# Original Article

# Multiple Variants in Vascular Endothelial Growth Factor (VEGFA) Are Risk Factors for Time to Severe Retinopathy in Type 1 Diabetes

## The DCCT/EDIC Genetics Study

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**OBJECTIVE**—We sought to determine if any common variants in the gene for vascular endothelial growth factor (VEGFA) are associated with long-term renal and retinal complications in type 1 diabetes.

**RESEARCH DESIGN AND METHODS**—A total of 1,369 Caucasian subjects with type 1 diabetes from the Diabetes Control and Complications Trial (DCCT)/Epidemiology of Diabetes Interventions and Complications (EDIC) Study had an average of 17 retinal photographs and 10 renal measures over 15 years. In the DCCT/EDIC, we studied 18 single nucleotide polymorphisms (SNPs) in VEGFA that represent all linkage disequilibrium bins (pairwise $r^2 \geq 0.64$) and tested them for association with time to development of severe retinopathy, three or more step progression of retinopathy, clinically significant macular edema, persistent microalbuminuria, and severe nephropathy.

**RESULTS**—In a global multi-SNP test, there was a highly significant association of VEGFA SNPs with severe retinopathy ($P = 6.8 \times 10^{-5}$)—the four other outcomes were all nonsignificant. In survival analyses controlling for covariate risk factors, eight SNPs showed significant association with severe retinopathy ($P < 0.05$). The most significant single SNP association was rs3025021 (hazard ratio 1.37 [95% CI 1.13–1.66], $P = 0.0017$). Family-based analyses of severe retinopathy provide evidence of excess transmission of C at rs699947 ($P = 0.029$), T at rs3025021 ($P = 0.013$), and the C-T haplotype from both SNPs ($P = 0.035$). Multi-SNP regression analysis including 15 SNPs, and allowing for pairwise interactions, independently selected 6 significant SNPs ($P < 0.05$).

**CONCLUSIONS**—These data demonstrate that multiple VEGFA variants are associated with the development of severe retinopathy in type 1 diabetes. *Diabetes* **56:2161–2168, 2007**

Diabetic retinopathy affects the majority of patients with >15 years of diabetes (1,2). Risk factors for diabetic retinopathy include poor glycemic control (as measured by A1C), longer diabetes duration, earlier age at diagnosis, higher waist-to-hip ratio, fasting triglyceride, BMI, blood pressure, and serum/plasma fibrinogen (3–5). The Diabetes Control and Complications Trial (DCCT) demonstrated familial clustering of severe retinopathy, but not for any retinopathy (6), which is consistent with others (7,8). The essential features of diabetic retinopathy include capillary microaneurysms and increased vascular permeability leading to macular edema, vascular occlusion and ischemia, neovascularization, and contraction of fibrovascular proliferation in the vitreous.

Vascular endothelial growth factor (VEGFA) is a mitogen that specifically acts on endothelial cells (9) and has various effects, including mediating increased vascular permeability (10) and inducing angiogenesis (11–13), cell growth, migration, and inhibition of apoptosis (14). Many retinal cells produce VEGFA, including pigment epithelial (15), capillary pericytes (15), endothelial (16), Mueller (17), ganglion (18), and glial (19) cells. VEGFA expression in retinal cells is increased 3- to 30-fold by hypoxia (16). VEGFA is significantly upregulated in diabetic retinopathy, particularly in retinal pigmented epithelial cells, glial cells, and vitreal fibroblasts (20). Moreover, higher plasma (21) and vitreous VEGFA (21–23) levels were found in individuals with proliferative diabetic retinopathy (PDR) when compared with nondiabetic control subjects (21) or with

TABLE 1
Distribution of the retinal and renal outcomes and results of the likelihood ratio test (LRT) based on multivariate Cox proportional hazards global test, including all 16 VEGFA SNPs

| | Number of events (%) | Sample size | LRT (16 d.f.) | $P$ |
|---|---|---|---|---|
| Retinal outcomes* | | | | |
| Progression to three or more steps | 724 (61) | 1,181 | 12.43 | 0.71 |
| Severe retinopathy | 220 (19) | 1,180 | 46.98 | $6.8 \times 10^{-5}$ |
| Clinical significant macular edema | 226 (19) | 1,181 | 23.34 | 0.105 |
| Renal outcomes† | | | | |
| Persistent microalbuminuria | 219 (20) | 1,123 | 14.76 | 0.54 |
| Severe nephropathy | 98 (8) | 1,181 | 18.21 | 0.31 |

*Retinal outcomes are from data up to EDIC year 10. †Renal outcomes include data up to EDIC year 8.

individuals with nonocular diseases (22). Phase II clinical trials have shown safety and efficacy of intraocular injections of pegaptanib, an anti-VEGFA aptamer in the treatment of diabetic macular edema (24). For these reasons, VEGFA is a functional candidate gene for the predisposition to diabetic retinopathy.

