Candidate Gene Association Study for Diabetic Retinopathy in Persons with Type 2 Diabetes: The Candidate Gene Association Resource (CARe)

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Purpose. To investigate whether variants in cardiovascular candidate genes, some of which have been previously associated with type 2 diabetes (T2D), diabetic retinopathy (DR), and diabetic nephropathy (DN), are associated with DR in the Candidate gene Association Resource (CARe).

Methods. Persons with T2D who were enrolled in the study (n = 2691) had fundus photography and genotyping of single nucleotide polymorphisms (SNPs) in 2000 candidate genes. Two case definitions were investigated: Early Treatment Diabetic Retinopathy Study (ETDRS) grades ≥14 and ≥30. The χ^2 analyses for each CARe cohort were combined by Cochran-Mantel-Haenszel (CMH) pooling of odds ratios (ORs) and corrected for multiple hypothesis testing. Logistic regression was performed with adjustment for other DR risk factors. Results from replication in independent cohorts were analyzed with CMH meta-analysis methods.

Results. Among 39 genes previously associated with DR, DN, or T2D, three SNPs in P-selectin (*SELP*) were associated with DR. The strongest association was to rs6128 (OR = 0.43, P = 0.0001, after Bonferroni correction). These associations remained significant after adjustment for DR risk factors. Among other genes examined, several variants were associated with DR with significant P values, including rs6856425 tagging α-L-iduronidase (*IDUA*) ($P = 2.1 \times 10^{-5}$, after Bonferroni correction). However, replication in independent cohorts did not reveal study-wide significant effects. The P values after replication were 0.55 and 0.10 for rs6128 and rs6856425, respectively.

Conclusions. Genes associated with DN, T2D, and vascular diseases do not appear to be consistently associated with DR. A few genetic variants associated with DR, particularly those in *SELP* and near *IDUA*, should be investigated in additional DR cohorts. (*Invest Ophthalmol Vis Sci.* 2011;52:7593–7602) DOI: 10.1167/iovs.11-7510

Diabetic retinopathy (DR) is the leading cause of blindness in working-age Americans[1,2] and is increasing in prevalence as rates of type 2 diabetes (T2D) soar worldwide.[3,4] The frequency and severity of DR are heterogeneous within and across ethnic groups,[5] even with adjustment for risk factors such as duration of diabetes and glycemic control.[2,6,7] There are people who have a long duration of diabetes without DR and those who have severe DR despite relatively good glycemic control. For these reasons, genetic risk factors are thought to play a role in DR. Heritability has been estimated to be as high as 27% for DR and 52% for proliferative diabetic retinopathy (PDR).[8–10] However, genetic association studies for DR have been thus far limited mostly to studies of one or a modest number of candidate genes.[11,12] Most reported associations have not been consistently reproduced.[11,13,14]

In contrast to DR, genetic association studies for T2D have revealed many consistently associated genes. Genes that increase T2D risk may also predispose to development of retinopathy. In the case of diabetic nephropathy (DN), a *TCF7L2* variant increases the risk of developing DN beyond the risk of diabetes.[15] Because there is evidence that DR shares risk factors and pathophysiological mechanisms with DN and macrovascular diabetic complications,[6,16–21] genes associated with DN and atherosclerotic vascular disease may also be associated with DR.

The Candidate gene Association Resource (CARe) is a collaboration for association analyses of genotypes and cardiovascular disease phenotypes.[22] It comprises >40,000 participants from nine cohorts who have been genotyped for 49,320 single nucleotide polymorphisms (SNPs) from approximately 2,000 candidate genes postulated or known to increase risk of cardiovascular, metabolic, and inflammatory diseases.[23] It includes 2691 T2D participants with fundus photographs of multiple ethnicities. Thus, the CARe framework provides an opportunity to investigate genetic associations for DR with a candidate gene approach. CARe genotyped many genes previously associated with DR,[24,25] DN,[25–27] and T2D.[28–34] The first purpose of this study was to investigate whether these genes are also associated with the presence of DR in CARe. The second purpose was to determine whether the remaining genes included in the CARe genotyping platform, which were also chosen as potential cardiovascular disease genes, are associated with DR.

Methods

Study Population and Fundus Photography Procedures

Four CARe cohorts have fundus photographs of T2D participants: Atherosclerosis Risk in Communities (ARIC) Study, Cardiovascular Health Study (CHS), Jackson Heart Study (JHS), and Multiethnic Study of Atherosclerosis (MESA).[35–38] T2D was defined according to the American Diabetes Association 2003 Criteria.[39] The fundus photography protocol for each cohort is described in Table 1.[40–42] In all studies, except for the JHS, fundus photographs were graded by masked readers at the University of Wisconsin Ocular Epidemiology Reading Center according to the modified Airlie House Classification system.[43] Fundus photographs for the JHS were graded by masked JHS ophthalmologist investigators according to the same criteria.

Definition of Diabetic Retinopathy

We examined two DR phenotypes. First we defined cases as participants with an Early Treatment Diabetic Retinopathy Study (ETDRS) grade ≥14 in the eye with the higher ETDRS grade or in the only eye photographed, depending on the study's protocol. These analyses were designed to detect associations with the presence of any DR. Our second phenotype defined cases as participants with ETDRS grade ≥30. The latter was intended to reduce misclassification of patients with minimal signs of DR, which may be seen even in persons without diabetes.[44–46] For all analyses, controls were defined as T2D participants with an ETDRS grade <14 (no DR).

Measurement of Other Variables

Data on DR risk factors were obtained from the study examination at which fundus photography was performed. These included duration of diabetes, fasting blood glucose, systolic and diastolic blood pressures, and fasting total cholesterol.[47–50] The procedures for measuring these variables are described in online documentation (www.cscc.unc.edu/aric/, www.chs-nhlbi.org, and www.mesa-nhlbi.org, provided by the National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD; and www.jsums.edu/jhs, JHS, Jackson State University, Jackson, MS). Some participants were unaware of a diabetes diagnosis and received the diagnosis based on their laboratory values at the study visit at which they also had fundus photography. For these patients, the duration of diabetes was calculated by halving the number of years between their prior study visit (when they did not meet criteria for T2D) and the visit at which they met criteria. If data on a risk factor were not measured at the fundus photography visit, the information was obtained from the visit closest in time to the fundus photography visit.

