effect sizes and p value of the associations with log transformed ISI values of each SNP in 829 normoglycemic untreated individuals are reported in Table 1, panel C. Of these, 347 had no risk variants, while 327, 128, 24 and 3 individuals had 1, 2, 3 and 4 risk variants, respectively. Distribution of weighted insulin sensitivity genotype risk score (IS-GRS) is shown in appendix Figure 2, panel C. When the joint associated effect of the three SNPs was investigated, a significant inverse relationship ($p=0.02$) was observed between IS-GRS and log ISI (Table 1, panel C), which remained significant also after adjusting for age, sex and BMI ($p=0.035$). Un-weighted genetic score gave similar results ($p=0.06$).

Subjects were then divided in relatively insulin-sensitive (i.e. with individual ISI in the higher tertile of the whole sample; $n=276$) and relative insulin-resistant (i.e. with individual

ISI in the lower tertile of the whole sample; $n=276$). IS-GRS was significantly associated with the chance to be insulin-resistant (OR, 95% CI, being 1.29, 1.07–1.55, $p=0.007$).

Studies on NOS activity in human endothelial cells

To assess the combined associated of *ENPP1* K121Q, *IRS1* G972R and *TRIB3* Q84R SNPs with *in vitro* insulin-stimulated NOS activity, conversion of L-[3H] arginine into L-[3H] citrulline was measured in cultured HUVECs from 40 donors.

Basal, insulin stimulated and percentage increase after insulin stimulation NOS activity across genotypes of each of the three SNPs is shown in appendix Table 3, fourth panel.

Effect sizes and p value of the associations with percentage increase insulin-stimulated NOS activity in all 40 cell lines are reported in Table 1, panel D. Of these cell lines, 17 had no risk variants, while 9, 10, 3 and 1 had 1, 2, 3 and 4 risk variants, respectively. Distribution of weighted insulin-stimulated NOS activity genotype risk score (NOS-GRS) is shown in appendix Figure 2, panel D. Mean NOS activity was significantly increased upon insulin stimulation in cells carrying no risk alleles (i.e. 44% increase over unstimulated cells, $p=0.023$). When the joint association of the three SNPs was investigated, a significant inverse relationship ($p=0.009$) was observed between NOS-GRS and percentage increase insulin-stimulated NOS activity (Table 1, panel D). Un-weighted genetic score gave similar results ($p=0.039$).

DISCUSSION

Our results indicate that the three non synonymous, functional SNPs *ENPP1* K121Q, *IRS1* G972R and *TRIB3* Q84R exert a combined effect on incident major cardiovascular events in high-risk patients. This effect is independent of that of age, sex, smoking habit, diabetes and hypertension. A significant association was also observed between these three SNPs and age at MI in cross-sectional studies, thus reinforcing our finding.

The data we here present strongly suggest that the effect through which insulin signaling regulating gene variants exert a joint effect in predicting cardiovascular events is mediated, at least partly, by their association with reduced whole body insulin sensitivity as well as with endothelial specific insulin resistance. Interestingly, insulin resistance at the endothelium level has been shown to directly prompt atherosclerosis progression in genetically modified animal models (30). Overall, our present finding convincingly points to defective insulin signaling as a molecular basis to the pathogenic role played by insulin resistance on CVD (10,11).

The joint prediction of *ENPP1* Q121, *IRS1* R84 and *TRIB3* R84 variants, seems to be more appreciable among obese individuals. Understanding this apparent preferential effect in the presence of fat accumulation is beyond the scope of the present investigation. One can here only hypothesize that obesity-related abnormalities, including chronic inflammation and abnormal adipokines production (31), are necessary for the combined effect of insulin signaling genetic variants to be fully effective in increasing cardiovascular risk.

The issue of whether and how genetic information may be used in the clinical set for predicting cardiovascular events is a matter of debate with more efforts needed to try fill the existing gaps of knowledge (32). In this context, it is of note that our study indicates that the use of a risk score derived from these three SNPs significantly increases the overall predictability of CVD events, especially among obese patients. In this latter group, the observed improvement is not only statistically significant but also clinically relevant allowing correct reclassification of high proportion of patients both with and without events.

