Combined Effect of Inflammatory Gene Polymorphisms and the Risk of Ischemic Stroke in a Prospective Cohort of Subjects With Type 2 Diabetes: A Go-DARTS Study

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OBJECTIVE—We have previously observed that genetic profiles determined by the combination of five functionally significant single nucleotide polymorphisms (SNPs) (*rs1800795*, *rs5498*, *rs5361*, *rs1024611*, and *rs679620*) of genes encoding prototypical inflammatory molecules are associated with history of ischemic stroke. Here we tested the ability of this multigenic model to predict stroke risk in a large population-based prospective cohort of subjects with type 2 diabetes.

RESEARCH DESIGN AND METHODS—This study was conducted using a prospective cohort of individuals with type 2 diabetes participating in the Go-DARTS (Genetics of Diabetes Audit and Research in Tayside Scotland) study, which includes genetic and clinical information of patients with diabetes within the Tayside region of Scotland, U.K. The above-mentioned inflammatory SNPs were investigated in 2,182 Go-DARTS participants. We created an inflammatory risk score (IRS), ranging from 0 to 5, according to the number of "at-risk" genotypes concomitantly carried by a given individual. The primary outcome was the occurrence of fatal or nonfatal stroke of any kind. Mean follow-up time was 6.2 ± 1.1 years.

RESULTS—The incidence of stroke increased according to the IRS. The IRS was significantly and independently associated with increased stroke risk after adjustment for other conventional risk factors (hazard ratio 1.34 [95% CI 1.1–1.7]; $P = 0.009$). The highest hazard ratio for stroke was found in subjects concomitantly carrying >3 proinflammatory variations and in subjects without previous cardiovascular diseases.

CONCLUSIONS—This large prospective cohort study provides evidence that SNPs of genes encoding prototypical inflammatory molecules may be used to create multigenic models that predict stroke risk in subjects with type 2 diabetes. ***Diabetes* 59: 2945–2948, 2010**

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Proinflammatory single nucleotide polymorphisms (SNPs) may contribute to the development and progression of pathological conditions, including cardiovascular diseases (1–7). Inflammatory gene variations may act synergistically, determining genetic profiles associated with increased risk for disease (8–13). We have previously demonstrated that genetic profiles determined by the combination of SNPs of five prototypical inflammatory genes are associated with history of ischemic stroke. In particular, we have found that the combined analysis of the *rs1800795*, *rs5498*, *rs5361*, *rs1024611*, and *rs679620* of the interleukin-6 (*IL-6*), monocyte chemoattractant protein-1 (*MCP-1*), intercellular adhesion molecule-1 (*ICAM-1*), selectin-E (*sel-E*), and matrix metalloproteinase-3 (*MMP-3*) genes may be used to create genetic profiles that are associated with different odds of stroke in a case-control scenario (10). A similar combination of inflammatory SNPs is also associated with peripheral artery disease (PAD) and critical limb ischemia (CLI) (11).

Here, we investigated whether this multigenic model may predict stroke risk in a large population-based prospective cohort of subjects with type 2 diabetes. We studied 2,182 diabetic individuals participating in the prospective Go-DARTS (Genetics of Diabetes Audit and Research in Tayside Scotland) study and found that risk for stroke is significantly and independently influenced by an inflammatory risk score (IRS) determined by the combination of the five SNPs mentioned above.

RESEARCH DESIGN AND METHODS

We studied 2,182 individuals with type 2 diabetes in the Go-DARTS study. Additional information on Go-DARTS is available in the online appendix available at http://diabetes.diabetesjournals.org/cgi/content/full/db09-1690/DC1. The study was approved by the local research ethics committee.

Genetic analyses and creation of the multigenic model. The following SNPs were analyzed: *IL-6* −174G/C *rs1800795*, *MCP* −2518A/G *rs1024611*, *ICAM-1* −469E/K *rs5498*, *sel-E* Ser128Arg *rs5361*, and *MMP-3* −117155A/6A *rs679620*. According to our previous study (10), the following genotypes were considered "at risk": *IL-6* GG and GC, *MCP-1* GG, *ICAM-1* EE, *sel-E* RR (ArgArg), and *MMP-3* 5A5A. The IRS model was created as a continuous variable, ranging from 0 to 5, based on the number of "at-risk" genotypes concomitantly carried by a given individual. The population was stratified prior to evaluation of the number of disease events and execution of the statistical analyses. Additional information is available in the online appendix.

