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Association Between a Genetic Variant Related to Glutamic Acid Metabolism and Coronary Heart Disease in Type 2 Diabetes

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AUTHOR CONTRIBUTIONS

L.Q. designed the study, acquired, analyzed, and interpreted the data, wrote the manuscript; **Q.Q**. acquired, analyzed, and interpreted data, reviewed the manuscript; **S.P.** acquired, analyzed, and interpreted data, reviewed the manuscript; **C.M.** acquired and analyzed data, reviewed the manuscript; **F.A.** acquired data, reviewed the manuscript; **N.d.P.** acquired data, reviewed the manuscript **M.S.** acquired data, reviewed the manuscript; **V.N.** acquired, analyzed, and interpreted data, reviewed the manuscript; **G.C.M.** acquired, analyzed, and interpreted data, reviewed the manuscript; **G.F.** acquired data, reviewed the manuscript; **E.V.G.** acquired, analyzed, and interpreted data, reviewed the manuscript; **T.H.H.** acquired, analyzed, and interpreted data, reviewed the manuscript;; **J.D.M** interpreted data, reviewed the manuscript; **M.A.N.** acquired, analyzed, and interpreted data, reviewed the manuscript; **A.S.K.** acquired, analyzed, and interpreted data, reviewed the manuscript **G.B.** acquired, analyzed, and interpreted data, reviewed the manuscript **A.P**. acquired, analyzed, and interpreted data, reviewed the manuscript; **E.R.** acquired, analyzed, and interpreted data, reviewed the manuscript; **G.S.** designed the study, acquired, analyzed, and interpreted the data, reviewed the manuscript **V.T.** designed the study, acquired, analyzed, and interpreted the data, wrote the manuscript; **F.H.** designed the study, acquired, analyzed, and interpreted the data, reviewed the manuscript; **A.D.** designed the study, acquired, analyzed, and interpreted the data, wrote the manuscript.

Competing Interests Statement
None

Databases
dbSNP: http://www.ncbi.nlm.nih.gov/projects/SNP/
Gene Entrez: http://www.ncbi.nlm.nih.gov/gene
HapMap: http://hapmap.ncbi.nlm.nih.gov/
MAGIC: http://www.magicinvestigators.org
DIAGRAM: http://diagram-consortium.org

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Abstract

IMPORTANCE—Diabetes is associated with an elevated risk of coronary heart disease (CHD). Previous studies have suggested that the genetic factors predisposing to excess cardiovascular risk may be different in diabetic and non-diabetic participants.

OBJECTIVE—To identify genetic determinants of CHD that are specific to diabetic patients.

DESIGN, SETTING, AND PARTICIPANTS—We studied five independent sets of CHD cases and CHD-negative controls from the Nurses Health Study (NHS; enrolled in 1976 and followed through 2008), Health Professionals Follow-up Study (HPFS; enrolled in 1986 and followed through 2008), Joslin Heart Study (enrolled in 2001-2008), Gargano Heart Study (enrolled in 2001-2008), and Catanzaro Study (enrolled in 2004-2010). Included were a total of 1,517 CHD cases and 2,671 CHD-negative controls, all with type 2 diabetes. Results in diabetic patients were compared with those in 737 non-diabetic CHD cases and 1,637 non-diabetic CHD-negative controls from the NHS and HPFS cohorts.

EXPOSURE—2,543,016 common genetic variants occurring throughout the genome.

MAIN OUTCOME—CHD defined as fatal or non-fatal myocardial infarction, coronary artery bypass grafting, percutaneous transluminal coronary angioplasty, or angiographic evidence of significant stenosis of the coronary arteries.

RESULTS—We identified a variant on chromosome 1q25 (rs10911021) consistently associated with CHD risk among diabetic participants with an odds ratio of 1.36 (95% confidence interval [CI] 1.22-1.51, $P=2\times10^{-8}$). No association between this variant and CHD was detected among non-diabetic participants (OR=0.99, P=0.89), consistent with a significant gene-by-diabetes interaction on CHD risk ($P=2\times10^{-4}$). As compared to protective allele homozygotes, rs10911021 risk allele homozygotes were characterized by a 32% decrease in the expression of the neighboring glutamate-ammonia ligase (*GLUL*) gene in human endothelial cells (P=0.0048). They also showed a decreased ratio between plasma levels of γ-glutamyl cycle intermediates pyroglutamic and glutamic acid in two independent studies (P=0.029 and P=0.003, respectively).