Several cross-sectional studies have investigated the genetic involvement of VEGFA in diabetic retinopathy (25–29). Three studies tested rs833061 (−460) for association with diabetic retinopathy in patients with type 2 diabetes (25–27): one observed an association with the C-allele and the C/C genotype (25), another found association with the C/T genotype (26), whereas the third one found no association (27). Two studies investigated the association of an 18-bp deletion in the promoter with diabetic retinopathy (28,29) in type 1 (29) and type 2 (28) diabetes but produced conflicting results. However, all of these studies (25–29) used a case-control design with various phenotype definitions and investigated only markers in the promoter region in relatively small sample sizes of Japanese type 2 diabetic patients (27) or Caucasians with a mix of type 1 and type 2 diabetes (29). Covariates, if available, were measured at a single time point when the phenotype was also determined.

The current study utilizes longitudinal data for retinal and renal complications in the Caucasian type 1 diabetic population from the DCCT/Epidemiology of Diabetes Interventions and Complications (EDIC) Study to examine the role of VEGFA polymorphisms (30).

## RESEARCH DESIGN AND METHODS

To reduce population stratification, a potential source of bias in genetic association studies, analyses were restricted to 1,369 Caucasian DCCT/EDIC subjects from whom DNA was collected (see online appendix supplementary text for details [available at http://dx.doi.org/10.2337/db07-0376]). The online appendix also provides the distribution of baseline characteristics at DCCT entry (online appendix Table 1) and clinical measures of available parents and siblings (online appendix Table 2).

**Phenotypic characterization of retinal and renal outcomes.** Based on repeated measurements of retinal and renal function (see online appendix text), we defined three retinal (three or more step progression, severe retinopathy, and clinically significant macular edema [CSME]) and two renal (persistent microalbuminuria and severe nephropathy) phenotypes. For each phenotype, a time-to-event outcome was derived as years from DCCT baseline until the event occurred or censoring. The number of events observed for all retinal and renal phenotypes is provided in Table 1.

Follow-up during DCCT/EDIC averaged 15.1 ± 2.8 (mean ± SD) years, during which time an average of 16.7 ± 3.7 retinal photographs were taken for each proband. Photos were scored using the Early Treatment Diabetic Retinopathy Study (ETDRS) system (31). The three or more step phenotype was defined as progression from DCCT baseline to three or more ETDRS steps. Criteria for severe retinopathy included an ETDRS level 53/<53 or greater or scatter laser treatment. The presence of CSME was defined according to ETDRS criteria (30). In addition, patients who underwent focal photocoagulation for macular edema were counted as having CSME thereafter.

Renal measurements of urinary albumin excretion rate (AER) were obtained yearly in the DCCT and biennially in the EDIC (see online appendix). The presence of persistent microalbuminuria was defined as AER >20.8 μg/min on two consecutive assessments. Severe nephropathy was assigned to subjects with persistent microalbuminuria who progressed to an AER >208 μg/min or renal replacement therapy (dialysis or transplant).

**Genotyping methods and quality control.** In total, we selected 18 SNPs for genotyping across the VEGFA gene (online appendix Table 3). Fifteen of these represent tagSNPs, selected from the SeattleSNP Project using LDSelect (http://pga.gs.washington.edu/VG2.html) using genotype data for 23 European American individuals and applying an $r^2 \geq 0.64$ cutoff (32). rs2010963, previously named −634 (27) or +405 (33), was selected from the promoter region. Two SNPs (rs699947 [−2578] [34] and rs1547651) that lie in the 5′ region were selected from the CEU data of phase I of the International HapMap project (35), since this region was not sequenced by the SeattleSNP project.

Eleven SNPs were genotyped using TaqMan assays and eight using the Illumina Goldengate bead array platform (36). Details regarding genotyping procedures, including sequences of primers and probes used in TaqMan (online appendix Table 4) and detailed quality control measurements (online appendix Table 3) are described in the online appendix. rs3025028 was genotyped on both Illumina and TaqMan platforms with a 1% genotype disagreement rate. Only genotypes that were identical between the two platforms were used in the analysis of this SNP. For the Illumina platform, the highest error rate based on duplicate plate was 4.4% for rs1547651 (online appendix Table 3). The lowest mean Gencall score, a measure of genotyping quality (38), was 0.7 for rs3025007 (online appendix Table 3). Among duplicate samples genotyped on TaqMan, the highest error rate was 12%, observed for rs833069. rs1413711 showed significant deviation from Hardy-Weinberg equilibrium ($P < 0.0001$) due to the absence of rare homozygous genotypes, likely a result of technical difficulties. Therefore, rs833069 and rs1413711 were replaced by rs833068 and rs833070, respectively, since they are in the same $r^2$ bin. All analyses with outcomes examined the 16 SNPs (Table 2) with good quality control results. In the family data, there were zero, one, two, and six Mendelian errors at rs1547651, rs699947, rs3025010, and rs3025021, respectively. Families with Mendelian errors were excluded from the family-based analysis. Linkage disequilibrium (LD) was calculated using Haploview version 3.2 (39) in 1,180 subjects with complete genotype data at all SNPs.