Disease events. All individuals were followed up until their first nonfatal or fatal stroke after recruitment. Nonfatal strokes were determined from both the hospital Scottish Morbidity Record and the DARTS program of nurse-facilitated validation. Fatal events were determined from death certificates obtained from the General Register Office for Scotland, as previously described (14–16). Additional information is available in the online appendix.

TABLE 1
Characteristics of the study cohort

Total sample size	2,182
Female subjects	1,035 (47.4%)
Male subjects	1,147 (52.6%)
Age (years)	64.5 ± 11.7
BMI (kg/m^2)	30.2 ± 5.45
Duration of diabetes (years)	7.7 ± 6.5
Age at diagnosis (years)	56.8 ± 12.2
Smoker (ever)	1,109 (50.8%)
Previous cardiovascular disease	720 (33.0%)
Previous stroke	200 (9.2%)
Follow-up (years)	6.2 ± 11
Strokes (fatal and nonfatal) after enrollment	108 (4.9%)

Data are means ± SD or *n* (%).

Statistical analyses. Cox regression was used to determine the association of genotype with stroke after recruitment. Survival functions were adjusted for age at recruitment and sex. For smoking, a composite variable was constructed (ever-smokers genotype+, ever-smokers genotype−, never-smokers genotype+, and never-smokers genotype−) and entered as indicator variables. Fully adjusted models, which included ratio of total-to-HDL cholesterol, mean arterial pressure, and years with diabetes, were also considered. STATA version 8 was used for all analyses. Additional information is available in the online appendix.

RESULTS

The demographic and clinical characteristics of the study population are presented in Table 1. The cohort consisted of 1,147 male and 1,035 female subjects (mean age 64.5 ± 11.7 years). Mean age at diagnosis was 56.8 ± 12.2 years. Mean duration of diabetes was 7.7 ± 6.5 years. A total of 1,109 subjects were "ever-smokers" (50.8%), 720 subjects had previous cardiovascular diseases (myocardial infarction and/or stroke) (33.0%), and 200 subjects had previous history of stroke (9.2%). During the follow-up period (6.2 ± 1.1 years), the overall incidence of new fatal and nonfatal strokes was 4.9% (108 events).

Genotype and allele frequencies of the individual variants in the study cohort are shown in supplemental Table 1. Allele frequencies of all variants were in Hardy-Weinberg equilibrium. The association of individual variants with prospective risk for stroke is shown in Table 2. In this analysis, we also considered whether the association between individual gene variations and stroke risk was influenced by smoking. None of the investigated gene variants increased the risk for stroke when considered alone. The *ICAM-1* EE genotype demonstrated a significant association with stroke in smokers (hazard ratio [HR] 2.6 [95% CI 1.2–6.0]; $P = 0.02$).

TABLE 2
Association of individual increased risk variants with prospective risk of stroke

Variant	Model	HR (95% CI)*	*P*
IL-6	GG/GC genotype	1.2 (0.6–1.9)	0.626
Smoking (ever)		0.9 (0.4–4.3)	0.683
MCP-1	GG genotype	1.4 (0.9–2.0)	0.102
Smoking (ever)		1.0 (0.4–2.1)	0.857
ICAM-1	EE genotype	1.2 (0.8–2.0)	0.365
Smoking (ever)		2.6 (1.2–6.0)	0.022
sel-E	RR genotype	2.0 (0.5–8.0)	0.346
Smoking (ever)		NA**	NA
MMP3	5A5A genotype	1.5 (1.0–2.2)	0.075
Smoking (ever)		1.6 (0.7–3.7)	0.273

*All models adjusted for age at study recruitment and sex. **Zero individuals in at least one cell. NA, not available.

TABLE 3
IRS genotypic model and risk of stroke in the Go-DARTS study

	HR (95% CI)	*P*
IRS	1.34 (1.1–1.7)	0.009
Age at enrollment	1.06 (1.0–1.1)	<0.001
Smoking (ever)	1.52 (1.0–2.3)	0.038
Previous cardiovascular disease	2.10 (1.4–3.1)	<0.001
Mean arterial pressure	1.02 (0.8–1.1)	0.087
Cholesterol ratio	3.93 (0.8–18.7)	0.085

Analyses were performed on 2,123 genotyped individuals with full covariates available for Cox proportional hazards study. Number of incident strokes is 104.