CONCLUSIONS AND RELEVANCE—A SNP was identified that was significantly associated with CHD among persons with diabetes but not in those without diabetes. This SNP was functionally related to glutamic acid metabolism, suggesting a mechanistic link.

Keywords

genetic; amino acids; coronary heart disease; diabetes

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INTRODUCTION

The prevalence of type 2 diabetes has reached epidemic proportions in the United States and other countries in the world, with the total number of affected people reaching over 370 million globally (http://www.idf.org/diabetesatlas/5e/Update2012). Long-term cardiovascular complications, and especially coronary heart disease (CHD), are the principal causes of morbidity and mortality among diabetic patients.[1] While mortality due to CHD has been overall declining during the past few decades in most industrialized countries,[2] the increasing prevalence of diabetes has made the number of CHD deaths attributable to this disease escalate.[3;4]

The role of genetic factors in modulating susceptibility to CHD has been known for many years[5] and more than 40 chromosomal loci associated with CHD have been identified to date in the general population by genome-wide association (GWA) studies.[6-11] Earlier analyses have shown considerable heterogeneity in genetic effects between diabetic patients and non-diabetic participants,[12] probably owing to the distinct mechanisms of atherogenesis in diabetes. This has led us to hypothesize that other, as yet undiscovered loci may exist that affect CHD risk only or mostly in the presence of diabetes. Finding these genes, if they exist, may point to atherogenic pathways that are specifically activated by the diabetic milieu and as such could be the target of new interventions aimed at preventing or treating CHD specifically among diabetic patients.

In this study, we performed a genome-wide association analysis of CHD targeted to type 2 diabetic participants, in order to identify genetic determinants of CHD that are specific to diabetic patients.

METHODS

Study populations

Detailed information on the study populations is provided in the **Supplementary Methods**. Briefly, **Stage I** included diabetic patients from the Nurses' Health Study (NHS) [13] and the Health Professional Follow-up Study (HPFS) [14] (Table 1; Supplemental Methods). CHD cases were defined as incident cases after the diagnosis of T2D to the end of 2008; controls were participants free of CHD events in the specified time period. These studies were approved by the Human Research Committee at the Brigham and Women's Hospital, Boston and all participants provided written informed consent. **Stage II** included diabetic CHD cases and CHD-negative controls from the Joslin Heart Study (JHS) [15] (Table 1; Supplemental Methods). The study protocol and informed consent procedures were approved by the Joslin Committee on Human Studies and the BIDMC Committee on Clinical Investigations. All subjects gave written informed consent. **Stage III** included diabetic CHD cases and CHD-negative controls patients from the Gargano Heart Study-cross sectional design (GHS) [16] and the Catanzaro Study (CZS) [17] (Table 1; Supplemental Methods). The study protocol and informed consent procedures were approved by the local human subject committees. All subjects gave written informed consent.

To compare the association between rs10911021 (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=10911021) and CHD risk in non-diabetic versus diabetic participants, we analyzed a separate non-diabetic CHD case-control GWA study, which included incident CHD cases and non-CHD controls from the NHS and HPFS cohorts,[18] after excluding individuals affected by diabetes (Supplemental Methods).We also obtained data on rs10911021 from the Coronary Artery Disease Genome-wide Replication and Meta-analysis (CARDIoGRAM).[10] To explore whether this variant may contribute to CHD through alterations of insulin-sensitivity, we interrogated the MAGIC database - a meta-analysis of

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genome-wide association data for metabolic traits.[19;20] We also interrogated the DIAGRAM database[21] to explore whether this variant might have pleiotropic associations with both type 2 diabetes and CHD.

Genotyping

Single nucleotide polymorphism (SNP) genotyping and imputation for Stage I have been described in detail elsewhere[22] and in the Supplementary Methods. Briefly, samples were genotyped using the Affymetrix Genome-Wide Human 6.0 array (Santa Clara, CA). A total of 704,409 and 706,040 SNPs passed quality control in the NHS and HPFS sets, respectively, and were used to impute the genotypes of other SNPs by means of MACH. [23] In Stage II and III, SNPs were genotyped by the Joslin DERC Genetics Core by means of TaqMan assays implemented on an ABI PRISM 7700 HT Sequence Detection System (Applied Biosystems, Foster City, CA).

