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Infrequent *TRIB3* coding variants and coronary artery disease in type 2 diabetes

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Web Resources:

dbSNP, http://www.ncbi.nlm.nih.gov/projects/SNP/

Exome Aggregation Consortium (ExAC) http://exac.broadinstitute.org

[January, 2015 accessed].

Mutation Assessor: http://www.ngrl.org.uk/Manchester/page/mutation-assessor

MutPred: http://mutpred.mutdb.org/

Mutation Taster: www.mutationtaster.org/

Polyphen-2: http://genetics.bwh.harvard.edu/pph2/

PROVEAN: http://provean.jcvi.org/index.php

SIFT: http://sift.jcvi.org/

SNPs&Go: http://snps.biofold.org/snps-and-go/index.html

Author Contributions

S.P.: designed the study, obtained, analyzed and interpreted the data and wrote the manuscript.
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G.S.: designed the study and edited the manuscript.
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V.T. is the guarantor of this work and, as such, had full access to all 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—Genes that modulate insulin sensitivity may also be involved in shaping the risk of coronary artery disease (CAD). The relatively common *TRIB3* Q84R polymorphism (rs2295490) has been associated with abnormal insulin signaling, endothelial dysfunction, insulin resistance, and pro-atherogenic phenotypes. The aim of our study was to investigate the association between low-frequency *TRIB3* coding variants and CAD in patients with type 2 diabetes (T2D).

Methods—Three case-control studies for CAD from Italy and US were analyzed, for a total of 1,565 individuals, all with type 2 diabetes. Infrequent variants were identified by re-sequencing *TRIB3* exons in 140 “extreme cases” and 140 “super-controls” and then genotyped in all study subjects.

Results—*TRIB3* infrequent variants (n=8), considered according to a collapsing rare variants framework, were significantly associated with CAD in diabetic patients from Italy (n=700, OR=0.43, 95% CI 0.20–0.91; p=0.027), but not from the US (n=865, OR==1.22, 95% CI 0.69–2.18; p=0.49). In the Italian sets, the association was especially strong among individuals who also carried the common R84 variant.

Conclusion—Although preliminary, our finding suggests a role of *TRIB3* low-frequency variants on CAD among Italian patients with T2D. Further studies are needed to address the role of *TRIB3* infrequent variants in other populations of both European and non-European ancestries.

Introduction

Coronary artery disease (CAD) is a leading cause of death worldwide, especially in patients with type 2 diabetes (T2D) (1). CAD, as many other complex diseases, is under the combined control of both genetic and environmental factors. While the latter are well known (2), the former are only partially understood as indicated by the fact that all the frequent variants discovered to date by genome-wide associations studies (GWAS) (3,4) account for only a small proportion of the CAD heritability. An additional proportion of the CAD-predisposing genetic background may be explained by low-frequency/rare variants (5–8). However, to date, no data have been made available in support of this hypothesis among patients with T2D - a condition characterized by high cardiovascular risk.

Insulin resistance is a well-established pathogenic factor for atherosclerosis and related cardiovascular disorders such as CAD (9,10). Since insulin resistance (11) and CAD (3) are both in part under genetic control, they might share some common genetic background, that is, genes that modulate insulin sensitivity may also modulate CAD risk. Indeed, we have recently reported that some common variants that affect insulin signaling and are associated with insulin resistance, are also associated with major cardiovascular events (12–14).

Among these variants is a relatively common amino acid substitution (Q84R; rs2295490) in *TRIB3* – an inhibitor of insulin-stimulated Akt phosphorylation and downstream signaling (15). This polymorphism, increasing TRIB3 inhibitory activity on insulin signaling (15–18),

has been associated with endothelial dysfunction *in vitro* (17, 19) and with several *in vivo* metabolic alterations including insulin resistance (16) and other pro-atherogenic phenotypes (16, 19, 20).

For these reasons, *TRIB3* is a prime candidate in the search for low-frequency coding variants predisposing to CAD among subjects with T2D.

Methods

Study participants

Individuals from three independent case-control studies of CAD among patients with T2D, namely the Gargano Heart Study-cross-sectional design (GHS, n=481), the Catanzaro Study (CS, n=219) and the Joslin Heart Study (JHS, n=865) were investigated. Briefly, patients from the first two studies, GHS and CS, were recruited in Italy, at the Institute "Casa Sollievo della Sofferenza" in San Giovanni Rotondo (FG) and at Magna-Graecia University in Catanzaro, respectively. JHS participants were recruited at the Joslin Diabetes Center and Beth Israel Deaconess Medical Center in Boston. Recruitment procedures for these three studies have been previously described (4). Briefly, CAD-cases were patients with T2D who had a stenosis greater than 50% in at least one major coronary artery or a main branch thereof that was documented by cardiac catheterization or had had a previous MI. CAD-control participants had no clinical evidence of CAD and had a normal ECG response to an exercise treadmill test; control participants from the JHS were recruited if they were older than 55 years and have had diabetes for more 5 years.

