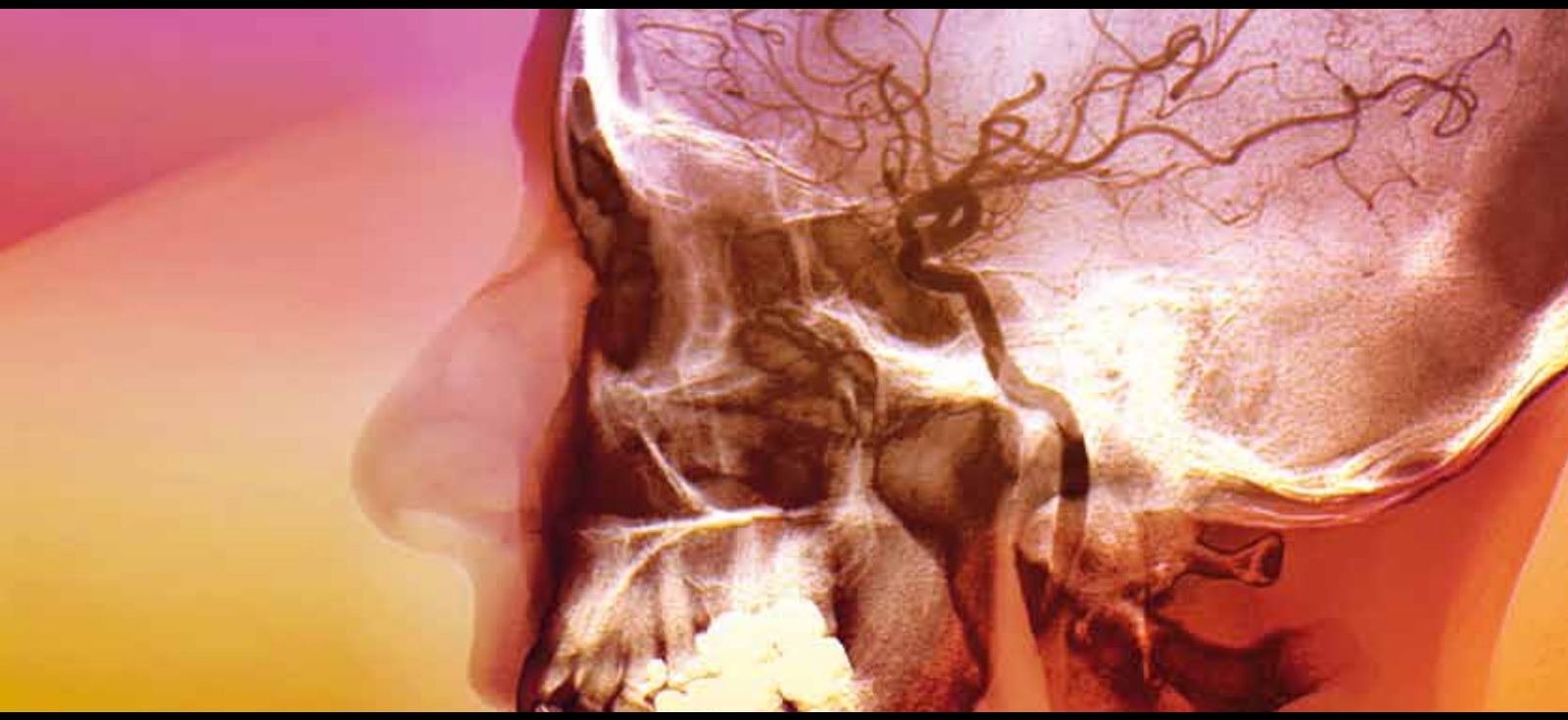
Stroke Research and Treatment

Genetics of Stroke

Guest Editors: Anna Bersano, Stephanie Debette, Leonardo Pantoni, Stephen Engelter, and Daniel Woo



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Research Article

Gene-Drug Interaction in Stroke

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Stroke is the third cause of mortality and one of most frequent causes of long-term neurological disability, as well as a complex disease that results from the interaction of environmental and genetic factors. The focus on genetics has produced a large number of studies with the objective of revealing the genetic basis of cerebrovascular diseases. Furthermore, pharmacogenetic research has investigated the relation between genetic variability and drug effectiveness/toxicity. This review will examine the implications of pharmacogenetics of stroke; data on antihypertensives, statins, antiplatelets, anticoagulants, and recombinant tissue plasminogen activator will be illustrated. Several polymorphisms have been studied and some have been associated with positive drug-gene interaction on stroke, but the superiority of the genotype-guided approach over the clinical approach has not been proved yet; for this reason, it is not routinely recommended.