A preferential combined effect of *ENPP1* Q121, *IRS1* R84 and *TRIB3* R84 variants was observed also in the cross-sectional study on MI. Given the poor ability to predict cardiovascular events among high-risk patients by using traditional predictors and the expected increased prevalence of obesity worldwide (33), a large number of individuals might be likely to benefit from the availability of such genetic information which is not costly and easy to obtain.

We acknowledge that both in the prospective and the cross-sectional study some individual SNP effect was very trivial (i.e. *IRS1* G972R); however, given the background upon which we designed our study and specified our aim, we believe it would not be fair to exclude any of the SNPs when analyzing their combined effects on our phenotypes of interest. Conversely, despite some individual SNP effect was larger than that obtained by the combined analysis, the level of statistical significance, was definitively higher for the latter approach. This is the consequence of a much larger number of patients who carry the risk genetic exposure when considering the combined GRS as well as the greater theoretical individual genetic load, ranging 0–2 or 0–6 risk variants when SNPs are considered either singly or combined.

We also acknowledge that the three cohorts of high risk individuals we studied were quite different at baseline in terms of presence/absence of diabetes, MI and end stage renal diseases and that this may be viewed as a limitation of our study. On the other hand, it should be recognized that the individual effect of each variant was not heterogeneous across the three samples and in all but one case (i.e. *IRS1* R972 in the GHS-prospective design) all associations were toward the same direction (Table 1). This increases the credibility of our finding and suggests it may be generalizable to all high-risk patients, irrespective of their clinical characteristics background.

A further limitation of this study is represented by the robustness of our p values which do not reach a genome-wide level of significance and are, therefore, still compatible with a false-positive result. Thus, although we were able to confirm in cross-sectional studies the combined effect of the three SNPs on cardiovascular risk we had observed prospectively, our present finding needs replication in larger prospective studies before it can be considered as established. Recently, several large GAWs on coronary artery disease have been published (3–9). Unfortunately, differences between our investigation and these studies, such as different end points (with our study comprising also stroke and cardiovascular death) and different baseline cardiovascular risk (with only the patients enrolled in our study being very high-risk as per selection criterion) makes problematic to use GWAS data as a proper replication set of our findings. In addition, to the best of our knowledge, neither the Q84R polymorphism nor good proxies that are in linkage disequilibrium with it were included in GWAS, thus making impossible to draw any information from these datasets.

We also recognize that our data on insulin-mediated endothelial function are obtained in HUVECs, which are not very responsive cells to insulin stimulation of NOS activity. On the other hand, it should be also recognized that this model is uniquely suited to study insulin action in human endothelial cells and, in fact, has been utilized to obtain most of the information we have today on the role of insulin signaling abnormalities on *in vitro* human endothelial dysfunction (16, 18,20).

Our study populations comprise only White individuals who (with the only exception of those from Boston) were recruited in a homogenous geographical region (i.e. central-south Italy). This might be considered a strength of our study. Conversely, it remains to be addressed whether or not our present findings can be extended to populations with different genetic and/or environmental background.

Finally, a composite end point comprising different cardiovascular events, which may recognize different pathogenic backgrounds, has been utilized. Though this approach may have weakened the genotype-phenotype association we have looked for, we recognize that further studies using unique clinical events as independent endpoints are likely to allow a deeper understanding of the biology underlying our finding. In conclusion, pending confirmation in further studies, our study indicates that functional non synonymous variants harbored by insulin signaling regulating genes jointly predict major cardiovascular events in high-risk patients, thus, strongly pointing to abnormal insulin signaling as a pathogenic factor of atherosclerosis development and progression. This association is likely to be mediated, at least partly, by a combined deleterious effect on both systemic and endothelial insulin resistance. Genotyping these variants improves the overall predictability of cardiovascular events, especially among obese patients and might become, therefore, a tool for identifying patients who are particularly vulnerable and need to be targeted with specific and even more aggressive preventive strategies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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- Combined role of three SNPs in insulin signaling genes on CVD has been investigated.
- Findings show a combined effect of the SNPs in predicting major cardiovascular events.
- Findings have been replicated in a cross-sectional study on age at MI.
- The role of the three SNPs has been evaluated on whole body insulin resistance.
- Insulin-stimulated NOS activity in HUVECs according to SNPs genotype has been evaluated.