In all three studies, participants were non-Hispanic Whites and were diagnosed with T2D according to the ADA 2003 criteria. Their clinical features are described in Table 1.

The study protocol and informed consent procedure were approved by the local human subject committees. All participants gave written informed consent.

Study design

Re-sequencing of the *TRIB3* coding region—The entire *TRIB3* coding region was re-sequenced in 280 individuals from GHS by the Sanger method as previously described (16). To increase the probability of detecting infrequent variants with biological effect (21) (i.e. that affect the risk of CAD), we selected 140 "extreme" cases that had had an MI (rather than simply a coronary stenosis) and 140 super-controls who were free of CAD despite being older than 60 years.

Genotyping of *TRIB3* infrequent coding variants—Genotyping of rs200422001, rs138380491, rs41281850, rs35051116, rs149447454, rs144632965, rs140801463, rs374473490, was carried out at the Joslin Genetics Core in a total of 1,565 individuals from GHS, JHS and CS by means of custom TaqMan assays implemented on an ABI PRISM 7700 HT Sequence Detection System (Life, Foster City, CA). Genotyping of rs2295490 was carried out at the Mendel Institute in 1,514 individuals, by means of custom TaqMan assays implemented on an ABI PRISM 7700 HT Sequence Detection System (Life, Foster City,

CA). Genotyping quality was tested by including six blinded duplicated samples in each 384-well assay. The average agreement rate was greater than 99%.

Statistical Analysis

Our discovery sample of 280 patients had 95 and 99% probability to identify *TRIB3* variants with minor allele frequency (MAF) equal to 0.5 and 0.8%, respectively.

The association between each individual SNP, as well as between all SNPs considered together (i.e. by a burden test which collapse rare variants in a genetic region into a single burden variable) and CAD was evaluated by Fisher exact test and by logistic regression analysis, respectively. Results were reported as odds ratios (OR) along with their 95% confidence interval (CI). The burden test was chosen since it is recognized as the most powerful statistical tool when the majority of variants can be considered as pathogenic and effects can be assumed to be in the same direction (21), as it was in our case (see results).

In the burden test, heterogeneity of *TRIB3* infrequent variants effect across sets or across strata in individual sets was tested by introducing the cross-product term (sample-by-number of variants, strata-by-number of variants).

With a cumulative variant frequency of 5.3%, equal to that observed among controls from our discovery sample, the entire case-control study comprising 1,565 individuals (715 cases and 850 controls) had 80% power to detect associations with an OR as low as 1.75 (or as high as 0.57), with a type I error (alpha) equal to 0.05.

Results

By sequencing all *TRIB3* exons in 280 subjects from the GHS, we identified 9 non-synonymous variants. The probability of these sequence differences to be functional, based on the most used and reliable prediction tools considered together to generate a collective predictive score, are shown in Table 2. Eight of these variants were genotyped in all study patients from the three samples. The remaining variant (rs146095753) was not investigated further as the adjacent coding sequence contains additional close variants, thus impeding the design of a custom TaqMan assay.

The distributions of all infrequent variants, considered individually, in controls and cases from each set are shown in Table 1, online supplemental material. None variant showed a distribution that was significantly different between samples from Italy and US. In the two Italian sets, all variants were observed among controls while only three were detected among cases; an opposite trend was observed in the US set.

All variants except one had a MAF < 0.5% in both Italian sets. By contrast, in the US set, rs35051116 (R153H) had a MAF of 1.62%, which is virtually identical to that reported by the Exome Aggregation Consortium (ExAC) in 60,706 unrelated individuals from a variety of disease-specific and population genetic studies. The same SNP had a MAF of 0.36% in the combined Italian samples. Given the very low MAF of all SNPs, testing of HWE was not carried out.

When all *TRIB3* variants were considered together according to a "collapsing variants" framework (i.e. by a burden test, which allows testing the combined effect of all variants rather than that of each variant as singly considered), these tended to be more frequent among controls than cases in both Italian sets (GHS and CS), but not in the set from the US (JHS) (Table 3). When the two Italian sets were pooled and analyzed together, a significant, inverse relationship was observed between the number of *TRIB3* infrequent alleles and the probability of being a case subject (additive OR=0.43, 95% CI 0.20–0.91; p=0.027) (Table 3). By contrast, no association between TRIB3 variants and case status was found in the JHS (additive O=1.22, 95% CI 0.69–2.18; p=0.49) (Table 3). The ORs in the Italian and US sets were statistically different (p for ORs heterogeneity=0.03).