Genotyping

CARe participant DNA samples were interrogated on a custom genotyping array (iSelect ITMAT-Broad-CARe [IBC] Chip; Illumina, San Diego, CA). Its design is described elsewhere.[23] SNP selection criteria and genotyping quality control (QC) procedures are explained in the Supplementary Methods (http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7510/-/DCSupplemental).[23]

Table 1. CARe Participants with T2D and DR Grading by Cohort and Ethnicity

Cohort/ Population	Eyes Photographed per Participant (*n*)	Fields Photographed per Eye (*n*)	Size of Each Field Photographed (deg)	Participants with T2D, DR Grading and IBC Chip Genotyping (*n*)	Participants with ETDRS Grade <14 (*n*)	Participants with ETDRS Grade ≥14(*n*)	Participants with ETDRS Grade ≥30 (*n*)
ARIC							
EA	One	One	45	885	732	153	91
AfrA				439	315	124	95
CHS							
EA	One	One	45	193	160	33	20
AfrA				54	35	19	15
JHS							
AfrA	Two	Seven	30	55	26	29	22
MESA							
EA	Two	Two	45	176	140	36	11
AfrA				275	176	99	57
AsA				79	54	25	14
HA				231	151	80	46
All							
EA				1254	1032	222	122
AfrA				823	552	271	189
AsA				79	54	25	14
HA				231	151	80	46

EA, European American; AfrA, African American; AsA, Asian American; HA, Hispanic American.

Statistical Analysis

We first investigated genes that have been previously associated with DR, DN, and T2D. For DR, we chose the genes that had the most robust evidence of association from a comprehensive review of the literature[24] and a subsequent strong association with the erythropoietin (*EPO*) gene promoter.[25] For DN, we chose genes that have shown nominal associations ($P < 0.05$) with DN or a related quantitative trait.[25–27] For T2D, we chose genes with SNPs that had met genome-wide significance.[28–31,33] Supplementary Table S1 (http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7510/-/DCSupplemental) lists the 39 genes included on the IBC chip that met these criteria. In the second phase, we investigated the remaining genes on the IBC chip which were primarily cardiovascular candidate genes.[23]

For genetic association testing, we used χ^2 analysis comparing European-American cases (participants with T2D and DR) to controls (participants with T2D and no DR) in each CARe cohort. Results were combined by Cochran-Mantel-Haenszel (CMH) pooling of the odds ratios (ORs).[51,52] This CMH method is a robust way of maintaining consistency with individual study ORs while estimating a single fixed-effects OR across all cohorts. We report correction for the multiple association tests performed with per gene Bonferroni correction and permutation testing. Per gene Bonferroni correction was performed because it is an intuitive, easily understood correction. This Bonferroni correction is not as conservative as the one that corrects for the total number of unique genetic loci tested, as would be represented by correcting for the total number of tag SNPs that are not in strong LD. For this reason, we also present the empiric permutation testing correction, which, although less intuitive, does account for this LD between SNPs. We defined statistical significance as $P < 0.05$ after per gene Bonferroni correction. We chose this threshold for the discovery phase of the experiment, although it is a less stringent threshold than one based on a correction that could completely account for LD between SNPs, to minimize type II error (false negatives) in this initial phase. We apply a more stringent threshold for the replication phase (see below), and the final decision of whether an SNP is truly associated is based on its final *P* value after replication. For SNPs that were statistically significant in the discovery phase, we performed haplotype analyses using the omnibus test and further examined the associations with logistic regression models that included other DR risk factors. Age and duration of diabetes were defined as continuous variables in years. Fasting glucose and total cholesterol were incorporated as continuous variables in milligrams per deciliter. Systolic and diastolic blood pressures were evaluated as continuous variables (in mm Hg). If a participant was taking antihypertension medication, 15 and 10 mm Hg were added to the systolic and diastolic blood pressure values, respectively.[53] Sex and study site were also incorporated. All statistical analyses were performed in PLINK.[54]

Replication

Top significant findings were pursued in the non-European American populations in CARe and in independent Caucasian cohorts with genome-wide genotyping results: Age, Gene/Environment Susceptibility (AGES) study[55]; Blue Mountains Eye Study (BMES)[56]; Genetics of Diabetes Audit and Research Tayside Study (Go-DARTS)[57]; Finnish Diabetic Nephropathy (FinnDiane) Study[9]; Family Investigation of Nephropathy and Diabetes-Eye (FIND-Eye) Study[10]; Singapore Malay Eye Study (SiMES)[58]; and the Singapore Prospective Study Program (SP2).[59] The Medical University of Lublin T2D cohort[60] performed de novo genotyping for replication. The phenotyping protocols for these studies are described in the Supplementary Methods and Supplementary Table S2 (http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7510/-/DCSupplemental). Because ETDRS grading was not used by all cohorts, phenotype data were harmonized into two categories that were analogous to ETDRS grade ≥14 and ETDRS grade ≥30. Meta-analysis of the discovery cohort and replication cohorts results was performed by CMH pooling of ORs. Statistical heterogeneity was assessed with Cochran's Q statistic.[61,62] The Q statistic calculates a

weighted sum of the square distances of the observed effects from the null hypothesis of equality of the effects. The weight for each study is the inverse of the variance of the effect estimator so that larger and more accurate studies are weighted more heavily. Statistical significance after replication was defined as a meta-analysis $P < 1 \times 10^{-6}$. This threshold was determined empirically on the basis of the number of genes tested, is analogous to the threshold for genome-wide significance of 5.0×10^{-8} for GWAS,[63] and corresponds approximately to a $P < 0.05$, after Bonferroni correction.

This research adhered to the tenets of the Declaration of Helsinki and was approved by the institutional review boards of the Massachusetts Eye and Ear Infirmary and the primary cohorts. Informed consent was obtained from all participants.

Results

Table 1 shows the number of T2D CARe participants by cohort and ethnicity. The prevalence of DR is similar among the cohorts, although it is higher in the JHS African-American population when compared to the other African-American populations. This discrepancy is probably secondary to the more precise phenotyping performed in the JHS with seven-field photography. Duration of diabetes and fasting glucose levels were not significantly different between the JHS cohort's and the other cohorts' African-American populations.

