

## Original Investigation

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

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**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 nondiabetic individuals.

**OBJECTIVE** To identify genetic determinants of CHD that are specific to patients with diabetes.

**DESIGN, SETTING, AND PARTICIPANTS** We studied 5 independent sets of CHD cases and CHD-negative controls from the Nurses' Health Study (enrolled in 1976 and followed up through 2008), Health Professionals Follow-up Study (enrolled in 1986 and followed up 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 1517 CHD cases and 2671 CHD-negative controls, all with type 2 diabetes. Results in diabetic patients were compared with those in 737 nondiabetic CHD cases and 1637 nondiabetic CHD-negative controls from the Nurses' Health Study and Health Professionals Follow-up Study cohorts. Exposures included 2 543 016 common genetic variants occurring throughout the genome.

**MAIN OUTCOMES AND MEASURES** Coronary heart disease—defined as fatal or nonfatal myocardial infarction, coronary artery bypass grafting, percutaneous transluminal coronary angioplasty, or angiographic evidence of significant stenosis of the coronary arteries.

**RESULTS** A variant on chromosome 1q25 (rs10911021) was consistently associated with CHD risk among diabetic participants, with risk allele frequencies of 0.733 in cases vs 0.679 in controls (odds ratio, 1.36 [95% CI, 1.22-1.51];  $P = 2 \times 10^{-8}$ ). No association between this variant and CHD was detected among nondiabetic participants, with risk allele frequencies of 0.697 in cases vs 0.696 in controls (odds ratio, 0.99 [95% CI, 0.87-1.13];  $P = .89$ ), consistent with a significant gene  $\times$  diabetes interaction on CHD risk ( $P = 2 \times 10^{-4}$ ). Compared with 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 ( $P = .0048$ ). A decreased ratio between plasma levels of  $\gamma$ -glutamyl cycle intermediates pyroglutamic and glutamic acid was also shown in risk allele homozygotes ( $P = .029$ ).

**CONCLUSION AND RELEVANCE** A single-nucleotide polymorphism (rs10911021) was identified that was significantly associated with CHD among persons with diabetes but not in those without diabetes and was functionally related to glutamic acid metabolism, suggesting a mechanistic link.

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The prevalence of type 2 diabetes has been steadily increasing in the United States and other countries, with the total number of affected people reaching more than 370 million globally.<sup>1</sup> Long-term cardiovascular complications, and especially coronary heart disease (CHD), are the principal causes of morbidity and mortality among diabetic patients.<sup>2</sup> Although mortality caused by CHD has been declining overall during the past few decades in most industrialized countries,<sup>3</sup> the increasing prevalence of diabetes has made the number of CHD deaths attributable to this disease escalate.<sup>4,5</sup>

The role of genetic factors in modulating susceptibility to CHD has been known for many years,<sup>6</sup> and more than 40 chromosomal loci associated with CHD have been identified to date in the general population by genome-wide association studies (GWAS).<sup>7-12</sup> Earlier analyses have shown considerable heterogeneity in genetic effects between diabetic and nondiabetic individuals,<sup>13</sup> 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 GWAS of CHD targeted to type 2 diabetic participants 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 eMethods in the Supplement. Briefly, stage 1 included diabetic patients from the Nurses' Health Study (NHS)<sup>14</sup> and the Health Professionals Follow-up Study (HPFS)<sup>15</sup> (eMethods in the Supplement). CHD cases were defined as those with incident cases after the diagnosis of type 2 diabetes to the end of 2008; controls were participants free of CHD events in the specified 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 2 included diabetic CHD cases and CHD-negative controls from the Joslin Heart Study<sup>16</sup> (eMethods in the Supplement). The study protocol and informed consent procedures were approved by the Joslin Committee on Human Studies and the BIDMC Committee on Clinical Investigations. All participants gave written informed consent.

Stage 3 included diabetic CHD cases and CHD-negative controls from the Gargano Heart Study (cross-sectional design)<sup>17</sup> and the Catanzaro Study<sup>18</sup> (eMethods in the Supplement). The study protocol and informed consent procedures were ap-

proved by the local human subject committees. All participants gave written informed consent.