We then examined the interaction between *TRIB3* infrequent variants and the *TRIB3* Q84R SNP (i.e. rs2295490) - a frequent polymorphisms that has been repeatedly reported to affect insulin signaling (15–18) and to be associated with insulin resistance and pro-atherogenic related traits (16, 19, 20, 22, 23). Data for this SNP were available for most study individuals, including 659 subjects from Italy and 855 from the JHS. No association was observed among these individuals between *TRIB3* Q84R and CAD (OR=0.90 95% CI 0.64–1.26, p=0.53 in the pooled Italian sets and OR=1.03 95% CI 0.77–1.37, p=0.84 in the JHS). In the pooled Italian samples, the association between *TRIB3* infrequent variants and CAD was statistically significant in the 203 individuals carrying the R84 allele (180 QR + 23 RR) than among the 457 Q84/Q84 individuals (OR=0.11, 95% CI= 0.00–0.57; p=0.019 vs. OR=0.60, 95% CI 0.28–1.30; p=0.20; p for interaction=0.08). Although no statistical significant was observed in either group, a difference toward a similar direction was observed in the US set (OR=0.82, 95% CI 0.22–3.13 in 287 XR vs. OR=1.32, 95% CI 0.69–2.52 in 568 QQ individuals).

Discussion

The main results of our study are that i) *TRIB3* infrequent variants are associated with CAD in patients with T2D of European ancestry from Italy and that ii) this finding could not be replicated in patients of the same ancestry from the Boston area.

It is important to note that the two Italian sets were recruited from two homogeneous regions, both from Central-Southern Italy and only few hundred kilometers apart. By contrast, JHS participants were from an urban area in the US, in which genetic and cultural differences, even among individuals of European ancestry, are much more pronounced. Although it is possible that the differences in findings between Italian and US samples were simply due to chance, it is plausible that these were due to the different environmental and/or genetic background of study samples.

While some data are available for the general population (5–8, 24), to the best of our knowledge, this is the first report about the role of infrequent variants on CAD in patients with T2D.

In an exploratory analysis, we observed that most of the protective effect of *TRIB3* infrequent variants on CAD observed among Italian subjects occurs among carriers of the

TRIB3 R84 variant, which has been previously associated with abnormal insulin signaling (15–18), insulin resistance (22, 23), and atherosclerosis-related traits (16, 19, 20). It can then be hypothesized that *TRIB3* infrequent variants preferentially exert their protective effect among individuals who are genetically predisposed to an insulin resistant, pro-atherogenic phenotype. In a broader perspective, these data confirm the tenet that the combination of both frequent and infrequent variants are involved in determining the risk of complex traits (25) and extend this concept to the circumstance of a combined effect of several variants that are harbored in the same gene.

Several limitations of our study should be acknowledged. First, the sample size used for the sequencing stage allowed us to identify 95% of the variants with a MAF of 0.5%, but only 40% of those with a MAF of 0.1% and 25% of those with a MAF of 0.05%. Indeed, while our study was ongoing, the ExAC reported a total of 206 TRIB3 infrequent or rare variants among which are the nine we found. Though, 176 of them are either singleton (i.e. seen only once; n=98) or observed in 1/100,000–1/10,000 alleles (n=78) and, therefore, unlikely to play a significant role at population level in a highly prevalent disorders as CAD, it remains to be established whether or not all the remaining less rare variants we have not detected in our screening procedure (n=21) play a role on CAD among patients with T2D. In the context of testing a larger number of variants in larger samples, it could be also possible to concentrate only on those, which, according to bioinformatic analysis, are suggested to be strongly functional. Second, the association stage was not powered to detect genetic effects with ORs smaller than 1.75 (or greater than 0.57, if protective). Thus, our study had a statistical power, which allowed us detecting an effect size, which is well below those so far reported for the role of combined rare variants on both cardiovascular (8) and metabolic (26) disorders. Nonetheless, larger collaborative studies would be useful not only to address the role of *TRIB3* rare variants in a more comprehensive way, but also to understand the nature of the discrepancies between Italian and US sets observed in our study. Finally, our study was limited to White subjects. Whether these or other infrequent variants may contribute to CAD in other racial groups remains to be investigated.