Table 2 shows the most significant associations for the analysis of the DR, DN, and T2D genes with any DR in European Americans. Only the associations to three SNPs in the P-selectin gene (*SELP*) were significant ($P < 0.05$, after Bonferroni correction). The three associated SNPs tagged the only associated haplotype (Supplementary Table S3, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7510/-/DCSupplemental). In logistic regression including other DR risk factors, the associations to rs6128, rs6133, and rs3917779 remained significant ($P = 0.026$, 0.022, and 0.026, respectively). The mean values for covariates are presented in Supplementary Table S4 (http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7510/-/DCSupplemental).

Table 3 shows the most significant associations from the analysis of the DR, DN, and T2D candidate genes with DR defined as an ETDRS grade ≥30 in European Americans. The three *SELP* SNPs, along with five SNPs in the fat mass and obesity-associated (*FTO*) gene, were significantly associated ($P < 0.05$, after Bonferroni correction). Variants rs12708942, rs9806929, and rs4783824 tagged the only associated haplotype (Supplementary Table S3, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7510/-/DCSupplemental). Because the effect of *FTO* on T2D risk is mediated by its effect on obesity, we executed a logistic regression model including age, sex, and body mass index (BMI). BMI was incorporated as a continuous variable (weight in kilograms divided by height in meters squared). Three *FTO* SNPs continued to be associated with DR ($P = 0.002$, 0.002, and 0.001 for rs9926180, rs7500562, and rs12149433, respectively). Of note, an *EPO* variant, rs551238, was associated with this more stringent definition of DR and had a $P < 0.05$ after permutation correction but not after Bonferroni correction.

We then examined the strength of these associations in the CARe non-European populations. For any DR, the associations with the *SELP* SNPs were not replicated in the African-, Hispanic-, or Asian-American populations (Table 4). When cases were defined as ETDRS grade ≥30, the SNPs in *SELP* and *FTO* were not associated, and in logistic regression models including age, sex, and BMI, no association with the *FTO* SNPs was detected (data not shown).

In the second phase of the analysis, we examined the remaining genes on the IBC chip in European Americans. The

Table 2. Top Association Results in European-American Samples with Cases Defined as ETDRS Grade ≥14 for Variants within Genes Previously Associated with DR, DN, or T2D

					ARIC				CHS				MESA				CMH Combined Analysis					P Correction	
					MAF				MAF				MAF				MAF						
SNP	Chr	Gene	Position	Minor Allele	Cases	Controls	OR	P	Cases	Controls	OR	P	Cases	Controls	OR	P	Cases	Controls	OR	L95	U95	P	Bonferroni	Permutation
rs6128	1	*SELP*	167829528	T	0.08	0.16	0.48	0.0007	0.06	0.18	0.30	0.02	0.08	0.20	0.36	0.02	0.08	0.17	0.43	0.31	0.62	3.1×10^{-6}	0.0001	0.0001
rs6133	1	*SELP*	167831970	A	0.05	0.12	0.39	0.0003	0.03	0.13	0.22	0.02	0.07	0.13	0.50	0.16	0.05	0.12	0.38	0.25	0.59	1.1×10^{-5}	0.0004	0.0001
rs3917779	1	*SELP*	167837472	A	0.05	0.12	0.40	0.0004	0.03	0.12	0.23	0.03	0.07	0.13	0.50	0.16	0.05	0.12	0.39	0.26	0.60	1.6×10^{-5}	0.0006	0.0001
rs12262390	10	*HHEX*	94436103	C	0.12	0.09	1.48	0.05	0.15	0.10	1.61	0.22	0.18	0.09	2.27	0.03	0.14	0.09	1.61	1.18	2.20	0.002	0.10	0.004
rs9356754	6	*CDKAL1*	20916721	G	0.51	0.43	1.37	0.01	0.53	0.44	1.43	0.18	0.40	0.38	1.09	0.74	0.49	0.42	1.32	1.08	1.63	0.007	0.29	0.008
rs9465904	6	*CDKAL1*	20929641	C	0.37	0.30	1.38	0.01	0.42	0.33	1.49	0.15	0.28	0.26	1.08	0.79	0.36	0.30	1.34	1.08	1.65	0.008	0.33	0.01
rs11466493	3	*TGFBR2*	30661786	G	0.01	0.04	0.14	0.002	0.06	0.05	1.23	0.72	0.04	0.07	0.59	0.41	0.02	0.05	0.41	0.21	0.80	0.009	0.35	0.01
rs17025862	3	*TGFBR2*	30660132	G	0.01	0.04	0.14	0.002	0.06	0.05	1.23	0.72	0.04	0.07	0.59	0.41	0.02	0.05	0.41	0.21	0.80	0.009	0.35	0.01
rs7747989	6	*CDKAL1*	20921354	C	0.37	0.30	1.35	0.02	0.44	0.33	1.60	0.09	0.28	0.26	1.08	0.79	0.36	0.30	1.33	1.07	1.65	0.009	0.36	0.01
rs2328572	6	*CDKAL1*	21323815	A	0.003	0.02	0.15	0.03	0	0.03	NA	NA	0.01	0.03	0.48	0.48	0.005	0.02	0.19	0.05	0.69	0.01	0.44	0.01
rs10214694	6	*CDKAL1*	21321533	T	0.003	0.02	0.15	0.03	0	0.03	NA	NA	0.01	0.03	0.48	0.48	0.005	0.02	0.19	0.05	0.69	0.01	0.45	0.01
rs12190631	6	*CDKAL1*	21020651	G	0.05	0.03	1.38	0.30	0.12	0.02	6.17	0.0001	0.01	0.01	1.29	0.83	0.05	0.03	1.85	1.14	3.01	0.01	0.51	0.02
rs2275729	10	*HHEX*	94442410	G	0.15	0.12	1.32	0.13	0.15	0.12	1.29	0.51	0.21	0.10	2.26	0.02	0.16	0.12	1.44	1.08	1.92	0.01	0.52	0.02
rs1511024	4	*FABP2*	120459629	T	0.05	0.03	1.74	0.07	0.05	0.02	3.00	0.12	0.06	0.03	1.76	0.35	0.05	0.03	1.86	1.13	3.06	0.01	0.55	0.01
rs9350294	6	*CDKAL1*	20978072	T	0.38	0.32	1.26	0.07	0.44	0.32	1.65	0.07	0.33	0.29	1.24	0.45	0.38	0.32	1.31	1.05	1.61	0.01	0.55	0.01

The minor allele is the effect allele for the ORs. Chr, chromosome; L95, lower 95% CI boundary; U95, upper 95% CI boundary; NA, not available.