1. Introduction

Stroke is the third cause of mortality and one of most frequent causes of long-term neurological disability. Well-established risk factors for stroke include increasing age, hypertension, diabetes mellitus, cigarette smoking, obesity, heart disease, atrial fibrillation and sedentary [1, 2]. However, a significant number of patients experience stroke in the absence of any risk factors; a hypothesis is that many risk factors have not been recognized yet, including genetic risk factors. The role of genetics has been evidenced through studies on twins and family history. Twin studies have shown that monozygotic twins are 1.6 more likely to be concordant for stroke than dizygotic twins [3]. Family history of stroke is a well-defined risk factor (OR 1.76 95% CI 1.7–1.9) [3].

Given these data, genetic studies have increasingly been performed with the objective of revealing the genetic basis of cerebrovascular diseases. Genetic studies have been proposed to (1) reveal the pathogenetic basis of stroke, which might become a therapeutic target for new drugs, (2) optimize risk assessment, (3) identify populations requiring more aggressive therapeutic strategies, and (4) choose the optimal drug therapy by assessing the risk/benefit ratio based on genetic characteristics [4]. The latter application has been extensively studied in pharmacogenetic studies [5–7]. Recently, genetic studies have moved to "pharmacogenomic" that involve a genome-wide association approach which scans the entire genome looking through thousands of genetic variants; these hypothesis-free studies have the aim of discovering novel genes associated with a specific disease. This review has the aim of reporting on the latest developments regarding pharmacogenetics and pharmacogenomics of stroke, focusing on the most commonly used drugs in the acute phase, for primary and secondary prevention.

2. Methods

This review was planned using key words such as "pharmacogenetics" or "pharmacogenomics" and "stroke" to search literature. These words were combined with "antihypertensive agents," "statins," "hydroxymethylglutaryl-CoA Reductase Inhibitors," "tissue plasminogen activator," "anticoagulants," "vitamin K antagonist," "antiplatelets," "cyclooxygenase Inhibitors," "aspirin," "clopidogrel," and "acetil salicylic acid/dipyridamole."

The following electronic databases were searched: MEDLINE (1995-June 11 2011) and EMBASE (1995-June 11 2011). One of the researchers (SA) read all the abstracts and

selected all articles that included either "stroke" as outcome in primary prevention studies or as the target population in acute stroke treatment or secondary prevention studies. If any doubt was raised on an article's relevance, a second opinion was formulated by VC.

3. Results

In this section, pharmacogenetic studies involving drugs currently used for ischemic stroke (prevention or acute phase therapy) are reviewed.

3.1. Antihypertensive Agents. Hypertension is the most common stroke risk factor [41]. β1 and β2 adrenergic receptor (AR) plays a major role in cardiac disease; their codifying genes have been associated with response to antihypertensive drugs. *β1-AR* gene interacted with beta-blocker (BB) therapy. Stroke risk has been shown to be higher in rs#2429511 carriers treated with BB (OR: 1.24, 95% CI: 1.03–1.50). On the contrary, BB therapy did not interact with *β2-AR* gene variants on the risks of ischemic stroke (Table 1) [14]. A large randomised trial on treated hypertensive patients, enrolled to add either verapamil SR or trandolapril (International Verapamil SR-Trandolapril Study, INVEST study), focused on the genetic component of hypertension (INVEST-GENES) (Table 1) [8, 9, 17, 18, 20]. One of the papers derived from this study examined the polymorphism of *α-adducin* (*ADD1*) *Gly460Trp* and race. The authors chose this polymorphism because α-adducin, a cytoskeleton protein related with sodium sensitivity and diuretics efficacy, has been linked to essential hypertension [42]. The results did not evidence any diuretic-genotype interaction [20]. On the contrary, a population-based case control study on the same polymorphism found that diuretics protected *ADD1 460 Trp* carriers from combined nonfatal MI/nonfatal stroke outcome. Other antihypertensive agents (e.g., beta blockers, ACE inhibitors, and calcium-channel blocker) did not show the same effect [19].