In conclusion, although preliminary, our results indicate a role of low-frequency coding variants in the *TRIB3* gene in modulating the risk of CAD among Italian patients with T2D. Further studies are needed to confirm these findings and determine whether they can be extended to other populations as well as to address the role of rare variants harbored by other genes and their possible combined effect with those we here described.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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REFERENCES

1. Nolan CJ, Damm P, Prentki M. Type 2 diabetes across generations: from pathophysiology to prevention and management. Lancet. 2011; 378:169–181. [PubMed: 21705072]
2. Anand SS, Islam S, Rosengren A, Franzosi MG, Steyn K, Yusufali AH, Keltai M, Diaz R, Rangarajan S, Yusuf S. INTERHEART Investigators. Risk factors for myocardial infarction in women and men: insights from the INTERHEART study. Eur Heart J. 2008; 29:932–940. [PubMed: 18334475]
3. Deloukas P, Kanoni S, Willenborg C, Farrall M, Assimes TL, Thompson JR, Ingelsson E, Saleheen D, Erdmann J, Samani NJ. CARDIoGRAMplusC4D Consortium. Large-scale association analysis identifies new risk loci for coronary artery disease. Nat Genet. 2013; 45:25–33. [PubMed: 23202125]
4. Qi L, Qi Q, Prudente S, Mendonca C, Andreozzi F, di Pietro N, Sturma M, Novelli V, Mannino GC, Formoso G, Gervino EV, Hauser TH, Muehlschlegel JD, Niewczas MA, Krolewski AS, Biolo G, Pandolfi A, Rimm E, Sesti G, Trischitta V, Hu F, Doria A. Association between a genetic variant related to glutamic acid metabolism and coronary heart disease in individuals with type 2 diabetes. JAMA. 2013; 310:821–828. [PubMed: 23982368]
5. Cohen JC, Boerwinkle E, Mosley TH, Hobbs HH. Sequence Variations in PCSK9 Low LDL, and Protection against Coronary Heart Disease. N Engl J Med. 2006; 354:1264–1272. [PubMed: 16554528]
6. Crosby J, Peloso GM, Auer PL, Crosslin DR, Stitziel NO, Lange LA, Lu Y, Tang ZZ, Zhang H, Hindy G, Masca N, Stirrups K, Kanoni S, Do R, Jun G, Hu Y, Kang HM, Xue C, Goel A, Farrall M, Duga S, Merlini PA, Asselta R, Girelli D, Olivieri O, Martinelli N, Yin W, Reilly D, Speliotes E, Fox CS, Hveem K, Holmen OL, Nikpay M, Farlow DN, Assimes TL, Franceschini N, Robinson J, North KE, Martin LW, DePristo M, Gupta N, Escher SA, Jansson JH, Van Zuydam N, Palmer CN, Wareham N, Koch W, Meitinger T, Peters A, Lieb W, Erbel R, Konig IR, Kruppa J, Degenhardt F, Gottesman O, Bottinger EP, O'Donnell CJ, Psaty BM, Ballantyne CM, Abecasis G, Ordovas JM, Melander O, Watkins H, Orho-Melander M, Ardissino D, Loos RJ, McPherson R, Willer CJ, Erdmann J, Hall AS, Samani NJ, Deloukas P, Schunkert H, Wilson JG, Kooperberg C, Rich SS, Tracy RP, Lin DY, Altshuler D, Gabriel S, Nickerson DA, Jarvik GP, Cupples LA, Reiner AP, Boerwinkle E, Kathiresan S. TG and HDL Working Group of the Exome Sequencing Project, National Heart, Lung, and Blood Institute. Loss-of-function mutations in APOC3, triglycerides, and coronary disease. N Engl J Med. 2014; 371:22–31. [PubMed: 24941081]
7. Jørgensen AB, Frikke-Schmidt R, Nordestgaard BG, Tybjærg-Hansen A. Loss-of-function mutations in APOC3 and risk of ischemic vascular disease. N Engl J Med. 2014; 371:32–41. [PubMed: 24941082]
8. Do R, Stitziel NO, Won HH, Jørgensen AB, Duga S, Angelica Merlini P, Kiezun A, Farrall M, Goel A, Zuk O, Guella I, Asselta R, Lange LA, Peloso GM, Auer PL, NHLBI Exome Sequencing Project. Girelli D, Martinelli N, Farlow DN, DePristo MA, Roberts R, Stewart AF, Saleheen D, Danesh J, Epstein SE, Sivapalaratnam S, Kees Hovingh G, Kastelein JJ, Samani NJ, Schunkert H, Erdmann J, Shah SH, Kraus WE, Davies R, Nikpay M, Johansen CT, Wang J, Hegele RA, Hechter E, Marz W, Kleber ME, Huang J, Johnson AD, Li M, Burke GL, Gross M, Liu Y, Assimes TL, Heiss G, Lange EM, Folsom AR, Taylor HA, Olivieri O, Hamsten A, Clarke R, Reilly DF, Yin W, Rivas MA, Donnelly P, Rossouw JE, Psaty BM, Herrington DM, Wilson JG, Rich SS, Bamshad MJ, Tracy RP, Adrienne Cupples L, Rader DJ, Reilly MP, Spertus JA, Cresci S, Hartiala J, Wilson Tang WH, Hazen SL, Allayee H, Reiner AP, Carlson CS, Kooperberg C, Jackson RD, Boerwinkle E, Lander ES, Schwartz SM, Siscovick DS, McPherson R, Tybjaerg-Hansen A, Abecasis GR, Watkins H, Nickerson DA, Ardissino D, Sunyaev SR, O'Donnell CJ, Altshuler D, Gabriel S, Kathiresan S. Exome sequencing identifies rare LDLR and APOA5 alleles conferring risk for myocardial infarction. Nature. 2015; 518:102–106. [PubMed: 25487149]
9. Howard G, O'Leary DH, Zaccaro D, Haffner S, Rewers M, Hamman R, Selby JV, Saad MF, Savage P, Bergman R. Insulin sensitivity and atherosclerosis. The Insulin Resistance Atherosclerosis Study (IRAS) Investigators. Circulation. 1996; 93:1809–1817. [PubMed: 8635260]