**Statistical analysis for genetic association.** Primary analyses were designed to evaluate the association of VEGFA SNPs with the time-to-event outcome for each retinal and renal phenotype (see online appendix text). To explain variability in diabetes complications, multivariate Cox proportional hazards models were developed to account for the DCCT/EDIC design (cohort and treatment, online appendix Table 5), as well as known risk factors and other potential confounding factors (Table 3 and online appendix Table 6). For reasons of validity, we elected to use the start of DCCT rather than diabetes diagnosis as the time-to-event baseline, including diabetes duration, along with DCCT baseline measures as explanatory covariates. DCCT participants met eligibility criteria based on disease duration and clinical progression, so they are not representative of an unselected population of newly diagnosed individuals with diabetes, precluding straightforward natural-history modeling. Diabetes duration before entry into DCCT and A1C at eligibility screening for DCCT were included in the multivariate Cox proportional hazards model to explain variation due to differences in duration and to capture prior glycemic exposure. Time-dependent covariates were included for updated A1C and hypertension to account for DCCT treatment effects. For parsimony, SNPs were coded additively, representing the number of copies of the minor allele. Although this strategy is expected to be robust to nonadditivity, we also examined models with both linear and quadratic genotype terms to assess this assumption

TABLE 2
Single-marker univariate and multivariate Cox proportional hazards analysis of VEGFA SNPs with severe retinopathy

| Marker | Minor allele (%) | Strand | Sample size | Univariate | | | Multivariate | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | HR | 95% CI | $P$ | HR | 95% CI | $P$ |
| rs1547651 | T (16.4) | Forward | 1,353 | 1.12 | 0.87–1.43 | 0.38 | 1.02 | 0.78–1.32 | 0.89 |
| rs699947 | A (49.5) | Forward | 1,344 | 0.90 | 0.75–1.07 | 0.23 | 0.83 | 0.69–1.00 | 0.045 |
| rs2010963 | G (31.8) | Reverse | 1,313 | 1.13 | 0.93–1.37 | 0.22 | 1.21 | 0.98–1.50 | 0.078 |
| rs3024987 | A (12.6) | Reverse | 1,362 | 1.04 | 0.81–1.33 | 0.78 | 1.05 | 0.81–1.36 | 0.72 |
| rs833068 | A (32.4) | Forward | 1,331 | 1.10 | 0.90–1.33 | 0.36 | 1.20 | 0.97–1.48 | 0.093 |
| rs833070 | A (49.7) | Forward | 1,329 | 0.88 | 0.74–1.05 | 0.16 | 0.82 | 0.68–1.00 | 0.045 |
| rs3024994 | A (4.9) | Reverse | 1,362 | 1.12 | 0.75–1.66 | 0.58 | 1.22 | 0.80–1.85 | 0.36 |
| rs2146323 | A (35.7) | Forward | 1,362 | 0.89 | 0.74–1.08 | 0.23 | 0.77 | 0.63–0.94 | 0.011 |
| rs3025007 | A (44.0) | Reverse | 1,358 | 0.80 | 0.67–0.96 | 0.017 | 0.80 | 0.66–0.96 | 0.017 |
| rs3025010 | C (37.8) | Forward | 1,340 | 0.87 | 0.72–1.05 | 0.13 | 0.75 | 0.62–0.92 | 0.0052 |
| rs3025020 | T (32.0) | Reverse | 1,361 | 0.82 | 0.67–0.99 | 0.039 | 0.75 | 0.61–0.92 | 0.0051 |
| rs3025021 | T (32.3) | Forward | 1,341 | 1.19 | 0.98–1.43 | 0.073 | 1.37 | 1.13–1.66 | 0.0017 |
| rs3025028 | G (43.4) | Reverse | 1,366 | 1.10 | 0.92–1.32 | 0.29 | 1.24 | 1.03–1.49 | 0.026 |
| rs3025030 | G (13.3) | Reverse | 1,307 | 1.06 | 0.81–1.40 | 0.66 | 1.06 | 0.80–1.42 | 0.67 |
| rs3025035 | A (7.2) | Reverse | 1,362 | 1.36 | 1.01–1.85 | 0.044 | 1.23 | 0.88–1.71 | 0.22 |
| rs3025053 | A (11.0) | Forward | 1,362 | 1.05 | 0.79–1.39 | 0.76 | 1.06 | 0.79–1.44 | 0.69 |

The results are shown for the available genotype dataset for each marker using an additive mode, with the major allele frequency (i.e., common homozygote) used as the reference. Results of the same analysis for the common dataset (1,180 individuals) are provided in online appendix Tables 9 and 12. The covariates included are listed in the online appendix supplementary text (and Table 3 for the multivariate model).