TABLE 3. Top Association Results in European-American Samples with Cases Defined as ETDRS Grade ≥30 for Variants in Genes Previously Associated with DR, DN, or T2D

					ARIC				CHS				MESA				CMH Combined Analysis					P Correction	
					MAF				MAF				MAF				MAF						
SNP	Chr	Gene	BP	Minor Allele	Cases	Controls	OR	P	Cases	Controls	OR	P	Cases	Controls	OR	P	Cases	Controls	OR	L95	U95	P	Bonferroni	Permutation
rs6133	1	*SELP*	167831970	A	0.04	0.12	0.34	0.002	0	0.12	NA	NA	0.09	0.12	0.74	0.69	0.05	0.12	0.32	0.17	0.58	2.3×10^{-4}	0.009	0.0002
rs3917779	1	*SELP*	167837472	A	0.04	0.12	0.35	0.003	0	0.12	NA	NA	0.09	0.12	0.74	0.69	0.05	0.12	0.32	0.17	0.59	2.9×10^{-4}	0.01	0.0001
rs9926180	16	*FTO*	52486108	T	0.33	0.25	1.51	0.01	0.38	0.25	1.81	0.08	0.32	0.17	2.32	0.07	0.34	0.24	1.65	1.24	2.18	5.0×10^{-4}	0.02	0.001
rs7500562	16	*FTO*	52488391	C	0.33	0.25	1.51	0.01	0.38	0.25	1.81	0.08	0.32	0.17	2.27	0.08	0.34	0.24	1.64	1.24	2.17	5.3×10^{-4}	0.02	0.001
rs12149433	16	*FTO*	52485580	G	0.15	0.09	1.75	0.01	0.20	0.08	2.84	0.01	0.05	0.04	1.07	0.95	0.15	0.09	1.93	1.33	2.81	5.5×10^{-4}	0.02	0.001
rs6128	1	*SELP*	167829528	T	0.08	0.16	0.49	0.009	0.05	0.17	0.26	0.05	0.09	0.18	0.45	0.27	0.08	0.16	0.44	0.27	0.70	5.6×10^{-4}	0.02	0.001
rs13335343	16	*FTO*	52488941	A	0.15	0.09	1.74	0.01	0.20	0.08	2.84	0.01	0.05	0.04	1.07	0.95	0.15	0.09	1.92	1.32	2.80	6.1×10^{-4}	0.02	0.001
rs12935710	16	*FTO*	52500306	T	0.32	0.23	1.55	0.01	0.33	0.25	1.48	0.28	0.27	0.15	2.09	0.14	0.32	0.22	1.61	1.21	2.14	0.001	0.04	0.002
rs10852525	16	*FTO*	52496582	A	0.17	0.12	1.52	0.05	0.20	0.10	2.29	0.05	0.09	0.05	2.09	0.34	0.17	0.11	1.71	1.20	2.45	0.003	0.12	0.005
rs9929152	16	*FTO*	52496904	G	0.33	0.25	1.45	0.03	0.35	0.26	1.53	0.23	0.32	0.20	1.89	0.18	0.33	0.25	1.52	1.15	2.02	0.004	0.14	0.006
rs4783824	16	*FTO*	52509162	T	0.13	0.09	1.50	0.09	0.20	0.08	2.95	0.01	0.05	0.03	1.69	0.62	0.14	0.08	1.78	1.21	2.64	0.004	0.15	0.005
rs1362570	16	*FTO*	52491048	C	0.14	0.10	1.52	0.07	0.20	0.09	2.45	0.03	0.05	0.04	1.07	0.95	0.14	0.09	1.70	1.16	2.48	0.007	0.26	0.009
rs4611524	17	*FTO*	58945384	T	0.31	0.41	0.67	0.02	0.33	0.42	0.68	0.27	0.36	0.40	0.86	0.74	0.32	0.41	0.69	0.52	0.91	0.009	0.34	0.009
rs12708942	16	*FTO*	52503705	A	0.14	0.10	1.46	0.1	0.20	0.09	2.63	0.02	0.05	0.04	1.15	0.89	0.14	0.09	1.68	1.14	2.47	0.009	0.35	0.01
rs9806929	16	*FTO*	52507417	A	0.14	0.10	1.46	0.1	0.20	0.09	2.63	0.02	0.05	0.04	1.15	0.89	0.14	0.09	1.68	1.14	2.47	0.009	0.35	0.01
rs551238	7	*EPO*	100159464	G	0.34	0.40	0.75	0.08	0.20	0.40	0.37	0.01	0.32	0.36	0.82	0.67	0.31	0.40	0.69	0.52	0.91	0.009	0.37	0.01

The minor allele is the effect allele for the ORs. Chr, chromosome; L95, lower 95% CI boundary; U95, upper 95% CI boundary; NA, not available.

TABLE 4. CHM Association Results for *SELP* SNPs in non-European-American CARe Populations, with Cases Defined as ETDRS Grade ≥14

SNP	Minor Allele	MAF		OR	L95	U95	P
		Cases	Controls				
African American							
rs6128	T	0.5	0.46	1.17	0.95	1.44	0.14
rs6133	C	0.42	0.45	0.89	0.72	1.09	0.26
rs3917779	G	0.48	0.50	0.94	0.76	1.15	0.55
Hispanic American							
rs6128	T	0.29	0.26	1.14	0.74	1.75	0.55
rs6133	A	0.16	0.18	0.91	0.55	1.52	0.72
rs3917779	A	0.14	0.16	0.89	0.52	1.52	0.67
Asian American							
rs6128	C	0.24	0.27	0.86	0.40	1.87	0.70
rs6133	A	0	0	NA	NA	NA	NA
rs3917779	A	0	0	NA	NA	NA	NA

L95, lower 95% CI boundary; U95, upper 95% CI boundary; NA, not available.

top association results for DR defined as ETDRS grade ≥14 and ETDRS grade ≥30 are shown in Tables 5 and 6, respectively. The lambdas for the quantile–quantile (Q–Q) plots were 1.01 and 1.00, respectively; Fig. 1). Supplementary Table S5 (http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7510/-/DCSupplemental) shows the results from the principal components analysis. There was no evidence of population stratification. Several variants in the two analyses had associations that were significant after Bonferroni correction. One variant, rs6856425, was significantly associated with DR in all three CARe cohorts for both definitions of DR with $P = 2.1 \times 10^{-5}$ after Bonferroni correction in the ETDRS grade ≥30 analysis. This association could not be replicated in the CARe African American cohorts (MAF 18%, OR = 0.94, $P = 0.69$).