The distribution of subjects according to the IRS is shown in supplemental Fig. 1. No subjects with an IRS of 5 were identified in our cohort. The occurrence of ischemic stroke over the follow-up period increased according to the IRS, with an incidence of 1.0, 4.5, 4.7, 6.4, and 10.9% among subjects with IRS 0, 1, 2, 3, and 4, respectively (supplemental Fig. 2). Cox proportional hazards analyses were performed to assess the relative risk for stroke according to the IRS. These analyses were performed on a total of 2,123 individuals for whom full covariates were available. In this subset of subjects, the incidence of stroke (4.8%, 104 events) did not differ from that observed in the overall study cohort ($n = 2,182$, incidence of stroke 4.9%, 108 events). After correcting for age, smoking habit, blood pressure, cholesterol ratio, and presence of previous cardiovascular diseases, the IRS was significantly and independently associated with increased stroke risk (HR 1.34 per risk allele [95% CI 1.1–1.7]; $P = 0.009$) (Table 3). The effect of IRS was mainly seen in individuals free of cardiovascular diseases at entry (supplemental Table 2). The percentage of subjects that survive without stroke differs according to the IRS, with individuals who have an IRS of 4 showing the lowest stroke-free survival (Fig. 1).

DISCUSSION

Multigenic approaches have been demonstrated to be valid tools for identifying subjects at risk for complex traits, such as type 2 diabetes (17) and coronary artery disease (18). A similar strategy was used in the present study, hypothesizing that, in subjects with type 2 diabetes, an

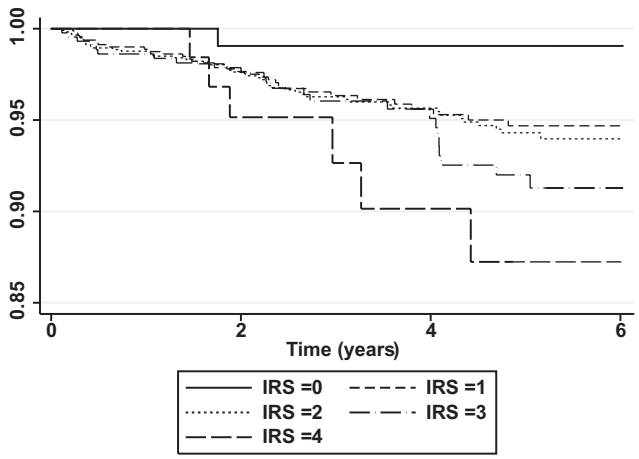

FIG. 1. Kaplan-Meier plot of stroke-free survival among the Go-DARTS population stratified by IRS.

increasing number of proinflammatory alleles may confer a significant risk of developing ischemic stroke. We have previously demonstrated the utility of the Go-DARTS cohort to study the contribution of gene variations to cardiovascular risk in subjects with diabetes (14–16). To our knowledge, this is the first prospective study that attempts to look at the impact of the combined effect of multiple gene variations on incident stroke. It is also the first prospective study considering the combination of multiple inflammatory SNPs in diabetic individuals.

The decision to study the *rs1800795*, *rs5498*, *rs5361*, *rs1024611*, and *rs679620* of the *IL-6*, *MCP-1*, *ICAM-1*, *sel-E*, and *MMP-3* genes was based on the following criteria. First, all five SNPs are functionally important. The *rs1800795* is a SNP of the *IL-6* promoter and influences the transcription rate of the gene and IL-6 plasma concentration (2). Similarly, the *rs1024611* is a SNP of the promoter and affects the expression rate of the *MCP-1* gene (1,4). Also the *rs679620* of the *MMP-3* gene is a SNP promoter and regulates transcription and protein levels in an allele-specific manner (5). Regarding the *rs5498* of the *ICAM-1* gene and the *rs5361* of the *sel-E* gene, these SNPs lead to changes in the amino acid sequence of the corresponding proteins that result in modifications of their biological activity. In particular, the *rs5498* of the *ICAM-1* gene affects the Ig-like domain 5 of the ICAM-1 protein, which is crucial for the interactions with lymphocyte function-associated antige-1 (LFA-1) and the adhesion of B-cells (6). On the other hand, the *rs5361* of the *sel-E* gene alters the ability of the E-sel protein to regulate leukocyte-endothelial interactions (3). Previous reports have implicated these SNPs in the pathobiology of atherosclerosis and stroke (6,19). We have previously reported that these SNPs are significantly and independently associated with history of ischemic stroke in a case-control scenario (10). Finally, we have shown that a combination of SNPs similar to that investigated in this study is associated with PAD and CLI (11). It should be noted that the synergistic and interdependent effects of these SNPs are mirrored by some important physiological interactions displayed by the corresponding proteins. For instance, MCP-1 is able to stimulate IL-6 secretion, and ICAM-1 synthesis is stimulated by MCP-1 in a time- and dose-dependent manner (20,21). On the other hand, ICAM-1 induces expression of several proinflammatory cytokines, including IL-6, which in turn induces the synthesis of various acute-phase proteins, thus maintaining and promoting the inflammatory phenotype (21,22). Taken together, these data indicate that the SNPs investigated in this study create a proinflammatory state that facilitates the development of atherosclerotic and ischemic disorders, such as stroke.