To adjust for multiple testing and select phenotypes for further analysis, we first performed a global test including all 16 VEGFA SNPs in the multivariate Cox proportional hazards model to test association between time-to-event and overall genetic variation at the VEGFA locus. The corresponding likelihood ratio test (16 d.f.) was corrected using a Bonferroni factor of 5, and only phenotypes with $P < 0.01$ were examined further. In additional single SNP analyses, the relative risk associated with each SNP was estimated by the hazard ratio (HR) from the Cox proportional hazards model. Single SNPs were examined in both univariate and multivariate Cox proportional hazards models. Multi-SNP selection analyses were also performed to determine the most likely etiological variant(s) and to estimate HRs associated with multiple SNP genotype combinations. These included multiple regression models with forward and backward selection of main effects and two-way interactions of VEGFA SNPs (details in the online appendix).

Martingale residuals, obtained from the multivariate Cox proportional hazards models without genotypes, were used as a continuous trait in subsequent family-based analyses of four SNPs genotyped in first-degree relatives (see online appendix Table 7 for family structure). This approach is conditional on the available parental genotypes to test for departures between the observed offspring genotype and those expected under Mendelian inheritance (38). Family-based analyses were performed assuming an additive genetic model and used the FBAT version 1.7.3 software to perform single SNP, multi-SNP, and haplotype analyses (39–41).

## RESULTS

**Marker-marker LD.** The genomic region encompassing VEGFA contains three distinct sets of SNPs that are in strong LD with each other. (D′ and $r^2$ values are shown in Fig. 1.) The first LD block extends from rs1547651 to rs2146323, the second contains rs3025020 and rs3025021, and the third block contains rs3025028, rs3025035, and rs3025053. The highest pairwise $r^2$ was observed between

TABLE 3
Covariate associations in multivariate Cox proportional hazards model for time to severe retinopathy

| Covariate | HR | 95% CI | $P$ |
|---|---|---|---|
| Secondary intervention vs. primary prevention cohort | 2.34 | 1.42–3.85 | $8.6 \times 10^{-4}$ |
| Intensive vs. conventional treatment | 0.82 | 0.42–1.59 | 0.56 |
| Cohort-by-treatment interaction | 0.93 | 0.45–1.91 | 0.85 |
| Age at diagnosis (years) | | | 0.25* |
|     Contrast between age of diagnosis at 30 vs. 20 years | 1.18 | 0.86–1.63 | 0.30† |
| Type 1 diabetes duration (years) at DCCT baseline | | | $<10^{-4}$* |
|     Contrast between 3.5 and 1.5 years of diabetes duration | 1.26 | 0.98–1.62 | 0.071† |
|     Contrast between 12 and 6 years of diabetes duration | 3.08 | 2.34–4.05 | $<10^{-4}$† |
| Female vs. male sex | 0.73 | 0.54–0.97 | 0.032 |
| BMI: 10% increase (25.7 vs. 23.4 kg/m²)§ | 0.99 | 0.88–1.10 | 0.79 |
| Mean arterial blood pressure: 10% increase (95.1 vs. 85.6 mmHg)§ | 1.00 | 1.00–1.00 | 0.66 |
| Triglyceride: 10% increase (90.0 vs. 81.8 mg/dl)§ | 1.02 | 0.98–1.05 | 0.37 |
| HDL cholesterol: 10% increase (55.4 vs. 50.4 mg/dl)§ | 1.03 | 0.96–1.10 | 0.36 |
| Total cholesterol: 10% increase (194.9 vs. 176.2 mg/dl)§ | 0.99 | 0.91–1.08 | 0.82 |
| A1C at DCCT eligibility: 10% increase (9.94 vs. 9.04%) | 1.15 | 1.12–1.19 | 0.0025 |
| Smoking at DCCT baseline (pack/year) | 0.97 | 0.94–0.99 | 0.0051 |
| Updated mean A1C measured during DCCT/EDIC: 1 unit increase (8.0 vs. 7.0%) | 2.63 | 2.22–3.11 | $<10^{-4}$ |
| Updated hypertension indicator during DCCT/EDIC | 1.75 | 1.26–2.43 | $8.9 \times 10^{-4}$ |

Sample size = 1,367. *P value from 2 d.f. Wald test for joint contribution of linear and quadratic trend. †P value from 1 d.f. Wald test derived from linear contrast of linear and quadratic trend. §Ten percent increase from the mean value at DCCT baseline.