To compare the association between rs10911021 (<http://www.ncbi.nlm.nih.gov/SNP/>) and CHD risk in nondiabetic vs diabetic participants, we analyzed a separate nondiabetic CHD case-control GWAS, which included incident CHD cases and non-CHD controls from the NHS and HPFS cohorts,<sup>19</sup> after excluding individuals affected by diabetes (eMethods in the Supplement). We also obtained data on rs10911021 from the Coronary Artery Disease Genome-wide Replication and Meta-analysis (CARDIoGRAM).<sup>11</sup> To explore whether this variant may contribute to CHD through alterations of insulin sensitivity, we interrogated the Meta-analyses of Glucose and Insulin-Related Traits Consortium (MAGIC) database, a meta-analysis of GWAS data for metabolic traits.<sup>20,21</sup> We also interrogated the Diabetes Genetics Replication and Meta-analysis (DIAGRAM) database<sup>22</sup> 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 1 have been described in detail elsewhere<sup>23</sup> and in the eMethods in the Supplement. Briefly, samples were genotyped with the Affymetrix Genome-Wide Human 6.0 array. 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 software.<sup>24</sup> In stages 2 and 3, 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).

### 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 messenger RNA levels of 8 neighboring genes (4 on the centromeric and 4 on the telomeric side) (eFigure 1 in the Supplement) in 124 human umbilical vein endothelial cell lines from nondiabetic 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 lines were established from the umbilical cords and cultured as described by Gorfien et al.<sup>25</sup> Cell lines were typed for the rs10911021 SNP with a TaqMan allelic discrimination assay (Applied Biosystems). Gene expression was assayed by means of real-time quantitative polymerase chain reaction-based TaqMan low-density arrays. 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 potential functional influence 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  $\gamma$ -glutamyl cycle) and glutamic acid in 100 diabetic patients from the Joslin Heart Study (50 rs10911021 risk allele C homozygotes and 50 T ho-

mozygotes). Plasma concentrations of glutamic acid and glutamine were assessed at the University of Trieste, Italy, by gas chromatography-mass spectrometry, using the internal standard technique, as previously described.<sup>26</sup> Known amounts of L-[<sup>15</sup>N]-glutamic acid and L-[<sup>15</sup>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 mass-to-charge ratio (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.

## Statistical Analyses

### GWAS and Validations

Analyses were carried out in 3 stages. In stage 1, 2 separate GWAS 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 with the ProbABEL package.<sup>27</sup> The genomic inflation factor  $\lambda$  was estimated from the median  $\chi^2$  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 2 GWAS scans was conducted by combining study-specific  $\beta$ -estimates from genome-wide associations, using inverse variance weights under a fixed-effect model in METAL software.<sup>28</sup> Variants yielding a  $P < 1 \times 10^{-4}$  in stage 1 were carried forward to stage 2, and those yielding a  $P < 1 \times 10^{-4}$  in stages 1 and 2 combined were carried forward to stage 3. In stages 2 and 3, sex-adjusted odds ratios (ORs) and their 95% CIs were estimated for each SNP and in each study by means of logistic regression according to an additive model. Associations across stages 1 and 2 studies and across all the studies in stages 1, 2, and 3 were summarized by meta-analyses with Stata version 7.0. The presence of heterogeneity among the 3 studies was tested by means of a  $\chi^2$  statistics. Because this test was not significant for any of the SNPs, we calculated summary ORs according to a fixed-effect model, ie, by averaging the natural logarithms of the ORs from individual studies, weighted by the inverses of their variances.<sup>29</sup>

The association between rs10911021 and CHD among nondiabetic 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  $\times$  diabetes cross-product to a logistic regression analysis of the combined diabetic and nondiabetic NHS/HPFS sets. The same approach was used to evaluate the interaction between rs10911021 and 36 established type 2-predisposing variants considered in the article by Qi et al.<sup>30</sup>

### Power of Genetic Studies

Power for the main SNP effects was estimated with the software CaTS,<sup>31</sup> assuming a risk allele frequency of 0.30. The GWAS

of type 2 diabetes participants had 80% power ( $\alpha = 5 \times 10^{-8}$ ) to detect associations with CHD, with summary ORs across the 3 stages as low as 1.35. The study of rs10911021 in nondiabetic participants had greater than 99% power ( $\alpha = .05$ ) 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

Change in threshold cycle ( $\Delta$ Ct) values were derived from the threshold cycle (Ct) data for each target gene with the equation  $\Delta$ Ct = Ct (target gene) - Ct (endogenous control).  $\Delta$ Ct values were then calculated for each sample and gene as the difference between the  $\Delta$ Ct and the mean  $\Delta$ Ct among rs10911021 T/T homozygotes. For each target gene, the association between rs10911021 and  $\Delta$ Ct was evaluated by linear regression with 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, sex,  $\gamma$ -glutamyl-transferase 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 GWAS analyses, 2-sided  $P$  values smaller than  $5 \times 10^{-8}$  were considered significant; for all other analyses, 2-sided  $P$  values smaller than .05 were considered significant.