We pursued replication of top findings from Tables 5 and 6 in independent cohorts of European ancestry with a fixed-effects meta-analysis model adjusted for age and sex (Table 7, Supplementary Fig. S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7510/-/DCSupplemental). None of the variants achieved significance in replication ($P < 1 \times 10^{-6}$). The smallest *P* value was for rs35260 ($P = 0.03$). For all SNPs examined in replication, there was a significant amount of heterogeneity ($P < 0.05$ for Q test). We performed a sensitivity analysis by removing the FinnDiane and Go-DARTS cohorts—the former because it had type 1 diabetes participants exclusively and both because they did not use ETDRS grading consistently for phenotyping (Supplementary Table S6, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7510/-/DCSupplemental). However, none of the associations were statistically significant in this analysis either, and significant heterogeneity remained for all SNPs with the exceptions of rs3917779 (Q test P = 0.06) and rs6856425 for the ETDRS grade ≥14 analysis (Q test P = 0.26). Given the significant residual heterogeneity, we then used a random effects model but found no significant difference in the results. We also performed meta-analyses without the CARe cohorts; there was no statistically significant result or any significant heterogeneity in these analyses. Of note, meta-analyses of the CARe cohorts alone also showed no significant heterogeneity.

In addition to the above replication efforts in European cohorts, we pursued replication of the same findings in two Asian cohorts, SiMES and SP2. None of the SNPs was statistically significant in these populations. We also investigated the *FTO* association in Go-DARTS; neither rs9926180 nor rs12935710 was significantly associated ($P = 0.84$ and 0.23, respectively).

Table 5. Top Association Results in European-American Samples with Cases Defined as ETDRS Score ≥14 for Variants in Genes on the IBC Chip

					ARIC				CHS				MESA				CMH Combined Analysis						*P* correction	
					MAF				MAF				MAF				MAF							
SNP	Chr	Gene	Position	Minor Allele	Cases	Controls	OR	*P*	Cases	Controls	OR	*P*	Cases	Controls	OR	*P*	Cases	Controls	OR	L95	U95	*P*	Bonferroni	Permutation
rs9332570	1	*F5*	167794699	G	0.09	0.18	0.44	8.8×10^{-5}	0.08	0.19	0.35	0.02	0.11	0.21	0.46	0.05	0.09	0.19	0.43	0.31	0.61	1.1×10^{-6}	4.3×10^{-5}	0.0001
rs7545236	1	*F5*	167796694	C	0.09	0.18	0.44	9.4×10^{-5}	0.08	0.19	0.35	0.02	0.11	0.21	0.46	0.05	0.09	0.19	0.44	0.31	0.61	1.2×10^{-6}	4.6×10^{-5}	0.0001
rs2298908	1	*F5*	167805168	T	0.09	0.18	0.45	0.0001	0.08	0.19	0.35	0.02	0.11	0.21	0.46	0.05	0.09	0.19	0.44	0.31	0.61	1.3×10^{-6}	4.9×10^{-5}	0.0001
rs1894701	1	*F5*	167797210	C	0.09	0.18	0.44	8.8×10^{-5}	0.08	0.19	0.36	0.03	0.11	0.21	0.46	0.05	0.09	0.19	0.44	0.31	0.61	1.3×10^{-6}	5.0×10^{-5}	0.0001
rs6427203	1	*F5*	167795655	C	0.09	0.18	0.45	0.0001	0.08	0.19	0.36	0.03	0.11	0.21	0.46	0.05	0.09	0.18	0.44	0.32	0.62	1.8×10^{-6}	7.1×10^{-5}	0.0001
rs35260	5	*PDE4D*	58668304	A	0.38	0.47	0.68	0.0003	0.36	0.48	0.61	0.07	0.24	0.50	0.31	7.4×10^{-5}	0.35	0.48	0.60	0.48	0.74	2.0×10^{-6}	7.6×10^{-5}	0.0001
rs6128	1	*SELP*	167829528	T	0.08	0.16	0.48	0.0007	0.06	0.18	0.30	0.02	0.08	0.20	0.36	0.02	0.08	0.17	0.43	0.31	0.62	3.1×10^{-6}	0.0001	0.0001
rs7168655	15	*PCSK6*	99782444	A	0.43	0.32	1.60	0.0002	0.47	0.33	1.76	0.04	0.43	0.33	1.50	0.13	0.44	0.33	1.61	1.31	1.98	7.6×10^{-6}	0.0003	0.0001
rs35259	5	*PDE4D*	58669348	T	0.39	0.47	0.70	0.005	0.36	0.48	0.61	0.07	0.26	0.50	0.36	0.0004	0.36	0.48	0.62	0.50	0.77	8.6×10^{-6}	0.0003	0.0001
rs6133	1	*SELP*	167831970	A	0.05	0.12	0.39	0.0003	0.03	0.13	0.22	0.02	0.07	0.13	0.50	0.16	0.05	0.12	0.38	0.25	0.59	1.1×10^{-5}	0.0004	0.0001
rs9332579	1	*F5*	167791936	T	0.06	0.14	0.37	8.9×10^{-5}	0.05	0.13	0.32	0.05	0.10	0.14	0.66	0.34	0.06	0.14	0.41	0.28	0.61	1.3×10^{-5}	0.0005	0.0001
rs3917779	1	*SELP*	167837472	A	0.05	0.12	0.40	0.0004	0.03	0.12	0.23	0.03	0.07	0.13	0.50	0.16	0.05	0.12	0.39	0.26	0.60	1.6×10^{-5}	0.0006	0.0002
rs6856425	4	*Near IDUA*	966918	C	0.04	0.02	2.36	0.01	0.11	0.02	6.17	0.0004	0.06	0.03	2.28	0.19	0.05	0.02	2.88	1.75	4.73	2.9×10^{-5}	0.001	0.0002
rs1824159	5	*PDE4D*	58654401	T	0.15	0.09	1.87	0.0006	0.11	0.09	1.15	0.76	0.19	0.08	2.81	0.004	0.15	0.09	1.88	1.39	2.53	3.5×10^{-5}	0.001	0.0001
rs1006273	15	*PCSK6*	99782862	A	0.52	0.43	1.47	0.002	0.59	0.43	1.93	0.02	0.51	0.42	1.48	0.14	0.53	0.43	1.53	1.24	1.88	5.6×10^{-5}	0.002	0.0001
rs16879334	5	*MTRR*	7944506	G	NA	NA	NA	NA	0.11	0.03	3.33	0.01	0.10	0.02	5.88	0.001	0.10	0.03	4.11	2.05	8.21	6.4×10^{-5}	0.002	0.0003
rs10036406	5	*PDE4D*	58588070	C	0.16	0.10	1.82	0.0007	0.12	0.10	1.29	0.55	0.18	0.09	2.14	0.04	0.16	0.10	1.78	1.34	2.38	9.1×10^{-5}	0.004	0.0001
rs7105871	11	*CADM1*	114717935	C	0.14	0.26	0.46	6.6×10^{-6}	0.24	0.25	0.98	0.94	0.25	0.28	0.87	0.65	0.17	0.26	0.59	0.46	0.77	9.4×10^{-5}	0.004	0.0002