The randomised INVEST-GENES study also investigated the relation between subunit β1 of the gene that encodes for a conductance calcium and voltage-dependent potassium channel (*KCNMB1*) genotype and response to calcium antagonists. The results showed that carriers of the *Leu 110* polymorphism have a reduced risk of combined death, MI, and stroke when assuming verapamil SR to treat hypertension [9]. In addition, the same research group focused on G-protein-coupled receptor kinases (GRKs), receptors involved in beta-adrenergic signalling. *GRK2* SNPs (*rs1894111 G > A*) and *GRK5 Gln41Leu* polymorphism were investigated in patients treated with atenolol or hydrochlorothiazide. The authors concluded that *GRK 41Leu* variant did not interact with any of the studied treatment regarding a combined cardiovascular outcome including death, MI, and stroke [8]. Finally, Pacawnosky investigated for an association between nitric oxide synthase (*NOS 3*) polymorphism [18], beta-adrenergic receptor gene (*ADRB1* and *ADRB2*) [17], and response to different antihypertensive agents. The first study focused on two *NOS 3* polymorphisms since nitric oxide regulates vascular tone and is associated with many cardiac diseases [43]; no outcome or drug interaction was associated with genotype [18]. Also the second study did not evidence any genotype-drug interaction on stroke [17].

A population-based prospective cohort study focused on the renin-angiotensin system which is affected by ACE-inhibitors and BB (Table 1) [15, 16]. Neither of the studies observed any interaction between drug use and genotype when stroke was considered as outcome [15, 16].

The genetics of hypertension-associated treatment (GenHAT) study investigated the ACE insertion/deletion (*ACE I/D*) polymorphism in a large population of hypertensive patients with one or more cardiovascular risk factors. This randomised study did not report any association between treatment, genotype, and primary or secondary outcomes [11].

The same result was replicated in a more articulated investigation on the *ACE* gene and 12 other polymorphisms (*ADD1 Gly460Trp, β1AR Gly389Arg, β2AR Arg16Gly, β2AR Gln27Glu, β3AR Trp64Arg, AGT Met235Thr, Aldosterone synthase promoter C-344T, Type 1 angiotensinogen receptor A1166C, bradykinin 2 receptor I/D, CYP2C9 ∗ 2 versus CYP2C9 ∗ 1, CYP2C9 ∗ 3 versus CYP2C9 ∗ 1, G protein β3-subunit C825T*) [10]. This study was the product of the randomised LIFE (Losartan Intervention for Endpoint reduction in Hypertension) study trial, which included patients with hypertension and left ventricular hypertrophy treated with losartan versus atenolol. The authors did not evidence any genetic-drug interaction on different outcomes such as blood pressure and heart rate control, composite adverse cardiovascular outcome, cardiovascular death, MI, and stroke; in fact, they concluded that the clinical superiority of losartan in 25% stroke reduction compared to atenolol was not explained by these susceptibility genes [10].

A role in modulating antihypertensive agents has been suggested for the gene which codes for the precursor of atrial natriuretic polypeptide (*NPPA* gene). The polymorphism of this gene was studied by the GenHAT study [12]. The objective was to demonstrate that minor *NPPA* alleles in the *T2238C* or *G664A* variants had lower rates of primary outcome events compared with common allele homozygotes, if treated with diuretics. Subjects randomly receiving amlodipine, chlorthalidone, lisinopril, or doxazosin were included in a genetic for treatment interaction analysis. Carriers of the minor *C* allele had more favourable stroke outcome when taking diuretics, whereas *TT* allele carriers had better stroke outcome when receiving a calcium channel blocker [12]. GenHAT [13] also showed that stroke risk was higher on lisinopril versus amlodipine in common *GG* homozygotes of fibrinogen beta (*FGB*) gene, which codes for a polypeptide of the coagulation factor fibrinogen. On the contrary, variant *A* allele carriers on lisinopril had slightly lower stroke risk. Finally, a pharmacogenetic study on perindopril failed to demonstrate a role for ACE I/D polymorphism on stroke [21].

3.2. Statins. The most currently used drugs for hypercholesterolemia are statins; although very effective, they induce a significant rate of adverse events such as myopathies and

Table 1: Antihypertensive agents.