## Results

### GWAS and Validations Among Diabetic Participants

A total of 1517 CHD cases and 2671 CHD-negative controls, all with type 2 diabetes, were included in the 3-stage genome-wide analysis: 350 cases and 976 controls from the NHS and 319 cases and 665 controls from the HPFS (stage 1), 420 cases and 431 controls from the Joslin Heart Study (stage 2), 314 cases and 384 controls from the Gargano Heart Study-cross-sectional design, and 114 cases and 215 controls from the Catanzaro Study (stage 3) (eMethods in the Supplement). 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 1, 26 met the criterion for promotion to stage 2 ( $P < .001$  in stage 1) and 3 of these further met the criterion for promotion to stage 3 ( $P < .001$  in stage 1 + stage 2). Detailed data on the variants associated with CHD at each stage can be found in eTable 1 and eFigures 2 and 3 in the Supplement. Of the 3 variants that were promoted to stage 3, 1 (rs10911021) showed an association with CHD that was nominally significant at each stage and exceeded genome-wide significance in the 3 stages combined ( $P = 2.0 \times 10^{-8}$ ) (**Table 2** and eTable 1 in the Supplement).

Table 1. Clinical Characteristics of the Discovery and Validation Studies

	No. (%)									
	Stage 1				Stage 2		Stage 3			
	Nurses' Health Study		Health Professionals Follow-up Study		Joslin Heart Study		Gargano Heart Study		Catanzaro Study	
	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 <sup>a</sup>	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	319 (100)	665 (100)	308 (73)	246 (57)	213 (68)	172 (45)	74 (65)	102 (47)
Diabetes duration, mean (SD), y <sup>b</sup>	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)
Body mass index, 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)
HbA <sub>1c</sub> , 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)

Abbreviations: HbA<sub>1c</sub>, hemoglobin A<sub>1c</sub>; HDL, high-density lipoprotein. SI conversion factors: To convert HDL cholesterol to mmol/L, multiply values by 0.0259; to convert triglycerides to mmol/L, multiply values by 0.0113. Body mass index is calculated as weight in kilograms divided by height in meters squared.

<sup>a</sup>Baseline age for Nurses' Health Study (NHS) and Health Professionals Follow-up Study (HPFS); age of enrollment for the other studies.

<sup>b</sup>Diabetes duration at coronary heart disease event (cases) or censoring (controls) for NHS and HPFS; diabetes duration at enrollment for the other studies.

Table 2. Association Between rs10911021 and CHD in the Presence of Type 2 Diabetes in 5 Independent Studies

	Stage 1		Stage 2	Stage 3		Combined <sup>a</sup>
	Nurses' Health Study	Health Professionals Follow-up Study	Joslin Heart Study	Gargano Heart Study	Catanzaro Study	
Risk allele	C	C	C	C	C	C
RAF controls	0.679	0.680	0.661	0.678	0.716	0.679
RAF cases	0.735	0.760	0.699	0.736	0.763	0.733
P value for HWE <sup>b</sup>	.69	.66	.70	.95	.22	
Odds ratio (95% CI)	1.36 (1.19-1.69) <sup>c</sup>	1.50 (1.21-1.87) <sup>d</sup>	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 value for association	.0059	.0003	.04	.0076	.18	2.04 × 10 <sup>-8</sup>
P value for heterogeneity						.82

Abbreviations: HWE, Hardy-Weinberg equilibrium; RAF, risk allele frequency. <sup>a</sup>Results were combined by using inverse variance weights under a fixed model. <sup>b</sup>P value for HWE in the control groups. <sup>c</sup>OR = 1.34; 95% CI, 1.09-1.61; and P = .0067 after adjustment for the top principal components.

<sup>d</sup>OR = 1.49; 95% CI, 1.20-1.86; and P = .0004 after adjustment for top principal components.

In a meta-analysis of the 5 case-control sets, the summary OR of CHD for each copy of the risk allele was 1.36 (95% CI, 1.22-1.51; risk allele frequencies, 0.697 in cases vs 0.696 in controls), with no evidence of heterogeneity across studies (I<sup>2</sup> = 0%; P = .82) (Table 2). The other 2 variants promoted to stage 3 (rs9361923 on chromosome 6 and rs7542837 on chromosome 1) had summary P values across the 5 sets in the 10<sup>-4</sup> range (eTable 1 in the Supplement). None of the loci previ-

ously associated with CHD in the general population were among the genetic variants promoted to stages 2 and 3, although 3 of them reached nominal significance at stage 1 (eTable 2 in the Supplement).