The minor allele is the effect allele for the ORs. Chr, chromosome; L95, lower 95% CI boundary; U95, upper 95% CI boundary; NA, not available.

Table 6. Top Association Results in European-American Samples with Cases Defined as ETDRS Grade ≥30 for Variants within All Genes on the IBC Chip

					ARIC				CHS				MESA				CMH Combined Analysis						*P* Correction	
					MAF				MAF				MAF				MAF							
SNP	Chr	Gene	Position	Minor Allele	Cases	Controls	OR	*P*	Cases	Controls	OR	*P*	Cases	Controls	OR	*P*	Cases	Controls	OR	L95	U95	*P*	Bonferroni	Permutation
rs6856425	4	Near *IDUA*	966918	C	0.05	0.02	3.01	0.002	0.15	0.02	8.50	1.8×10^{-5}	0.09	0.03	3.54	0.10	0.07	0.02	3.83	2.26	6.46	5.3×10^{-7}	2.1×10^{-5}	0.0001
rs1555179	6	*MTHFDIL*	151313049	A	0.19	0.35	0.45	3.0×10^{-5}	NA	NA	NA	NA	0.18	0.32	0.47	0.17	0.19	0.34	0.45	0.32	0.65	1.3×10^{-5}	0.0005	0.0002
rs7078169	10	*CUBN*	17202320	T	0.09	0.03	2.86	0.0002	0.10	0.03	3.71	0.02	0.05	0.05	0.93	0.94	0.09	0.04	2.66	1.65	4.27	5.3×10^{-5}	0.002	0.0001
rs3901540	5	*PDE4D*	58465490	T	0.15	0.28	0.44	0.0001	0.18	0.29	0.52	0.13	0.32	0.32	0.98	0.96	0.17	0.29	0.50	0.35	0.70	5.6×10^{-5}	0.002	0.0002
rs6663966	1	*GLISI*	53842601	C	0.04	0.01	5.16	5.2×10^{-5}	0.00	0.01	NA	NA	0.09	0.02	6.46	0.01	0.04	0.01	4.15	2.07	8.34	6.2×10^{-5}	0.002	0.0009
rs4697843	4	*HS3ST1*	10704856	A	0.21	0.33	0.52	0.0005	0.20	0.33	0.51	0.10	0.18	0.31	0.50	0.21	0.20	0.33	0.52	0.38	0.72	6.3×10^{-5}	0.002	0.0001
rs4521758	8	*PRKDC*	48904123	T	0.17	0.11	1.59	0.03	0.30	0.11	3.47	0.0007	0.23	0.09	2.92	0.04	0.20	0.11	1.98	1.41	2.77	7.3×10^{-5}	0.003	0.0006
rs10752067	10	*CUBN*	17188898	C	0.09	0.04	2.76	0.0003	0.10	0.03	3.73	0.02	0.05	0.05	0.93	0.94	0.09	0.04	2.59	1.62	4.16	7.9×10^{-5}	0.003	0.0001
rs3917462	1	Near *SELE*	167958291	C	0.03	0.01	4.03	0.006	0.05	0.01	9.05	0.009	0.05	0.01	5.16	0.12	0.03	0.01	4.75	2.17	10.36	9.3×10^{-5}	0.004	0.001
rs2508450	11	*IL10RA*	117369039	T	0.34	0.44	0.65	0.008	0.28	0.49	0.40	0.01	0.23	0.44	0.38	0.06	0.32	0.45	0.58	0.44	0.76	0.0001	0.004	0.0001
rs2195611	2	*PDE1A*	182964812	C	0.59	0.44	1.83	0.0001	0.58	0.45	1.69	0.12	0.45	0.48	0.91	0.83	0.58	0.45	1.68	1.29	2.19	0.0001	0.005	0.0001
rs11163323	1	Near *PRKACB*	81759046	T	0.33	0.19	2.05	1.7×10^{-5}	0.23	0.23	0.98	0.96	0.23	0.19	1.24	0.69	0.30	0.20	1.76	1.32	2.35	0.0001	0.005	0.0002
rs11155762	6	*MTHFDIL*	151314489	T	0.19	0.35	0.45	3.0×10^{-5}	0.38	0.34	1.17	0.64	0.18	0.32	0.47	0.17	0.22	0.34	0.55	0.40	0.75	0.0001	0.006	0.0005
rs17186372	1	*NOTCH2*	120302474	G	NA	NA	NA	NA	0.25	0.07	4.28	0.0002	0.14	0.07	2.08	0.26	0.21	0.07	3.45	1.81	6.56	0.0002	0.006	0.001
rs6583305	3	*UBXN7*	197603018	T	0.47	0.36	1.63	0.002	0.55	0.34	2.39	0.008	0.36	0.36	1.00	0.99	0.48	0.35	1.66	1.27	2.16	0.0002	0.007	0.0003
rs6764533	3	*UBXN7*	197572861	A	0.47	0.36	1.63	0.002	0.55	0.34	2.36	0.009	0.36	0.36	1.00	0.99	0.48	0.35	1.65	1.27	2.15	0.0002	0.007	0.0004
rs11545078	8	*GGH*	64101318	A	0.02	0.10	0.15	0.0003	0.08	0.11	0.64	0.47	0.05	0.11	0.37	0.33	0.03	0.10	0.26	0.13	0.53	0.0002	0.008	0.0002