Gene expression in endothelial cell lines

To investigate whether the association between rs10911021 and CHD risk could be mediated by gene expression changes, we measured mRNA levels of 8 neighboring genes (4 on the centromeric and 4 on the telomeric side, 'Supplementary Figure 1') in 124 human umbilical vein endothelial cell lines from non-diabetic mothers. Umbilical cords were obtained from randomly selected healthy mothers who delivered at the Pescara Town Hospital (Italy) and gave written consent to this procedure. Primary human umbilical vein endothelial cell (HUVEC) lines were established from the umbilical cords and cultured as described by Gorfien et al.[24] Cell lines were typed for the rs10911021 SNP using a TaqMan allelic discrimination assay (Applied Biosystems, Foster City, CA). Gene expression was assayed by means of real-time quantitative PCR-based (qPCR) TaqMan Low Density Arrays (TLDAs). Eight target genes neighboring rs10911021 along with a housekeeping gene (GUSB; NCBI Entrez Gene NG_016197.1) as endogenous control were included in the array.

Amino acid measurements

To obtain further insights into the functional impact of rs10911021, we measured plasma glutamine and glutamic acid as well as the ratio between pyroglutamic acid (the immediate precursor of glutamic acid in the γ-glutamyl cycle) and glutamic acid in 100 diabetic patients from the JHS (50 rs10911021 risk allele C homozygotes and 50 T homozygotes). Plasma concentrations of glutamic acid and glutamine were assessed at the University of Trieste (Italy) by gas chromatography- mass spectrometry (GC-MS), using the internal standard technique, as previously described.[25] Known amounts of L-[^{15}N]-glutamic acid and L[^{15}N]-glutamine (Cambridge Isotope Laboratories) were added as internal standards to a known volume of plasma. Silylated derivatives were measured under electron-impact ionization by selective ion monitoring at a nominal m/z of 432/433 for glutamic acid and 431/432 for glutamine. The pyroglutamic acid derivative was also monitored at a nominal m/z of 300. The pyroglutamic/ glutamic acid peak area ratio was determined at nominal m/z of 300 and 432, respectively. In addition, the relationship between rs10911021 and these three metabolic indices was evaluated by using existing metabolomic data concerning 60 individuals with type 2 diabetes from the Joslin Diabetes Center.[26]

Statistical analyses

Genome-wide association analyses and validations—Analyses were carried out in three stages. In Stage I, two separate GWA analyses for CHD across 2,543,016 genotyped or imputed SNPs (with imputed SNPs expressed as allele dosage) were performed in the NHS and HPFS sets by means of logistic regression under an additive genetic model using the

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ProbABEL package.[27] The genomic inflation factor λ was estimated from the median χ^2 statistic. To control for potential confounding by population stratification, we performed further analyses by including the top principal components of genetic variation chosen for each study in the models (top 3 and 4 eigenvectors for NHS and HPFS, respectively). Meta-analysis of the two GWA scans was conducted by combining study-specific β-estimates from genome wide associations using inverse variance weights under a fixed-effect model in METAL.[28] Variants yielding a p value $<1\times10^{-4}$ in Stage I were carried forward to Stage II, and those yielding a p value $<1\times10^{-4}$ in Stage I and II combined were carried forward to Stage III. In stage II and stage III, sex-adjusted odds ratios and their 95% confidence intervals were estimated for each SNP and in each study by means of logistic regression according to an additive model. Associations across stage I and II studies, and across all the studies in stage I, II and III were summarized by meta-analyses using STATA (STATA, College Station, TX, Version 7.0). The presence of heterogeneity among the three studies was testedby means of a chi-square statistics. Since this test was not significant for any of the SNPs, we calculated summary ORs according to a fixed-effect model, i.e. by averaging the natural logarithms of the ORs from individual studies, weighted by the inverses of their variances.[29] The association between rs10911021 and CHD among non-diabetic participants from the NHS and HPFS cohorts was evaluated as described above for diabetic participants. The interaction between rs10911021 and diabetes on CHD risk was evaluated by adding the rs10911021 × diabetes cross-product to a logistic regression analysis of the combined diabetic and non-diabetic NHS/HPFS sets. The same approach was used to evaluate the interaction between rs10911021 and 36 established type 2-predisposing variants considered in the paper by Qi et al. [30]