| | rs1547651 | rs699947 | rs2010963 | rs3024987 | rs833068 | rs833070 | rs3024994 | rs2146323 | rs3025007 | rs3025010 | rs3025020 | rs3025021 | rs3025028 | rs3025030 | rs3025035 | rs3025053 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| rs1547651 | | 0.98 | 1.00 | 0.94 | 1.00 | 0.96 | 1.00 | 0.94 | 0.81 | 0.94 | 0.03 | 0.03 | 0.07 | 0.32 | 0.24 | 0.52 |
| rs699947 | 0.44 | | 0.98 | 0.99 | 0.98 | 0.98 | 1.00 | 0.98 | 0.42 | 0.94 | 0.26 | 0.20 | 0.09 | 0.00 | 0.18 | 0.18 |
| rs2010963 | 0.30 | 0.66 | | 1.00 | 0.99 | 0.99 | 1.00 | 0.99 | 0.97 | 0.99 | 0.02 | 0.14 | 0.02 | 0.13 | 0.01 | 0.86 |
| rs3024987 | 0.16 | 0.37 | 0.26 | | 1.00 | 1.00 | 1.00 | 1.00 | 0.94 | 0.94 | 0.69 | 0.18 | 0.04 | 0.28 | 0.09 | 0.59 |
| rs833068 | 0.31 | 0.67 | 0.99 | 0.26 | | 1.00 | 1.00 | 0.99 | 0.97 | 0.98 | 0.02 | 0.14 | 0.03 | 0.11 | 0.01 | 0.87 |
| rs833070 | 0.43 | 0.16 | 0.67 | 0.38 | 0.69 | | 1.00 | 0.99 | 0.43 | 0.95 | 0.25 | 0.20 | 0.08 | 0.00 | 0.16 | 0.17 |
| rs3024994 | 0.10 | 0.16 | 0.16 | 0.09 | 0.16 | 0.22 | | 1.00 | 1.00 | 1.00 | 0.66 | 0.71 | 0.64 | 0.74 | 0.03 | 0.65 |
| rs2146323 | 0.56 | 0.74 | 0.51 | 0.28 | 0.51 | 0.75 | 0.17 | | 0.09 | 0.98 | 0.28 | 0.35 | 0.43 | 0.24 | 0.01 | 0.45 |
| rs3025007 | 0.32 | 0.38 | 0.59 | 0.40 | 0.60 | 0.38 | 0.20 | 0.08 | | 0.12 | 0.02 | 0.13 | 0.03 | 0.04 | 0.39 | 0.14 |
| rs3025010 | 0.54 | 0.75 | 0.53 | 0.28 | 0.53 | 0.76 | 0.18 | 0.93 | 0.10 | | 0.30 | 0.35 | 0.37 | 0.23 | 0.05 | 0.40 |
| rs3025020 | 0.02 | 0.18 | 0.01 | 0.18 | 0.01 | 0.17 | 0.10 | 0.26 | 0.01 | 0.26 | | 0.99 | 0.93 | 0.67 | 0.97 | 0.70 |
| rs3025021 | 0.02 | 0.14 | 0.14 | 0.10 | 0.14 | 0.14 | 0.11 | 0.18 | 0.08 | 0.19 | 0.47 | | 0.97 | 0.96 | 0.94 | 1.00 |
| rs3025028 | 0.03 | 0.08 | 0.01 | 0.02 | 0.01 | 0.07 | 0.17 | 0.28 | 0.02 | 0.26 | 0.56 | 0.77 | | 0.99 | 0.95 | 0.78 |
| rs3025030 | 0.06 | 0.00 | 0.07 | 0.04 | 0.06 | 0.00 | 0.07 | 0.12 | 0.02 | 0.11 | 0.18 | 0.26 | 0.34 | | 1.00 | 1.00 |
| rs3025035 | 0.15 | 0.05 | 0.00 | 0.06 | 0.005. | 0.04 | 0.00 | 0.00 | 0.10 | 0.02 | 0.19 | 0.18 | 0.23 | 0.11 | | 0.52 |
| rs3025053 | 0.80 | 0.06 | 0.21 | 0.08 | 0.21 | 0.06 | 0.42 | 0.12 | 0.06 | 0.11 | 0.17 | 0.24 | 0.31 | 0.14 | 0.06 | |

FIG. 1. LD between VEGFA SNPs genotyped in white DCCT/EDIC probands. D′ values are above the diagonal and $r^2$ below the diagonal. Dark gray boxes indicate >0.85 and light gray boxes 0.7–0.8. The triangles identified by black borders represent LD blocks (D′ > 0.8).

rs2010963 and rs833068 (0.988) in the first LD block (Fig. 1).

**Proportional hazards model with covariates.** A number of significant ($P < 0.05$) associations were detected between DCCT baseline covariates, specifically triglycerides and HDL, with certain VEGFA SNPs (online appendix Table 8). These were considered as possible confounders in subsequent multivariate association analyses. Before inclusion of SNPs, multivariate Cox proportional hazards analysis of time to severe retinopathy revealed the following covariates to be significant at $P < 0.05$: secondary versus primary cohort, sex, prior diabetes duration (joint effect of linear and quadratic terms), A1C at screening for DCCT eligibility, smoking (pack years) at DCCT baseline, updated mean A1C, and updated hypertension (see Table 2 for univariate and multivariate outcomes; for other outcomes, see online appendix Tables 5 and 6).