The minor allele is the effect allele for the ORs; Chr, chromosome; L95, lower 95% CI boundary, U95, upper 95% CI boundary, NA, not available.

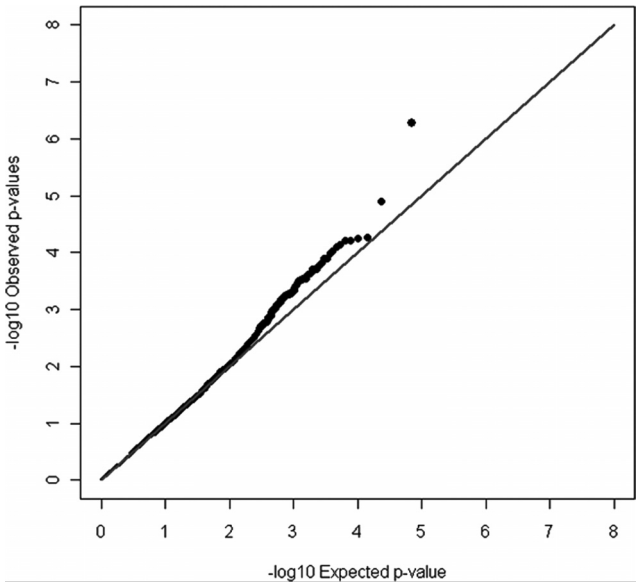

FIGURE 1. Quantile–quantile plot of all single SNPs examined on the IBC chip and their CMH association analysis to diabetic retinopathy, defined as an ETDRS score ≥30.

DISCUSSION

In this large international collaborative study, genes previously linked with T2D, DR, and DN and vascular diseases were not generally associated with DR. In the CARe European American population, among genes that have been previously associated with DR, DN, and T2D, three SNPs in *SELP* were associated with DR, even after adjustment for DR risk factors. However, we were unable to replicate this finding in other ethnic groups in CARe or in independent Caucasian cohorts. The *SELP* SNPs associated with DR in the present study were not in LD with rs6131, the SNP initially associated with diabetic albuminuria (Supplementary Fig. S2, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7510/-/DCSupplemental).[64] P-selectin plays a role in leukocyte adhesion to endothelium during inflammation, and thus there is a biological rationale for its role in both diabetic microalbuminuria and retinopathy.[65] With regards to *FTO*, the SNPs associated with DR in CARe were not in significant LD with rs9939609, the SNP associated with T2D (Supplementary Fig. S2, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7510/-/DCSupplemental).[66]

Importantly, we were unable to confirm an association to most genes that have previously been associated with DR and were included on the IBC chip. We note that for several of these genes, the IBC chip did not include SNPs in LD to the previously associated variants because the selection of tag SNPs may not have densely covered those genes (Supplementary Table S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7510/-/DCSupplemental). For some genes, most notably *EPO*, we did have excellent proxies to the initially reported variants. The chip included two perfect proxies (rs551238 and rs1734907) for the *EPO* SNP originally associated to PDR, rs1617640.[25] When we defined cases as having an ETDRS grade ≥30, rs551238 had a significant effect consistent with that found in the previous study, where the minor allele is protective (OR = 0.69, $P = 0.009$); the P value remains significant after correction by permutation ($P = 0.01$) but not by the Bonferroni method ($P = 0.37$). It is possible that with a larger sample size, the association would withstand the Bonferroni correction. We were also unable to detect an association to DR in other genes previously associated with DN and T2D. For the

TABLE 7. Replication Results in European Samples

			ARIC		CHS		MESA		AGES		BMES		FIND-Eye		FinnDiane		Go-DARTS		Lublin		Meta-analysis (Fixed Effects)		
Controls, *n*			732		160		140		249		175		627		570		774		620				
Cases ETDRS ≥14, *n*			153		33		36		92		67		105		2009		923		576				
Cases ETDRS ≥30, *n*			91		20		11		37		26		NA		1399		923		138				
SNP	Minor Allele	Definition of Cases	OR	*P*	OR	*P*	OR	*P*	MAF	OR	*P*	MAF	OR	*P*	MAF	OR	*P*	MAF	OR	*P*	MAF	OR	*P*	*z* Score	OR	*P*
rs9332570	G	ETDRS ≥14	0.43	0.0001	0.33	0.02	0.49	0.06	NA	0.75	0.26	0.21	1.24	0.42	0.19	0.79	0.26	0.27	1.16	0.05	NA	NA	NA	NA	0.002	1	0.99
rs35260	A	ETDRS ≥14	0.68	0.002	0.59	0.08	0.29	0.0001	NA	1.01	0.98	0.49	1.23	0.32	0.48	0.9	0.45	0.5	0.96	0.59	0.44	0.98	0.77	NA	NA	NA	−2.24	0.91	0.03
rs6128	T	ETDRS ≥14	0.48	0.0007	0.27	0.02	0.39	0.03	0.09	1.27	0.32	0.21	1.27	0.37	0.17	0.73	0.14	0.24	1.16	0.07	NA	1.13	0.21	0.16	1.01	0.96	0.6	1.03	0.55
rs7168655	A	ETDRS ≥14	1.59	0.0004	1.78	0.04	1.48	0.15	NA	0.91	0.57	0.34	0.96	0.83	0.36	1.08	0.63	0.34	1.02	0.82	0.34	0.96	0.6	NA	NA	NA	1.55	1.07	0.12
rs6133	A	ETDRS ≥14	0.39	0.0005	0.19	0.03	0.51	0.16	0.15	0.73	0.32	0.14	1.37	0.28	0.13	0.72	0.17	0.09	1.13	0.31	NA	1.08	0.45	0.08	1.03	0.84	−0.42	0.98	0.68
rs3917779	A	ETDRS ≥14	0.40	0.0007	0.20	0.03	0.51	0.16	0.09	0.73	0.32	0.14	1.25	0.45	0.12	0.72	0.19	0.09	1.14	0.29	NA	NA	NA	NA	NA	NA	−1.39	0.88	0.17
rs6856425	C	ETDRS ≥14	2.48	0.01	5.21	0.004	2.63	0.16	0.04	0.89	0.86	0.02	1.8	0.45	0.02	1.18	0.75	0.03	0.97	0.87	0.02	1.34	0.27	0.05	0.7	0.07	1.1	1.13	0.27
rs7105871	C	ETDRS ≥14	0.47	1.7×10^{-5}	0.97	0.91	0.86	0.62	NA	0.81	0.2	0.25	1.16	0.54	0.22	0.88	0.47	0.31	1	0.96	NA	NA	NA	NA	NA	NA	−1.85	0.9	0.07
rs6856425	C	ETDRS ≥30	3.19	0.003	6.8	0.002	3.25	0.19	NA	NA	NA	NA	NA	NA	0.03	0.79	0.39	0.02	1.34	0.27	0.05	0.46	0.06	1.65	1.28	0.1