10. Rutter MK, Meigs JB, Sullivan LM, D'Agostino RB Sr, Wilson PW. Insulin resistance, the metabolic syndrome, and incident cardiovascular events in the Framingham Offspring Study. Diabetes. 2005; 54:3252–3257. [PubMed: 16249452]

11. Scott RA, Lagou V, Welch RP, Wheeler E, Montasser ME, Luan J, Mägi R, Strawbridge RJ, Rehnberg E, Gustafsson S, Kanoni S, Rasmussen-Torvik LJ, Yengo L, Lecoeur C, Shungin D, Sanna S, Sidore C, Johnson PC, Jukema JW, Johnson T, Mahajan A, Verweij N, Thorleifsson G, Hottenga JJ, Shah S, Smith AV, Sennblad B, Gieger C, Salo P, Perola M, Timpson NJ, Evans DM, Pourcain BS, Wu Y, Andrews JS, Hui J, Bielak LF, Zhao W, Horikoshi M, Navarro P, Isaacs A, O'Connell JR, Stirrups K, Vitart V, Hayward C, Esko T, Mihailov E, Fraser RM, Fall T, Voight BF, Raychaudhuri S, Chen H, Lindgren CM, Morris AP, Rayner NW, Robertson N, Rybin D, Liu CT, Beckmann JS, Willems SM, Chines PS, Jackson AU, Kang HM, Stringham HM, Song K, Tanaka T, Peden JF, Goel A, Hicks AA, An P, Müller-Nurasyid M, Franco-Cereceda A, Folkersen L, Marullo L, Jansen H, Oldehinkel AJ, Bruinenberg M, Pankow JS, North KE, Forouhi NG, Loos RJ, Edkins S, Varga TV, Hallmans G, Oksa H, Antonella M, Nagaraja R, Trompet S, Ford I, Bakker SJ, Kong A, Kumari M, Gigante B, Herder C, Munroe PB, Caulfield M, Antti J, Mangino M, Small K, Miljkovic I, Liu Y, Atalay M, Kiess W, James AL, Rivadeneira F, Uitterlinden AG, Palmer CN, Doney AS, Willemsen G, Smit JH, Campbell S, Polasek O, Bonnycastle LL, Hercberg S, Dimitriou M, Bolton JL, Fowkes GR, Kovacs P, Lindström J, Zemunik T, Bandinelli S, Wild SH, Basart HV, Rathmann W, Grallert H, DIAbetes Genetics Replication and Meta-analysis (DIAGRAM) Consortium. Maerz W, Kleber ME, Boehm BO, Peters A, Pramstaller PP, Province MA, Borecki IB, Hastie ND, Rudan I, Campbell H, Watkins H, Farrall M, Stumvoll M, Ferrucci L, Waterworth DM, Bergman RN, Collins FS, Tuomilehto J, Watanabe RM, de Geus EJ, Penninx BW, Hofman A, Oostra BA, Psaty BM, Vollenweider P, Wilson JF, Wright AF, Hovingh GK, Metspalu A, Uusitupa M, Magnusson PK, Kyvik KO, Kaprio J, Price JF, Dedoussis GV, Deloukas P, Meneton P, Lind L, Boehnke M, Shuldiner AR, van Duijn CM, Morris AD, Toenjes A, Peyser PA, Beilby JP, Körner A, Kuusisto J, Laakso M, Bornstein SR, Schwarz PE, Lakka TA, Rauramaa R, Adair LS, Smith GD, Spector TD, Illig T, de Faire U, Hamsten A, Gudnason V, Kivimaki M, Hingorani A, Keinanen-Kiukaanniemi SM, Saaristo TE, Boomsma DI, Stefansson K, van der Harst P, Dupuis J, Pedersen NL, Sattar N, Harris TB, Cucca F, Ripatti S, Salomaa V, Mohlke KL, Balkau B, Froguel P, Pouta A, Jarvelin MR, Wareham NJ, Bouatia-Naji N, McCarthy MI, Franks PW, Meigs JB, Teslovich TM, Florez JC, Langenberg C, Ingelsson E, Prokopenko I, Barroso I. Large-scale association analyses identify new loci influencing glycemic traits and provide insight into the underlying biological pathways. Nat Genet. 2012; 44:991–1005. [PubMed: 22885924]