**Proportional hazards model for severe retinopathy with SNPs.** Only the severe retinopathy phenotype showed significant association in the global test, which included all 16 VEGFA SNPs ($P = 6.8 \times 10^{-5}$) (Table 1). Therefore, we focus further analyses solely on severe retinopathy. (Results of single SNP analyses of the renal phenotypes, three or more steps progression, and CSME are provided in the online appendix Tables 9–12.) Single SNP multivariate Cox proportional hazards analyses detected nominally significant ($P < 0.05$) associations between severe retinopathy and each of rs699947, rs833070, rs2146323, rs3035007, rs3025010, rs3025020, rs3025021, and rs3025028 (Table 3). In contrast, only three SNPs (rs3025007, rs3025020, and rs3025035) achieved this criterion when examined in a single SNP univariate model, suggesting that inclusion of covariates increases the sen-

sitivity of the analysis (online appendix Table 10 and online appendix supplementary material). The greater extremity of HR estimated in the multivariate model can be explained as a consequence of adjustment for imbalances in covariate distributions across genotypes. However, if strict Bonferroni adjustment are applied within VEGFA, then only rs3025021 would achieve significance ($P < 0.0031$). SNP rs3025021 provided the highest HR estimate and the most significant association (HR 1.37 [95% CI 1.13–1.66], $P = 0.0017$). rs2010963, which lies in the promoter region, approached nominal 5% significance ($P = 0.078$). Except for rs699947 ($P = 0.074$), similar results were obtained when analyses were repeated using the subset of 1,180 probands with complete genotype data for all 16 SNPs (online appendix Table 12).

To assess whether a nonadditive association may have been overlooked, we tested a quadratic term for the gene effect in the multivariate Cox proportional hazards model (online appendix Table 13). Evidence for nonadditivity was observed for rs3025030 ($P = 0.019$), likely due to the rare homozygous genotypes, and to a lesser extent for rs833068 and rs3025007 ($P = 0.041$ and 0.042, respectively). Given these marginal results, the additive model was applied to all subsequent analyses.

**Multivariate multi-SNP analysis.** To identify the most likely etiological variant(s) among the SNPs, we performed variable selection ($P < 0.05$) in multivariate Cox proportional hazards regression models for severe retinopathy. Due to high LD ($r^2 = 0.998$) between rs2010963 and rs833068, the latter was excluded, leaving 15 SNPs for backward and forward variable selection analyses. Six SNPs were retained in the final backward model, whereas two were included in the forward selection (online appen-

TABLE 4
Family-based association analyses between VEGFA markers and Martingale residuals from the Cox proportional hazards model of severe retinopathy via FBAT version 1.70.3 software, assuming an additive model of inheritance

| Marker(s) | Allele (frequency) | Number of informative families | $Z$ statistic | $P$ |
|---|---|---|---|---|
| Single-marker analyses | | | | |
| rs1547651 | T (0.182) | 291 | 0.66 | 0.51 |
| rs699947 | C (0.502) | 441 | 2.19 | 0.029 |
| rs3025010 | T (0.614) | 427 | 1.32 | 0.19 |
| rs3025021 | T (0.33) | 548 | 2.49 | 0.013 |
| Multimarker analyses* | | | | |
| 1 | A-C (0.372) | 435 | −2.052 | 0.040 |
| 2 | C-C (0.321) | 421 | 0.067 | 0.94 |
| 3 | C-T (0.184) | 328 | 2.098 | 0.035 |
| 4 | A-T (0.123) | 251 | 0.286 | 0.77 |

*Haplotype analyses of rs699947 and rs3025021. For multimarker analysis, four-marker test of rs1547651, rs699947, rs3025010, and rs3025021: S_MM = 12.30; 4 d.f.; $P$ = 0.015; two-marker test of rs699947 and rs3025021: S_MM = 9.97; 2 d.f.; $P$ = 0.0068.

dix Table 13). Of note, rs3025007 and rs3025021 were retained in both models. Application of a conservative, crude Bonferroni adjustment for 15 tests ($P < 0.0033$) to the final backward selection model suggests that rs699947, rs2146323, rs3024987, rs3025007, and rs3025021 are unlikely to be associated with severe retinopathy by chance alone. Thus the SNPs selected represent the first (rs699947, rs3024987, and rs2146323) and second (rs3025021) LD blocks, as well as the region between these two blocks (rs3025007) (Fig. 1). While global tests for two-way SNP interactions in exploratory Cox proportional hazards analyses yielded consistent evidence for more complex combined SNP effects and/or potential information in haplotype phase (42), conservative Bonferroni-adjusted $P$ values for single interaction terms were somewhat equivocal (online appendix Tables 14 and 15). HRs for genotype combinations estimated in a six-SNP model with three two-way interactions are classified into protective, neutral, and risk categories (online appendix Table 16), corresponding to a broad range of risks for severe retinopathy.