**Interaction With Diabetes Status**

No association between rs10911021 and CHD was found among 737 nondiabetic CHD cases and 1637 nondiabetic CHD-

**Table 3. Endothelial Cell Expression of Genes Adjacent to rs10911021 According to the Genotype at This Locus<sup>a</sup>**

Gene	Location	Position (Mb)	T/T (n = 16)	C/T (n = 42)	C/C (n = 60)	P Value
<i>MRI</i>	Centromeric	179.27-179.2	1.00 (0.74-1.35)	0.76 (0.60-0.96)	0.76 (0.66-0.88)	.24
<i>IERS</i>	Centromeric	179.32-179.33	1.00 (0.66-1.51)	0.98 (0.82-1.18)	0.90 (0.82-1.00)	.39
<i>CACNA1E</i>	Centromeric	179.72-180.04	UD	UD	UD	
<i>ZNF648</i>	Centromeric	180.29-180.30	UD	UD	UD	
<i>GLUL</i>	Telomeric	180.62-180.63	1.00 (0.62-1.62)	0.81 (0.71-0.92)	0.68 (0.62-0.76)	.0048
<i>TEDDM1</i>	Telomeric	180.63-180.64	1.00 (0.34-2.95)	1.11 (0.77-1.61)	1.01 (0.75-1.37)	.90
<i>LINC00272</i>	Telomeric	180.61-180.68	UD	UD	UD	
<i>RGSL1</i>	Telomeric	180.69-180.80	UD	UD	UD	

Abbreviation: UD, undetectable.

<sup>a</sup>Data are geometric means (95% CI) of the  $-2^{\Delta\Delta CT}$  values obtained by real-time polymerase chain reaction. The  $-2^{\Delta\Delta CT}$  value is a measure of the messenger

RNA level in each endothelial cell line normalized to the average messenger RNA level in T/T homozygous lines.

negative controls from the NHS and HPFS cohorts (eTable 3 in the Supplement). The OR among these nondiabetic individuals was not significantly different from 1 (OR = 0.99; 95% CI, 0.87-1.13;  $P = .89$ ; risk allele frequencies, 0.697 in cases vs 0.696 in controls) while being significantly different from the OR in diabetic participants (OR = 1.36; 95% CI, 1.22-1.51;  $P$  for diabetes  $\times$  genetic variant interaction,  $2.6 \times 10^{-4}$ ). 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<sup>30</sup> (all  $P > .05$ ).

In CARDIoGRAM, which comprises 22 233 CHD cases and 64 762 controls from the general population, rs10911021 showed a nominally significant association with CHD in the same direction as among the diabetic participants in our study (OR = 1.04; 95% CI, 1.01-1.07;  $P = .011$ ) but was significantly weaker ( $I^2 = 96\%$ ;  $P$  for heterogeneity =  $2.2 \times 10^{-6}$ ; 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<sup>32-35</sup>—the OR observed in the CARDIoGRAM population corresponded to the weighted average of the ORs observed in our study in diabetic and nondiabetic participants (OR = 1.36 and 0.99, respectively). No other variant neighboring rs10911021 showed associations at genome-wide significance level in this data set (eFigure 4 in the Supplement).

### Genotype Association With the Expression of Neighboring Genes

Variant rs10911021 is located between 2 genes, *ZNF648* ( $\approx 51$  kb in centromeric direction; NCBI Entrez Gene 127665) and *GLUL* ( $\approx 270$  kb in telomeric direction; NCBI Entrez Gene NG\_013347.1), and neighbors several other genes (eFigure 1 in the Supplement). No missense variants in linkage disequilibrium 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 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

**Table 4. Plasma Glutamine, Glutamic Acid, and Pyroglutamic/Glutamic Ratio According to rs10911021 Genotype**

Metabolite	Median (IQR)		P Value
	T/T (n = 49)	C/C (n = 49)	
Glutamine, $\mu\text{mol/L}$	501 (417-568)	519 (463-573)	.30
Glutamic acid, $\mu\text{mol/L}$	111 (91-138)	115 (102-132)	.18
Pyroglutamic/glutamic acid	0.94 (0.76-1.14)	0.79 (0.67-0.98)	.029