12. Prudente S, Morini E, Trishitta V. Insulin signaling regulating genes: effect on T2DM and cardiovascular risk. Nat Rev Endocrinol. 2009; 5:682–693. [PubMed: 19924153]

13. Bacci S, Rizza S, Prudente S, Spoto B, Powers C, Facciorusso A, Pacilli A, Lauro D, Testa A, Zhang YY, Di Stolfo G, Mallamaci F, Tripepi G, Xu R, Mangiacotti D, Aucella F, Lauro R, Gervino EV, Hauser TH, Copetti M, De Cosmo S, Pellegrini F, Zoccali C, Federici M, Doria A, Trischitta V. The ENPP1 Q121 variant predicts major cardiovascular events in high-risk individuals: evidence for interaction with obesity in diabetic patients. Diabetes. 2011; 60:1000–1007. [PubMed: 21282363]

14. Bacci S, Prudente S, Copetti M, Spoto B, Rizza S, Baratta R, Di Pietro N, Morini E, Di Paola R, Testa A, Mallamaci F, Tripepi G, Zhang YY, Mercuri L, Di Silvestre S, Lauro R, Malatino L, Consoli A, Pellegrini F, Pandolfi A, Frittitta L, Zoccali C, Federici M, Doria A, Trischitta V. Joint effect of insulin signaling genes on cardiovascular events and on whole body and endothelial insulin resistance. Atherosclerosis. 2013; 226:140–145. [PubMed: 23107043]

15. Prudente S, Sesti G, Pandolfi A, Andreozzi F, Consoli A, Trischitta V. The mammalian tribbles homolog TRIB3, glucose homeostasis, and cardiovascular diseases. Endocr Rev. 2012; 33:526–546. [PubMed: 22577090]

16. Prudente S, Hribal ML, Flex E, Turchi F, Morini E, De Cosmo S, Bacci S, Tassi V, Cardellini M, Lauro R, Sesti G, Dallapiccola B, Trischitta V. The Functional Q84R Polymorphism of Mammalian Tribbles Homolog TRB3 Is Associated With Insulin Resistance and Related Cardiovascular Risk in Caucasians From Italy. Diabetes. 2005; 54:2807–2811. [PubMed: 16123373]

17. Andreozzi F, Formoso G, Prudente S, Hribal ML, Pandolfi A, Bellacchio E, Di Silvestre S, Trischitta V, Consoli A, Sesti G. TRIB3 R84 variant is associated with impaired insulin-mediated

nitric oxide production in human endothelial cells. Arterioscler Thromb Vasc Biol. 2008; 28:1355–1360. [PubMed: 18436806]