All results are adjusted for age and sex. NA, not available.

DN genes, again the IBC chip may not have included SNPs in LD with the previously reported variants. For T2D, however, the IBC chip variants were specifically those previously associated at genome-wide significant levels.

Another explanation for the inability to replicate previous DR associations lies in the heterogeneity among studies regarding DR definitions and participants' mean duration of diabetes. We attempted to mitigate the heterogeneity of DR definitions by examining two different definitions. However, this may not be sufficient to account for all the possible phenotype heterogeneity. The studies from which we selected genes deemed to be previously associated with DR all used controls that were diabetic patients without DR, as we did; however, some of them were performed in type 1 diabetic patients, which is another potential source of heterogeneity. There was also great variability in the duration of T2D among cases and controls in CARe cohorts. In particular, there were participants with short durations of diabetes who were included. There is the potential for misclassification of controls if these participants did not have DR at the time of study inclusion but are at risk for significant DR with longer duration of diabetes. We attempted to correct for this by including duration of disease as a covariate in logistic regression, but these issues could still bias the results toward the null. However, our ability to detect an association with *EPO* indicates that the amount of control misclassification in CARe is not significant enough to prevent detection of associations of this effect size.[25,67] Finally, while the current investigation is the largest candidate gene study for DR to date, it still has limited power to detect genetic associations of modest or small effects (Supplementary Table S7, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7510/-/DCSupplemental). In particular, CARe includes a modest number of European-American cases (122) defined as ETDRS grade ≥30. Examining milder degrees of DR as outcomes may have further decreased our ability to detect associations as the development of early DR has a lower heritability. Of note, the ARIC cohort is larger than the other cohorts, and the top findings in the analyses were often driven by the results in ARIC.

In the second phase of the analysis, we took an unbiased approach at the remaining genes available on the IBC chip. Although we found several strong associations in our discovery cohort, replication in independent samples did not yield variants with consistent effects nor any variants that met the replication significance threshold ($P = 1 \times 10^{-6}$). The rs6856425 association was initially compelling because it was consistent within each CARe cohort. Furthermore, the strength of the association was greater when DR was defined as ETDRS grade ≥30 vs. ETDRS grade ≥14, which is in line with the expected greater heritability of more advanced DR phenotypes. The rs35260 variant had the lowest *P* value in the replication meta-analyses ($P = 0.03$), but this was still far below the replication threshold for significance ($P = 1 \times 10^{-6}$).

Failure to replicate a genetic association can be explained broadly, either as a false positive in the discovery cohort or a false negative in the replication cohort. For rs6856425, a rare variant, the initial estimate was based on limited instances of the minor allele: 9 in cases and 20 in controls. Small numbers of observations can lead to unstable effect estimates that represent chance statistical fluctuations rather than true associations. This underscores the importance of large sample sizes in both discovery and replication cohorts, particularly for rare variants. Another possible reason for false positives is population stratification, but there was no significant population stratification in this study.

False negatives in the replication cohort can be due to a lack of power, genotyping/imputation imprecision or heterogeneity between cohorts. Our aggregate replication cohort sample was well powered (Supplementary Table S7, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7510/-/DCSupplemental). Of note, imputation was used by most replication cohorts for at least one of the SNPs. Imputation quality scores were greater than 0.90 for all SNPs with the exception of rs6856425 in FIND-Eye where the imputation quality score was 0.67. Errors in imputed genotype calls could lead to false-negative results, particularly for rare SNPs, which are more susceptible to genotyping artifacts. In addition, there was significant heterogeneity among samples that could not be explained by excluding type 1 diabetes participants and cohorts that did not use ETDRS grading consistently. Because the heterogeneity was not present when meta-analysis was restricted to the replication cohorts alone or the CARe cohorts alone, the "winner's curse" effect of large effect sizes in the discovery cohorts likely explains most of this heterogeneity.[68] Some heterogeneity might also derive from the different DR ascertainment methods and case–control definitions. Cohorts differed in their photography protocols, with some cohorts having one field of only one eye for phenotype determination. This introduces misclassification bias for participants for whom the DR grade in the one or two fields photographed may not accurately represent the DR grade in other fields or the contralateral eye. It is therefore possible that some of the variants associated with DR in CARe may eventually be replicated in larger studies with direct genotyping and better phenotype harmonization.

In summary, in this candidate gene analysis of DR with data from the CARe consortium, with replications in a several large, well-powered samples, we found little evidence of a major DR gene. This is the largest number of candidate genes studied for DR to date. Although no association could be confirmed with a high threshold for significance, the results are hypothesis generating and the genes associated with DR in CARe could be prioritized in studies. The importance of well-powered replication and phenotype harmonization are highlighted by our study. These issues will continue to be important as results from genome-wide association studies for DR become increasingly available.

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