Power of genetic studies—Power for the main SNP effects was estimated using the software CaTS[31] assuming a risk allele frequency of 0.30. The GWA analysis of T2D participants had 80% power ($\alpha=5\times10^{-8}$) to detect associations with CHD with summary ORs across the three stages as low as 1.35. The study of rs10911021 in non-diabetic participants had >99% power ($\alpha=0.05$) to detect an association with CHD with an OR similar to that observed among diabetic participants (OR=1.36) and 80% power to detect an association with an OR as low as 1.19.

Gene expression studies—ΔCt values were derived from the threshold cycle (Ct) data for each target gene using the equation ΔCt = Ct (target gene) – Ct (endogenous control)]. $\Delta\Delta$Ct values were then calculated for each sample and gene as the difference between the ΔCt and the mean ΔCt among rs10911021 T/T homozygotes. For each target gene, the association between rs10911021 and $\Delta\Delta$Ct was evaluated by linear regression using an additive genetic model.

Amino acid studies—The association between plasma amino acid levels and rs10911021 genotype or CHD case-control status was evaluated by means of linear regression models with the amino acid levels as the dependent variables and age, gender, γGT levels, rs10911021 genotype, and CHD case-control status as the independent variables. Glutamic acid and the pyroglutamic/glutamic ratio were evaluated after log transformation because of their non-normal distributions.

Significance thresholds—For GWA analyses, two-sided *P*-values smaller than 5×10^{-8} were considered as significant; for all other analyses, two-sided *P*-values smaller than 0.05 were considered as significant.

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RESULTS

Genome-wide association analyses and validations among diabetic participants

A total of 1,517 CHD cases and 2,671 CHD-negative controls, all with type 2 diabetes, were included in the three-stage genome-wide analysis: 350 cases and 976 controls from the NHS and 319 cases and 665 controls from the HPFS (Stage I), 420 cases and 431 controls from the JHS (Stage II), 314 cases and 384 controls from the GHS and 114 cases and 215 controls from the CZS (Stage III) (Supplemental Methods). The clinical characteristics of the case-control sets analyzed at each stage are summarized in Table 1. Of the 2,543,016 genetic variants that were tested for association with CHD in Stage I, 26 met the criterion for promotion to Stage II (p<0.0001 in Stage I) and 3 of these further met the criterion for promotion to Stage III (p<0.0001 in Stage I+ Stage II). Detailed data on the variants associated with CHD at each stage can be found in Supplementary Table 1 and Supplementary Figures 2 and 3. Of the three variants that were promoted to Stage III, one (rs10911021) showed an association with CHD that was nominally significant at each stage and exceeded genome-wide significance in the three stages combined (P=2.0×10^{-8}, Table 2 and Supplementary Table 1). In a meta-analysis of the five case-control sets, the summary odds ratio of CHD for each copy of the risk allele was 1.36 (95% CI 1.22-1.51), with no evidence of heterogeneity across studies (I^2=0%, P=0.82, Table 2). The other two variants promoted to Stage III (rs9361923 on chr 6 and rs7542837 on chr 1) had summary P values across the five sets in the 10^{-4} range (Supplementary Table 1). None of the loci previously associated with CHD in the general population were among the genetic variants promoted to Stages II and III, although three of them reached nominal significance at Stage I (Supplementary Table 2).