Abbreviation: IQR, interquartile range.

a high DNase I sensitivity cluster binding to the RE1-silencing transcription factor 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 compared with protective allele (T/T) homozygotes, with heterozygotes having intermediate levels ( $P$  for trend = .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 $\gamma$ -Glutamyl Cycle

In a sample of 100 Joslin Heart Study 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  $\gamma$ -glutamyl cycle) and glutamic acid was significantly lower in C/C as compared to T/T carriers ( $P = .03$ ) (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; interquartile range 0.62-0.97) than in the 56 who were CHD negative (median, 0.92; interquartile range, 0.78-1.14) ( $P = .02$ ). The OR of CHD for the rs10911021 C/C genotype in this subsample decreased from 1.83 to 1.39 ( $\approx 50\%$  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.

### Association With Other Cardiovascular Risk Factors

No significant association between rs10911021 and serum fasting insulin, homeostatic assessment model insulin-resistance index, or 2-hour glucose during an oral glucose tolerance test was found in the MAGIC database including data on more than 35 000 nondiabetic individuals. Similarly, no significant association was found with type 2 diabetes in the DIAGRAM database (OR = 1.01; 95% CI, 0.97-1.04;  $P = .76$ ).

## 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 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 type 2 diabetes participants meets genome-wide significance ( $P < 5 \times 10^{-8}$ ), namely, 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 geographic origin, reaching nominal significance in 4 of the 5 sets that were considered. Third, the difference in ORs between diabetic and nondiabetic participants was supported by a robust  $P$  value for interaction. Finally, an association 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 according to the effects detected in our study in diabetic and nondiabetic 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.<sup>36</sup> Both amino acids play important roles in human physiology. Glutamic acid is a key intermediate of several metabolic pathways, most notably of the  $\gamma$ -glutamyl cycle, through which the antioxidant glutathione is generated<sup>37</sup>; glutamine is involved in the regulation of cell proliferation, inhibition of apoptosis, and cell signaling.<sup>38</sup> 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.<sup>39</sup> In diabetic patients, it was found that glutamine reduced glucose excursions when administered before oral glucose<sup>40</sup> and effectively increased circulating incretin and insulin concentrations.<sup>41</sup> Several clinical trials also suggest cardioprotective effects of glutamine used parenterally and enterally.<sup>42,43</sup> In epidemiologic studies, abnormal metabolism of these amino acids has been shown to be related to insulin resistance, type 2 diabetes, and cardiovascular disorders.<sup>44-46</sup>

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. 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, homeostatic assessment model insulin-resistance index, or 2-hour glucose during a glucose tolerance test 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  $\gamma$ -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<sup>47</sup> and potentially explaining the fact that this genetic effect can be observed only 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 ( $P = .00096$  in KORA; <http://metabolomics.helmholtz-muenchen.de/gwa>).<sup>48</sup> However, additional contributions by pathways that are not directly related to glutamate and glutamine may also be present because other metabolites implicated in vascular biology and atherogenesis, such as the long-chain  $\omega$ 3-polyunsaturated fatty acid eicosapentaenoate (20:5n3) and a variety of lysophospholipids,<sup>49,50</sup> are associated with rs10911021 in those same databases.

Further studies are 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 because this may provide clues about whether the gene  $\times$  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. However, 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 validation design with 5 independent cohorts of diabetic patients, a rigorous definition of CHD, and a sample size that was adequate for the detection of additive genetic effects of the magnitude reported. Nonetheless, some limitations should be acknowledged. First, although our study was powered to detect major genetic effects such as that described in this article, 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 analytic methods based on biological pathways such as gene ontology<sup>51</sup> might lead to the identification of additional genetic determinants of CHD in diabetic patients and pro-

vide 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 remains to be determined. Also, according to 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 article in other racial groups. Third, although the achieved level of statistical significance meets genome-wide significance, it still corresponds to a 5% probability of a false-positive result. Although the likelihood of such an event is reduced by the validation of the association in CARDIOGRAM, further studies are needed before this CHD locus can be considered as fully validated.

## Conclusion

In summary, through a 3-stage GWAS in 4188 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 nondiabetic participants, consistent with a gene  $\times$  diabetes synergism. Preliminary evidence suggests that this locus may modulate CHD risk by affecting glutamate/glutamine metabolism and the activity of the  $\gamma$ -glutamyl cycle, but further studies are needed to fully understand the biological mechanisms linking it to CHD in diabetes.

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