Table 1

Individual effect of each SNP on: prediction of major cardiovascular events, age at myocardial infarction, insulin sensitivity and NOS activity in endothelial cells.

SNP	PANEL A Major cardiovascular events		PANEL B Age at myocardial infarction		PANEL C Insulin sensitivity		PANEL D NOS activity	
	Sample	HR (95%CI), p	Sample	Effect size (SE), p	Effect size (SE), p	Effect size (SE), p	Effect size (SE), p	Effect size (SE), p
<i>ENPP1</i> K121Q (rs1044498)	GHS	1.29 (0.78–2.11), 0.32						
	TVAS	1.80 (1.11–2.92), 0.02	GHS - cross sectional	0.48 (1.4) ^a , 0.73				
	CREED	1.26 (0.85–1.86), 0.25	JHS	–3.9 (1.5) ^a , 0.009	–0.01 (0.04) ^a , 0.80	–22.49 (12.09) ^a , 0.07		
	Pooled	1.40 (1.08–1.81), 0.01	Pooled	–1.45 (1.0) ^a , 0.15				
<i>IRS1</i> G972R (rs1801278)	GHS	0.96 (0.50–1.85), 0.91						
	TVAS	1.17 (0.41–3.32), 0.77	GHS - cross sectional	–3.02 (2.0) ^a , 0.13				
	CREED	1.05 (0.65–1.68), 0.85	JHS	–2.5 (2.3) ^a , 0.28	–0.06 (0.06) ^a , 0.32	–0.14 (21.12) ^a , 0.99		
	Pooled	1.02 (0.72–1.46), 0.89	Pooled	–2.8 (1.49) ^a , 0.06				
<i>TRIB3</i> Q84R (rs2295490)	GHS	1.40 (0.87–2.26), 0.17						
	TVAS	1.66 (0.90–3.08), 0.10	GHS - cross sectional	–2.98 (1.5) ^a , 0.049				
	CREED	1.18 (0.81–1.72), 0.38	JHS	1.2 (1.4) ^a , 0.38	–0.10 (0.05) ^a , 0.04	–29.58 (14.94) ^a , 0.05		
	Pooled	1.33 (1.02–1.73), 0.03	Pooled	–0.73 (0.47) ^a , 0.47				
Weighted GRS		1.18 (1.07–1.30), 0.0009		–1.25 (0.46)^b, 0.006	–0.04 (0.02)^b, 0.02	–11.76 (4.25)^b, 0.009		

Risk variants are *ENPP1* Q121, *IRS1* R972 and *TRIB3* R84.

GHS=Gargano Heart Study; TVAS=Tor Vergata Atherosclerosis Study; CREED=Cardiovascular Risk Extended Evaluation in Dialysis database; JHS= Joslin Heart Study.

SNP: single nucleotide polymorphism.

All analyses in each individual sample are unadjusted.

Pooled analyses are adjusted for “study sample” as indicated in the Methods.

^a Effect size on continuous traits is given for each risk allele according to additive genetic model.

^b Effect size is given for one score unit, considering GRS as a continuous trait

Table 2

Multivariate analysis on prediction of major cardiovascular events in 733 individuals from GHS-prospective design, TVAS and CREED.

Covariate	HR	95%CI	p-value
CV-GRS (one unit)	1.128	1.021–1.246	0.017
Smoking habit (yes vs. no)	1.539	1.061–2.231	0.023
Gender (male vs. female)	1.199	0.817–1.761	0.354
Hypertension (yes vs. no)	1.451	0.929–2.266	0.101
Diabetes (yes vs. no)	1.685	1.119–2.538	0.013
Age (one year)	1.038	1.024–1.053	2×10^{-7}

GHS=Gargano Heart Study; TVAS=Tor Vergata Atherosclerosis Study; CREED=Cardiovascular Risk Extended Evaluation in Dialysis database.

CV-GRS= cardiovascular genotype risk score.

CV-GRS and age have been analyzed as continuous traits.