Interaction with diabetes status

No association between rs10911021 and CHD was found among 737 non-diabetic CHD cases and 1,637 non-diabetic CHD-negative controls from the NHS and HPFS cohorts (Supplementary Table 3). The OR among these non-diabetic individuals was not significantly different from 1 (OR=0.99, 95% CI 0.87-1.13, P=0.89) while being significantly different from the OR in diabetic participants (1.36, 95% CI 1.22-1.51; P for diabetes × genetic variant interaction = 2.6×10^{-4}). Among the NHS and HPFS diabetic participants, no significant interaction on CHD risk was observed between rs10911021 and established type 2 diabetes-predisposing variants, considered individually or in combination as a genetic predisposition score[30] (all p>0.05). In CARDIoGRAM, which comprises 22,233 CHD cases and 64,762 controls from the general population, rs10911021 showed a nominally significant association with CHD that went in the same direction as among the diabetic participants of our study (OR=1.04, 95%CI 1.01-1.07, P=0.011) but was significantly weaker (I^2=96%, P for heterogeneity = 2.2×10^{-6}; fixed-effect model). If we assume a 15% average prevalence of diabetes – an estimate based on the CARDIoGRAM studies for which data on the occurrence of diabetes are available[32-35] – the OR observed in the CARDIoGRAM population corresponded almost exactly to the weighted average of the ORs observed in our study in diabetic and non-diabetic participants (OR=1.36 and OR=0.99, respectively). No other variant neighboring rs10911021 showed associations at genome-wide significance level in this dataset (Supplementary Figure 4).

Genotype association with the expression of neighboring genes

Variant rs10911021 is located between two genes, *ZNF648* (~51 kb; NCBI Entrez Gene 127665) and *GLUL* (~270 kb; NCBI Entrez Gene NG_013347.1), and neighbors several other genes (Supplementary Figure 1). No missense variants in linkage disequilibrium (LD) with rs10911021 were identified in the HapMap or the 1000 Genome Projects databases, suggesting an effect on gene regulation as the mechanism underlying the observed

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association with CHD. In support of this hypothesis, rs10911021 is listed in the Regulome DB as occurring in an E-box binding site for basic helix-loop-helix transcription factors and ENCODE data indicate that a variant in linkage disequilibrium with this variant (rs7517310, r^2=0.72 in the HapMap database) is placed in a high DNAse I sensitivity cluster binding to the RE1-Silencing Transcription Factor (REST) in a variety of cell types. As shown in Table 3, the expression of *GLUL* - the closest gene in telomeric direction – was significantly associated with rs10911021 in endothelial cells, being 32% lower in risk allele (C/C) homozygotes as compared to protective allele (T/T) homozygotes, with heterozygotes having intermediate levels (*P* for trend = 0.0048). *ZNF648* – the closest gene on the 5' side - was not expressed in endothelial cells and none of the other neighboring genes were significantly associated with rs10911021.

Association with plasma markers of glutamic acid metabolism and the γ-glutamyl cycle

In a sample of 100 JHS participants, no significant differences in plasma glutamic acid or glutamine (the substrate and the product, respectively, of the enzyme encoded by *GLUL*) were observed between risk allele C homozygotes and allele T homozygotes (Table 4). However, the ratio between plasma pyroglutamic acid (the immediate precursor of glutamic acid in the γ-glutamyl cycle) and glutamic acid was significantly lower in C/C as compared to T/T carriers (*P*=0.029) (Table 4). In this sample, the pyroglutamic-to-glutamic ratio was also significantly lower in the 44 participants who had developed CHD (median=0.79, IQR 0.62-0.97) than in the 56 who were CHD-negative (median=0.92, IQR 0.78-1.14) (*P*=0.02). Of note, the OR of CHD for the rs10911021 C/C genotype in this subsample decreased from 1.83 to 1.39 (a ~50% reduction in the log scale) after adjustment for the pyroglutamic-to-glutamic acid ratio, suggesting that the effect of this locus on CHD was at least in part mediated by its effect on this parameter. The association between rs10911021 and pyroglutamic-to-glutamic acid ratio was confirmed in an independent sample of 60 Joslin patients with T2D who had undergone a metabolomic study, with the median ratio being 1.44 in 6 T/T, 1.18 in 29 C/T and 0.92 in 25 C/C participants (*P* for trend=0.003).

Association with other cardiovascular risk factors

No significant association between rs10911021 and serum fasting insulin, insulin-resistance index HOMA-IR, or 2 hr-glucose was found in the MAGIC database including data on >35,000 non-diabetic individuals. Similarly, no significant association was found with type 2 diabetes in the DIAGRAM database (OR=1.01, 95% 0.97-1.04, p=0.76).