18. Liew CW, Bochenski J, Kawamori D, Hu J, Leech CA, Wanic K, Malecki M, Warram JH, Qi L, Krolewski AS, Kulkarni RN. The pseudokinase tribbles homolog 3 interacts with ATF4 to negatively regulate insulin exocytosis in human and mouse beta cells. J Clin Invest. 2010; 120:2876–2888. [PubMed: 20592469]
19. Formoso G. The TRIB3 R84 variant is associated with increased carotid intima–media thickness in vivo and with enhanced MAPK signalling in human endothelial cells. Cardiovascular Research. 2011; 89:184–192. [PubMed: 20693163]
20. Lange LA, Bowden DW, Langefeld CD, Wagenknecht LE, Carr JJ, Rich SS, Riley WA, Freedman BI. Heritability of carotid artery intima-medial thickness in type 2 diabetes. Stroke. 2002; 33:1876–1881. [PubMed: 12105369]
21. Lee S, Abecasis GR, Boehnke M, Lin X. Rare-variant association analysis: study designs and statistical tests. Am J Hum Genet. 2014; 95:5–23. [PubMed: 24995866]
22. Prudente S, Scarpelli D, Chandalia M, Zhang YY, Morini E, Del Guerra S, Perticone F, Li R, Powers C, Andreozzi F, Marchetti P, Dallapiccola B, Abate N, Doria A, Sesti G, Trischitta V. The TRIB3 Q84R polymorphism and risk of early-onset type 2 diabetes. J Clin Endocrinol Metab. 2009; 94:190–196. [PubMed: 18984671]
23. Prudente S, Morini E, Marselli L, Baratta R, Copetti M, Mendonca C, Andreozzi F, Chandalia M, Pellegrini F, Bailetti D, Alberico F, Shah H, Abate N, Sesti G, Frittitta L, Marchetti P, Doria A, Trischitta V. Joint effect of insulin signaling genes on insulin secretion and glucose homeostasis. J Clin Endocrinol Metab. 2013; 98:E1143–E1147. [PubMed: 23633196]
24. Stitziel NO, Won HH, Morrison AC, Peloso GM, Do R, Lange LA, Fontanillas P, Gupta N, Duga S, Goel A, Farrall M, Saleheen D, Ferrario P, König I, Asselta R, Merlini PA, Marziliano N, Notarangelo MF, Schick U, Auer P, Assimes TL, Reilly M, Wilensky R, Rader DJ, Hovingh GK, Meitinger T, Kessler T, Kastrati A, Laugwitz KL, Siscovick D, Rotter JI, Hazen SL, Tracy R, Cresci S, Spertus J, Jackson R, Schwartz SM, Natarajan P, Crosby J, Muzny D, Ballantyne C, Rich SS, O'Donnell CJ, Abecasis G, Sunyaev S, Nickerson DA, Buring JE, Ridker PM, Chasman DI, Austin E, Ye Z, Kullo IJ, Weeke PE, Shaffer CM, Bastarache LA, Denny JC, Roden DM, Palmer C, Deloukas P, Lin DY, Tang ZZ, Erdmann J, Schunkert H, Danesh J, Marrugat J, Elosua R, Ardissino D, McPherson R, Watkins H, Reiner AP, Wilson JG, Altshuler D, Gibbs RA, Lander ES, Boerwinkle E, Gabriel S, Kathiresan S. Myocardial Infarction Genetics Consortium Investigators. Inactivating mutations in NPC1L1 and protection from coronary heart disease. N Engl J Med. 2014; 371:2072–2082. [PubMed: 25390462]
25. Wang J, Ban MR, Zou GY, Cao H, Lin T, Kennedy BA, Anand S, Yusuf S, Huff MW, Pollex RL, Hegele RA. Polygenic determinants of severe hypertriglyceridemia. Hum Mol Genet. 2008; 17:2894–2899. [PubMed: 18596051]
26. Bonnefond A, Clément N, Fawcett K, Yengo L, Vaillant E, Guillaume JL, Dechaume A, Payne F, Roussel R, Czernichow S, Hercberg S, Hadjadj S, Balkau B, Marre M, Lantieri O, Langenberg C, Bouatia-Naji N, Meta-Analysis of Glucose and Insulin-Related Traits Consortium (MAGIC). Charpentier G, Vaxillaire M, Rocheleau G, Wareham NJ, Sladek R, McCarthy MI, Dina C, Barroso I, Jockers R, Froguel P. Rare *MTNR1B* variants impairing melatonin receptor 1B function contribute to type 2 diabetes disorders. Nature Genetics. 2012; 44:297–301. [PubMed: 22286214]