In a recent prospective study, the *rs1800795* of the *IL-6* gene has been associated with fasting glucose levels and development of diabetes (23). This raises the possibility that at least some of the SNPs composing our IRS affect the severity of diabetes. According to this hypothesis, increased risk of stroke might depend on subjects with high IRS having more severe diabetes. However, this hypothesis cannot be tested in the Go-DARTS study, because parameters of diabetes severity are not available. The analysis of our multigenic model in prospective cohorts in which outcomes of diabetes severity are available might address this issue.

None of the SNPs evaluated in our study have been associated with stroke in recent genome-wide association studies (GWASs) (24–27). Although this might appear disappointing, it is not inconsistent with our findings, if one considers that, in our study also, none of the five investigated SNPs was significantly and independently associated with incidence of stroke when considered alone. It was the combinatorial analysis of these five SNPs that provided evidence of their association with increased stroke risk, and no GWASs have so far investigated the possibility that this or similar proinflammatory multigenic models are associated with stroke or other cardiovascular diseases. In this respect, it will be interesting to test our multigenic model in existing GWAS databases, in order to understand whether individuals carrying multiple proinflammatory SNPs are more common in the groups of subjects with stroke than in control groups. This would also indicate that GWASs should take into consideration the fact that SNPs that fall below the threshold of statistical significance when considered alone might instead be associated with a disease when evaluated in combination. This concept suggests that future studies should use genome-wide data to evaluate specific types of functional associations within biologically relevant pathways, thus combining the unbiased approach of GWASs with the hypothesis-driven approach of candidate gene studies.

Further studies are also needed to understand if the addition of multigenic models to conventional risk factors improves measures of calibration, discrimination, and risk reclassification for cardiovascular events. We have performed a receiver operating characteristic analysis to compare the predictive value of our multigenic profile with the conventional risk profile for stroke and observed only a small increase (1.1%) in predictive value (supplemental Fig. 3). However, our current model is based on a limited number of functional gene variations centered around an inflammatory hypothesis, and it is highly unlikely that we have exhausted all the possible genetic contributory factors. In its current state, the IRS model represents a work in progress that may improve as additional alleles are added, but the model is, as currently formulated, predictive of future risk of stroke in subjects with type 2 diabetes. Further validation will be necessary, and its predictive ability in the general population and for additional cardiovascular indications remains to be tested, but this study suggests a viable strategy for developing genetic profiles at risk.

This study has some potential limitations. First, our results need to be replicated in additional cohorts. Second, we did not perform an analysis of the different subtypes of ischemic stroke. Therefore, it is possible that our multigenic model predicts the risk only of some stroke subtypes. Finally, we cannot exclude that the observed associations depend on the effect of genes in linkage disequilibrium with those investigated in our study.

In conclusion, we demonstrate that variations of genes encoding prototypical inflammatory molecules synergistically interact to determine proinflammatory genetic profiles that significantly influence the risk of stroke in subjects with type 2 diabetes. This multigenic model of stroke risk merits further investigation as a new, potential, independent predictor of individual risk for cardiovascular diseases.

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C.N.A.P. researched data, contributed to the discussion,

and wrote/reviewed/edited the manuscript. C.H.K., A.S.P., and A.D.M. researched data. A.S.F.D. wrote/reviewed/edited the manuscript. E.G. and R.C.S. contributed to the discussion and wrote/reviewed/edited the manuscript. M.Q. reviewed/edited the manuscript. R.P. designed the study and wrote/reviewed/edited the manuscript.

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