Discussion

In this study, we have identified a previously unknown genetic locus associated with increased CHD risk among type 2 diabetic patients. The locus is placed in the region of the *GLUL* gene on chromosome 1q25 and may affect CHD risk by reducing the expression of this gene and affecting glutamate and glutamine metabolism in endothelial cells. This genetic variant appeared to be specifically associated with CHD in the diabetic population and showed a significant gene-by-diabetes synergism on CHD risk.

Several pieces of evidence suggest that these findings are unlikely to be due to chance. First, the *P* value for the association between this locus and CHD in T2D participants meets genome-wide significance ($P<5\times10^{8}$), that is, withstands adjustment for the large number of comparisons that are made in a genome-wide analysis. Second, the association was consistent across multiple samples of type 2 diabetic participants of different ethnic and geographical origin, reaching nominal significance in four of the five sets that were considered. Third, the difference in odds ratios between diabetic and non-diabetic participants was supported by a robust *P* value for interaction. Finally, an association

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between this locus and CHD was also found in a large study of the general population (CARDIoGRAM) with a magnitude similar to what one would expect based on the effects detected in our study in diabetic and non-diabetic participants and the prevalence of diabetes in CARDIoGRAM.

GLUL – the gene whose expression is decreased in risk allele carriers – encodes glutamate-ammonia ligase (also known as glutamine synthase), which catalyzes the conversion of glutamic acid and ammonia into glutamine.[36] Both amino acids play important roles in human physiology. Glutamic acid is a key intermediate of several metabolic pathways, most notably of the γ-glutamyl cycle through which the anti-oxidant glutathione is generated;[37] glutamine is involved in the regulation of cell proliferation, inhibition of apoptosis, and cell signaling.[38] Evidence from experimental and human studies points to glutamine/glutamic acid metabolism as contributing to the regulation of insulin secretion and glucose metabolism. In islets, glutamine enhances both mitochondrial metabolism and insulin secretion.[39] In diabetic patients, it was found that glutamine reduced glucose excursions when given before oral glucose[40] and effectively increased circulating incretin and insulin concentrations.[41] Several clinical trials also suggest cardioprotective effects of glutamine used parenterally and enterally.[42;43] In epidemiological studies, abnormal metabolism of these amino acids has been shown to be related to insulin resistance, type 2 diabetes, and cardiovascular disorders.[44-46]

The mechanisms through which alterations of glutamate and glutamine metabolism, such as those that one would expect from the reduced *GLUL* expression observed in risk allele carriers, may lead to increased CHD risk are unclear at this time. The newly identified CHD risk variant was not associated with risk of type 2 diabetes in DIAGRAM, suggesting that the pathways underlying the association with CHD are distinct from those involved in the etiology of type 2 diabetes. Similarly, the absence of association between the risk variant and serum fasting insulin, HOMA-IR, or 2 hr-glucose in the MAGIC database seems to exclude insulin-resistance as the underlying mechanism. Rather, our finding of association between the risk variant and a lower pyroglutamic-to-glutamic acid ratio in plasma, and the fact that the association between risk allele and CHD was attenuated after adjustment for this variable, suggest an impairment of the γ-glutamyl cycle, of which pyroglutamic acid is an intermediate, as a possible mechanism. Such alteration might increase CHD risk by limiting the availability of the natural antioxidant glutathione, compounding the known negative effect of diabetes on this metabolite[47] and potentially explaining the fact that this genetic effect can only be observed among diabetic participants. Consistent with this hypothesis, an association between rs10911021 and pyroglutamine (expressed as the ratio with the fatty acid sebacate) is also found in the KORA/Twins UK metabolomic databases (p=0.00096 in KORA, http://metabolomics.helmholtzmuenchen.de/gwa).[48] However, additional contributions by pathways that are not directly related to glutamate and glutamine may also be present as other metabolites implicated in vascular biology and atherogenesis, such as the long chain ω3-polyunsaturated fatty acid eicosapentaenoate (EPA; 20:5n3) and a variety of lysophospholipids,[49;50] are associated with rs10911021 in those same databases. Further studies are clearly needed to dissect the mechanisms linking this locus to the development and progression of atherosclerosis in diabetes. As part of these efforts, it would be useful to extend the study to type 1 diabetes as this may provide clues on whether the gene-×-diabetes interaction involves hyperglycemia or instead concerns factors that are specific to type 2 diabetes such as insulin-resistance or some of the genes predisposing to this form of diabetes, even though the lack of interaction in our study between rs10911021 and genetic variants predisposing to type 2 diabetes makes the latter hypothesis unlikely.