Table 1

Clinical features of study subjects from the three different samples

	GHS		CS		JHS	
	Control	**Case**	**Control**	**Case**	**Control**	**Case**
N	264	217	138	81	448	417
Male/Female	116/148	145/72	62/76	55/26	266/182	296/121
Age at examination (yrs)	64.8±8.1	59.8±8.5	63.4±8.9	60.6±11.3	65±7	64±6
Age at Diabetes (yrs)	50.6±11	48.6±9.6	50.3±11.9	60.6±12.3	52±10	52±8
BMI (kg/m^2)	30.1±4.7	31.2±5.6	30.2±4.6	31.1±5.9	32.2±6.4	32.3±5.9
Diabetes Duration (yrs)	14.3±9.3	11.3±8.1	13.1±9.8	9.8±8.8	13±9	12±7
HbA1C (%)	8.5±1.9	8.6±1.9	7.7±3.9	8.5±4.2	7.3±1.2	7.5±1.4
Glucose-lowering therapy						
Diet only (%)	13	10	35.5	24.7	52.8	43.8
Oral Agents (%)	47	46	42.2	40.6	16.6	22.3
Insulin ± Oral Agents (%)	40	44	22.3	34.7	30.6	33.9
Antihypertensive therapy (%)	48.5	51.5	68.4	85.1	70.8	82.2
Lipid-lowering therapy (%)	38.2	61.8	27.4	52.5	38.4	67.5
Ever smoked						
Current smokers (%)	38.7	61.3	16.9	15.8	6.3	8.2
Former smokers (%)	50.8	49.2	25.6	42.6	32.1	59.3

GHS: Gargano Heart Study; CS: Catanzaro Study; JHS: Joslin Heart Study.

Continuous variables are reported as means ± SD, categorical variables as percentages. BMI: body mass index; HbA1c: glycated hemoglobin; yrs: years.

Table 2

Description of infrequent non-synonymous variants detected by *TRIB3* re-sequencing, which were further tested for association with CAD

				Bioinformatics Assessment							
Variant	**dbSNP ID**	**nt change**	**MAF**	**PolyPhen-2**	**SIFT**	**SNPs&GO**	**Mutation Assessor**	**MutPred**	**PROVEAN**	**Mutation Taster**	**Predictive score** *
A10V	rs200422001	c.29 C>T	0.02	Benign	Tolerated	Neutral	Low	Neutral	Neutral	Neutral	7
V107M	rs138380491	c.319 G>A	0.24	Possibly damaging	Tolerated	Neutral	Low	Neutral	Neutral	Neutral	7.5
S146N	rs41281850	c.437 G >A	0.48	Probably damaging	Tolerated	Disease	Medium	Neutral	Deleterious	Disease causing	11.5
R153H	rs35051116	c.458 G>A	1.61	Benign	Tolerated	Disease	Medium	Neutral	Deleterious	Neutral	9.5
R181C	rs149447454	c.541 C>T	0.06	Probably damaging	Disease	Disease	High	Neutral	Deleterious	Disease causing	13
R234Q	rs144632965	c.701 G>A	0.01	Benign	Tolerated	Disease	Low	Neutral	Neutral	Neutral	8
R275H	rs140801463	c.824 G>A	0.02	Possibly damaging	Disease	Disease	Medium	Neutral	Deleterious	Disease causing	12
H328R	rs374473490	c.983 A>G	0.01	Benign	Tolerated	Neutral	Neutral	Neutral	Neutral	Neutral	7

TRIB3 (NM_021158.3) re-sequencing was carried out in DNA of 280 individuals (140 “extreme” controls and 140 “extreme” cases) from the GHS.

nt change: nucleotide change; MAF: Minor Allele Frequency, in percentage, evaluated by Exome Aggregation Consortium (ExAC) browser.

* The collective Predictive score, ranging 7–14, was calculated as the sum of individual scores of the seven tools here utilized, each being 1 or 1.5 or 2, according to a progressively increase in mutation severity.

Table 3

Association between total number of infrequent *TRIB3* variants and CAD in the three study sets.

Study name	Study country	Variants (n)	Control n (%)	Case n (%)	OR (95% CI)	p value for OR heterogeneity
GHS	*Italy*	*0*	*242 (91.7)*	*208 (95.9)*	*0.47(0.22–1.00)*	
		1	*20 (7.6)*	*9 (4.1)*		
		2	*2 (0.7)*	*0*	*p=0.05*	*NE*
CS	*Italy*	*0*	*135 (97.8)*	*81 (100)*		
		1	*3 (2.2)*	*0*	*NE*	
		2	*0*	*0*	*p=0.30**	
GHS+CS	Italy	0	377 (93.8)	289 (97.0)	0.43 (0.20–0.91)	
		1	23 (5.7)	9 (3.0)	p=0.03	
		2	2 (0.5)	0		0.03
JHS	U.S.	0	426 (95.1)	393 (94.2)	1.22 (0.69–2.18)	
		1	22 (4.9)	23 (5.6)	p=0.49	
		2	0	1 (0.2)		

GHS: Gargano Heart Study; CS: Catanzaro Study; JHS: Joslin Heart Study.
NE: not estimable, due to the lack of variants among cases.

* as estimated by Fisher exact test