**Family-based analysis.** Family-based analyses were performed for four SNPs that were genotyped in available first-degree relatives (online appendix Table 2). To adjust for covariates, Martingale residuals from the multivariate Cox proportional hazards model for severe retinopathy without genotypes were treated as a quantitative trait. Excess transmission was observed for alleles C of rs699947 ($Z = 2.19$, $P = 0.029$) and T of rs3025021 ($Z = 2.49$, $P = 0.013$) (Table 4), confirming the significant association of these SNPs that we observed in multi-SNP regression analyses. Therefore, we also examined rs699947 and rs3025021 jointly (2 d.f., $P = 0.0068$) and in haplotype analyses: haplotype C-T showed significant excess transmission ($P = 0.035$), whereas A-C was significantly less transmitted ($P = 0.040$). Association with the prevalence of severe retinopathy was also examined in relatives with diabetes who were genotyped at the four SNPs (see above), but Fisher's exact tests detected no significant association (online appendix Table 17).

## DISCUSSION

**Multivariate single and multi-SNP analysis for severe retinopathy.** We examined the association between VEGFA SNPs and time to event for three retinal and two renal outcomes in the DCCT/EDIC cohort. SNPs were selected from HapMap, SeattleSNPs, and the literature

(27) to capture common variation spanning ~22 kb encompassing VEGFA.

Our approach, capturing associations due to the most common variations in VEGFA and taking into account factors known to be associated with the development of complications, is more comprehensive than previous studies of diabetic retinopathy that examined a single or few variations in only the promoter region of VEGFA. Our use of tagSNPs, representative of bins with high LD, facilitates detection of association between polymorphic variation in and around the gene with relative efficiency in genotyping.

We note the importance of including known risk factors in analyses aiming to identify gene effects. Single SNP analyses identified eight SNPs that were significantly associated with severe retinopathy in the multivariate analyses ($P < 0.05$), while only three of these were significant in the univariate analysis, underscoring the importance of inclusion of measured covariates.

As described in the online appendix, a number of associations between DCCT baseline covariates and SNPs were observed (online appendix Table 6), and we considered possible collinearity and/or confounding effects. The association of rs2146323 and rs3025010 with severe retinopathy was sustained when either triglycerides or other lipids were excluded from the multivariate model (data not shown), arguing against mediating effects.

We attempted to reduce false-positive results due to multiple testing by using strict Bonferroni correction criteria both for global tests of all SNPs with the five outcomes as well as for the single and multi-SNP analyses for severe retinopathy. In spite of this conservative correction, which assumes independence among markers (Fig. 1), the strongest association signal observed for rs3025021 nevertheless retains significance at the overall 5% level.

In the backward selection of 15 SNPs, including all covariates, six SNPs remained in the model (Table 3). Both rs3025007 and rs3025021 were selected in backward and forward selection models. The finding that multiple SNPs make independent contributions suggests two possible explanations: that either one or both of these SNPs are in high LD with an as yet unidentified variant or that allelic heterogeneity in VEGFA predisposes to higher risk of developing severe retinopathy. As pointed out by Clayton et al. (42), functional studies are necessary to distinguish the indirect effect of an unobserved locus from the direct effect of two loci on the same chromosome. Due to the low

LD between these two SNPs (D′ = 0.13, $r^2$ = 0.08), it is likely that at least two etiologic variants exist. In this case, different possible mechanisms need to be investigated concerning how these variants are involved in the pathological process of retinopathy, which include effects on VEGFA gene expression, splicing, or other mechanisms.

**Family-based single SNP and haplotype analysis.** This is the first study to perform family-based analysis of VEGFA SNPs with severe retinopathy in type 1 diabetes. Using Martingale residuals from the multivariate Cox proportional hazards models as a quantitative trait, alleles at rs699947 and rs3025021 exhibited significant excess transmission with severe retinopathy. rs3025021 was most strongly associated with severe retinopathy in the individual-level single SNP analysis, while rs699947 approached nominal 5% significance (HR 0.83, $P$ = 0.045). Family-based haplotype analysis of rs699947 and rs3025021 confirmed that the haplotype consisting of their risk alleles (C-T) was transmitted more often to those with high risk of developing severe retinopathy ($P$ = 0.035), whereas the haplotype consisting of protective alleles (A-C) was transmitted less often ($P$ = 0.040). Association of rs3025010 was detected in the individual-level single-marker analysis but not in the family-based analysis. However, this SNP was not retained in the multi-SNP selection model, suggesting that its association in the single SNP analysis is an indirect result of its high LD ($r^2$ = 0.93) with rs2146323.

In summary, both rs699947 and rs3025021 met criteria for significance in individual-based single SNP analysis, were retained in the backward selection of the multi-SNP regression analysis, and showed excess allele and haplotype transmission with the Martingale residuals in family-based analysis. The consistency of these findings argues for a true positive finding that cannot be explained by population stratification.