Our study has several strengths, namely the replication design with five independent cohorts of diabetic patients, a rigorous definition of CHD, and a sample size that was adequate for

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the detection of additive genetic effects of the magnitude reported. Nonetheless, some limitations should be acknowledged. First, while our study was powered to detect major genetic effects such as that described in this report, larger studies would be necessary to detect loci having smaller but still relevant effects on CHD risk in diabetes. In this context, the use of analytical methods based on biological pathways such as Gene Ontology (GO)[51] might lead to the identification of additional genetic determinants of CHD in diabetic patients and provide further insights on the links between diabetes and atherogenesis. Second, our study was restricted to non-Hispanic Whites and whether these findings can be generalized to other races remain to be determined. Also, based on the known differences in linkage disequilibrium patterns among races, different genetic markers may be more effective in capturing the predisposing effect of the locus described in this paper in other racial groups. Finally, one should consider that the achieved level of statistical significance, while it meets genome-wide significance, still corresponds to a 5% probability of a false-positive result. Although the likelihood of such an event is reduced by the replication of the association in CARDIoGRAM, further studies are needed before this CHD locus can be considered as fully validated.

In summary, through three-stage genome-wide association analyses in 4,188 type 2 diabetic patients, we have identified a novel susceptibility locus for CHD in the region of the *GLUL* gene. This locus appears to be associated with CHD very weakly or not at all among non-diabetic participants, consistent with a gene-by-diabetes synergism. Preliminary evidence suggests that this locus may modulate CHD risk by affecting glutamate/glutamine metabolism and the activity of the γ-glutamyl cycle, but further studies are needed to fully understand the biological mechanisms linking it to CHD in diabetes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Clinical characteristics of the discovery and validation studies.

	STAGE I				STAGE II		STAGE III			
	NHS		HPFS		JHS		GHS		CZS	
	Cases	Controls	Cases	Controls	Cases	Controls	Cases	Controls	Cases	Controls
No of Participants	350	976	319	665	420	431	314	384	114	215
Age, mean (SD), y	46 (6)	42 (7)	58 (8)	54 (8)	65 (7)	64 (6)	65 (8)	60 (8)	64 (9)	60 (10)
Age at diagnosis of diabetes, mean (SD), y	55 (11)	60 (10)	63 (9)	64 (8)	52 (10)	52 (8)	50 (11)	49 (10)	51 (12)	50 (12)
Male	0 (0)	0 (0)	319 (100)	665 (100)	308 (73)	246 (57)	213 (68)	172 (45)	74 (65)	102 (47)
Diabetes duration, mean (SD), y	14 (8.7)	14 (9.4)	7 (5.1)	10 (5.4)	13 (8.7)	12 (6.8)	14 (9.0)	11 (8.1)	13 (9.0)	11 (9.7)
Smoking status										
Ever	95 (27)	264 (27)	166 (52)	313 (47)	276 (66)	164 (38)	134 (43)	115 (30)	60 (53)	96 (45)
Current	109 (31)	264 (27)	32 (10)	80 (12)	32 (8)	22 (5)	53 (17)	62 (16)	17 (15)	33 (15)
History of hypertension	130 (37)	176 (18)	144 (45)	173 (26)	359 (85)	313 (73)	268 (85)	254 (66)	92 (81)	156 (73)
History of hypercholesterolemia	35 (10)	39 (4)	64 (20)	100 (15)	365 (87)	348 (81)	206 (65)	131 (34)	87 (76)	152 (71)
BMI, mean (SD)	28.9 (5.6)	27.0 (4.6)	28.1 (4.4)	27.6 (4.0)	32.1 (5.9)	32.3 (5.6)	30.4 (4.7)	31.3 (5.2)	30.7 (4.6)	31.0 (5.8)
HDL cholesterol, mean (SD), mg/dl	47 (13)	51 (15)	38 (10)	41 (11)	39 (11)	46 (19)	43 (13)	46 (12)	45 (13)	48 (15)
Triglycerides, mean (SD), mg/dl	242 (154)	204 (164)	201 (103)	192 (99)	187 (146)	178 (119)	154 (94)	151 (93)	161 (107)	160 (96)
HbA1c, mean (SD), %	7.2 (1.8)	6.6 (1.7)	7.5 (1.6)	7.1 (1.5)	7.5 (1.4)	7.3 (1.2)	8.7 (1.9)	8.5 (1.9)	8.1 (2.0)	7.8 (2.0)

Data are expressed as No. of participants (%) unless otherwise indicated.

*Baseline age for NHS and HPFS; Age of enrollment for JHS, SGR and CZS.

†Diabetes duration at CHD event (cases) or censoring (controls) for NHS and HPFS; Diabetes duration at diabetes enrollment for JHS, SGR and CZS.

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Table 2

Association between rs10911021 and CHD in the presence of type 2 diabetes in five independent studies.

	Stage I		Stage II	Stage III		Combined*
	NHS (n=1,326)	HPFS (n=984)	JHS (n=851)	GHS (n=698)	CZS (n=329)	
Risk Allele	C	C	C	C	C	
RAF Controls	0.679	0.680	0.661	0.678	0.716	
RAF Cases	0.735	0.760	0.699	0.736	0.763	
P for HWE†	0.69	0.66	0.70	0.95	0.22	
Odds ratio (95% CI)	1.36‡ (1.19–1.69)	1.50§ (1.21–1.87)	1.25 (1.01–1.55)	1.38 (1.09–1.74)	1.27 (0.89–1.81)	**1.36 (1.22 – 1.51)**
P for association	0.0059	0.0003	0.042	0.0076	0.18	**2.04×10^{-8}**
P for heterogeneity						**0.82**

RAF, Risk Allele Frequency

†P for HWE in the Control groups.

‡OR=1.34, 95% CI 1.09–1.61, p=0.0067 after adjustment for the top principal components (PCs).

§OR=1.49, 95% CI 1.20–1.86, p=0.0004 after adjustment for top PCs.

*Results were combined using inverse variance weights under a fixed model.

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Table 3

Endothelial cell expression of genes adjacent to rs10911021 according to the genotype at this locus.

Gene	Location	Position (Mb)	rs10911021			P value
			T/T (n=16)	C/T (n=42)	C/C (n=60)	
MR1	Centromeric	179.27–179.2	1.00 (0.74–1.35)	0.76 (0.60–0.96)	0.76 (0.66–0.88)	0.24
IER5	Centromeric	179.32–179.33	1.00 (0.66–1.51)	0.98 (0.82–1.18)	0.90 (0.82–1.00)	0.39
CACNA1E	Centromeric	179.72–180.04	UD	UD	UD	-
ZNF648	Centromeric	180.29–180.30	UD	UD	UD	-
GLUL	Telomeric	180.62–180.63	1.00 (0.62–1.62)	0.81 (0.71–0.92)	0.68 (0.62–0.76)	0.0048
TEDDM1	Telomeric	180.63–180.64	1.00 (0.34–2.95)	1.11 (0.77–1.61)	1.01 (0.75–1.37)	0.90
LINC00272	Telomeric	180.61–180.68	UD	UD	UD	-
RGSL1	Telomeric	180.69–180.80	UD	UD	UD	-

Data are geometric means (95% CI) of the $-2^{\Delta\Delta CT}$ values obtained by RT-PCR. The $-2^{\Delta\Delta CT}$ value is a measure of the mRNA level in each endothelial cell line normalized to the average mRNA level in T/T homozygous lines.

UD, undetectable

Table 4

Plasma glutamine, glutamic acid and pyroglutamic/glutamic ratio according to rs10911021 genotype

	rs10911021		
Metabolite	**T/T (n=49)**	**C/C (n=49)**	**P value**
Glutamine (μmol/l)	501 (417–568)	519 (463–573)	0.30
Glutamic Acid (μmol/l)	111 (91–138)	115 (102–132)	0.18
Pyroglutamic/Glutamic Acid	0.94 (0.76–1.14)	0.79 (0.67–0.98)	0.029

Data are medians (IQR).

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