**Comparison with previous studies of VEGFA and retinopathy.** Three studies (25,27,28) investigated rs2010963, a promoter variant, of which two studies were performed in type 2 diabetes (27,28) and one in both type 1 and type 2 diabetes (25). Two studies (25,28) found no association between this SNP and diabetic retinopathy. While rs2010963 (C on the forward strand) approached nominal significance with time to severe retinopathy in the DCCT/EDIC probands in our single-marker analysis (HR 1.21 [95% CI 0.98–1.50], $P$ = 0.078), it was not retained in backward or forward selection models. rs2010963 (C) and rs833070 have been reported to be associated with a severe neovascular/exudative form of age-related macular degeneration (AMD) (43) using family- and haplotype-based analysis. Another study found a significant association of rs1413711 C genotype with AMD (44). This marker falls in the same bin as rs833070, which we found to be weakly associated with severe retinopathy in our single SNP analysis. We speculate that there may be a real signal in the first block underlying the association with severe retinopathy observed in our study, as well as in the other studies of diabetic retinopathy and AMD. Inconsistencies as to which marker is most strongly associated in each study may depend on which SNPs were genotyped from the first block (Fig. 1). Our multi-SNP results, however, support rs699947, rs2146323, and rs3024987 being independently responsible for the signal seen in the first LD block, which contains the promoter and the 5′ region. The global multi-SNP test results in DCCT/EDIC for the two other retinal outcomes (three or more step progression and macular edema) were nonsignificant: for the three or more

step progression, this was likely due to it measuring change from baseline; the effect size for macular edema may be smaller, for which we have low power.

**Functional relevance.** In ex vivo transfection experiments using COS cells (45), vectors containing the entire 5′ untranslated region in frame with the cDNA encoding the VEGFA[189] isoform produced more VEGFA protein in the presence of the C-allele of rs2010963 in comparison with the G-allele. rs2010963 C/C genotype was found to be associated with higher fasting VEGFA levels in healthy subjects (27). There is substantial evidence supporting the association between VEGFA levels and development of diabetic retinopathy. Transgenic nondiabetic mice expressing even minimally elevated levels of human VEGFA protein in the eye were found to develop clinical and pathological changes consistent with those seen in non-PDR and very early stages of PDR (46). There was also a positive correlation between VEGFA expression and severity of retinopathy (46). We speculate that the production of higher VEGFA levels through the variants significantly associated with severe retinopathy (or variants that are in high LD with them) is one possible mechanism by which these variants mediate their effect on diabetic retinopathy. Because several variants survived the multi-SNP association model with severe retinopathy, yet are not in LD with known promoter variants that affect VEGFA expression, other mechanisms are possible including mRNA stability or splice site variation. To date, seven splice forms of VEGFA have been identified, including VEGFA165, which is missing exon 6 and appears to predominate quantitatively and functionally in most angiogenic states. A recently described family of VEGFA isoforms, termed VEGFAb (47), is formed by alternative splicing, which results in the inclusion of an 18-bp fragment, called exon8b, in place of exon 8. This family produces proteins of the same length as other forms but with a different COOH-terminal amino acid sequence (48), including VEGFA165b. VEGFA165b lacks exon7b, but, despite the presence of the receptor-binding domain, it does not stimulate angiogenesis. Further, it inhibits the proliferative, migratory, and vasodilator effects of VEGFA165. VEGFAb inhibits VEGFA-induced angiogenesis in a one-to-one stochiometric manner. Decreased expression of VEGFA165b has been observed in human eye tissues from patients with diabetes (49). Interfering with the splice format could be one mechanism for the association; however, none of the associated SNPs are located in conserved splice sites.

In conclusion, our study extends previous reports in several respects. The retinal phenotypes in the DCCT/EDIC Study have been measured 17 times over 15 years, leading to a more sensitive measure of the risk of complications. A strength of prospective studies such as DCCT/EDIC is the reliability of outcome and covariates measured repeatedly over an extended period of time. In contrast to case-control studies, this allows inclusion of important covariates measured before development of the outcome. All known risk factors for retinopathy were measured and included in the multivariate time-to-event analysis. As expected, longitudinal measures of A1C over the period at risk had a strong relationship with the development of severe retinopathy. This is in stark contrast to cross-sectional studies that typically only have a single measure at the time of observation. Moreover, unlike previous studies that focused only on variants in the promoter region, we investigated tagSNPs that cover the

entire gene. Under the assumption of prior evidence for association with VEGFA, the current study provides strong evidence that more than one SNP in VEGFA is independently associated with the risk of developing severe diabetic retinopathy in patients with type 1 diabetes. This suggests that allelic heterogeneity is operating through one or more pathological mechanisms or that an as yet unidentified variant is underlying the observed association. The generalizability of these findings must be interpreted with some caution, given the extensive inclusion and exclusion criteria of the original DCCT Study, specifically the exclusion of severe retinopathy at DCCT baseline (30). These findings add to the growing literature concerning the important role that VEGFA plays in this diabetes complication. Addressing the mechanisms by which VEGFA variants exert effects on diabetic retinopathy should be a priority for further research.

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