Name	Outcome	Gene and variant	Sample size/drugs used	Effect estimates and significance levels
INVEST-GENES [8]	Death/MI or stroke	GRK2 SNPs (rs1894111 $G > A$) GRK5 Gln41Leu	48/Verapamil SR, atenolol	GRK5 41Leu decreased the risk of adverse cardiovascular outcome adjusted independently of treatment (OR 0.535, 95% CI: 0.313–0.951)
INVEST-GENES [9]	Death/MI or stroke	KCNMB1 Glu65Lys KCNMB1 Val110Leu	5979 with HTN/Verapamil SR, atenolol	KCNMB1 110Leu had reduced risk of composite outcome (HR 0.68 (95% CI 0.47–0.998); this effect was higher in Verapamil SR (HR 0.587, 95% CI 0.33–1.04) than atenolol-treated patients (HR 0.946, 95% CI 0.56–1.59)
LIFE substudy [10]	Cardiovascular events	13 polymorphisms (angiotensin-converting enzyme I/D, α-adducin Gly460Trp, β1-adrenergic receptor Gly389Arg, β2-adrenergic receptor Arg16Gly, β2-adrenergic receptor Gln27Glu, β3-adrenergic receptor Trp64Arg, angiotensinogen Met235Thr, aldosterone synthase promoter C-344T, type 1 angiotensinogen receptor A1166C, bradykinin 2 receptor I/D, CYP2C9 $*$ 2 versus CYP2C9 $*$ 1, CYP2C9 $*$ 3 versus CYP2C9 $*$ 1, G protein β3-subunit C825T)	3503/Losartan, atenolol	No significant genotype-drug interaction on the outcome
GEN-HAT [11]	Primary: fatal CHD/nonfatal MI. Secondary: stroke, all-cause mortality, combined CHD, and combined cardiovascular disease	ACE I/D	37,939/chlorthalidone, amlodipine, lisinopril, or doxazosin	No significant association with the outcome was reported; no significant gene-drug interaction was found
GEN-HAT [12]	Primary: fatal CHD/nonfatal MI. Secondary: stroke, all-cause mortality, combined CHD, and 6-mos systolic and diastolic BP changes	NPPA SNP T2238C (rs5065) NPPA SNP G664A (rs5063)	38,462 with HTN/chlorthalidone, amlodipine, lisinopril, or doxazosin	NPPA T2238C TT variant x "chlorthalidone versus amlodipine" interaction was significantly associated stroke (HR 1.09 95% CI 0.95–1.26). NPPA T2238C TT variant x "chlorthalidone versus amlodipine + lisinopril" interaction was significantly associated with stroke (HR 1.09 95% CI 0.95–1.26). NPPA T2238C CC variant x "chlorthalidone versus amlodipine" interaction was significantly associated with stroke (HR 1.18 95% CI 0.72–1.90). Either NPPA T2238C variant or NPPA G664A was not significantly associated with stroke and "chlorthalidone versus lisinopril," "chlorthalidone versus doxazosin"

Table 1: Continued.

Name	Outcome	Gene and variant	Sample size/drugs used	Effect estimates and significance levels
GEN-HAT [13]	Primary: fatal CHD/nonfatal MI. Secondary: stroke, heart failure, all-cause mortality, end-stage renal disease	FGB G455A	30 076 with HTN/chlorthalidone, amlodipine, lisinopril	Common GG homozygotes had higher stroke risk on lisinopril versus amlodipine (HR 1.38, $P < 0.001$); variant A allele carriers had slightly lower risk on lisinopril versus amlodipine (HR 0.96, P value for interaction = 0.03)
Lemaitre et al. [14]	MI, ischemic stroke	ADRB1 (Seven SNPs plus haplotype), ADRB2 (five SNPs plus haplotypes)	938 cases with MI or stroke/beta blocker	beta1-AR gene variation and beta-blocker use showed a positive interaction on ischemic stroke risk ($P = 0.04$). Homozygosis or heterozygosis for rs#2429511 variant was associated with higher MI/stroke combined risk in beta-blocker users (OR 1.24 95% CI 1.03–1.50). No interaction of ADRB2 with beta-blocker use and outcomes
Rotterdam study [15]	MI, stroke	AGT (M235T)	4097 with HTN/ACEI, BB	No significant gene-drug interaction was found on stroke
Rotterdam study [16]	MI, stroke	AGTR1 (C573T) ACE (I/D)	4097 with HTN/ACEI, BB	No significant AGTR1 and ACE I/D interaction on stroke risk with ACEI or BB
INVEST-GENES [17]	Death/nonfatal MI/nonfatal stroke	ADRB1 (Ser49GLy, Arg389Gly) and ADRB2 (Gly16Arg, Gln27Glu, 523 C > A)	5,895 CAD patients/Verapamil SR, atenolol	No association between any haplotype and treatment on stroke
INVEST-GENES [18]	Death/nonfatal MI/nonfatal stroke	NOS3-786T > C (rs2070744), NOS3 Glu298 > Asp (rs1799983)	258 death/MI/stroke versus 774 control	No genetic interaction with drugs and composite outcome
Psaty et al. [19]	MI/nonfatal stroke	ADD1 (Gly460Trp)	Cases versus controls	ADD1 Trp460 variant had lower stroke risk on diuretics (OR, 0.49; 95% CI, 0.32–0.77). The point estimate of diuretic-adducin interaction was SI 0.45 (95% CI 0.26–0.79) for the combined outcome MI and stroke; separate analyses yielded similar results: MI (SI 0.41 95% CI 0.21–0.80) and stroke (SI 0.53 95% CI 0.24–1.19)
INVEST-GENES [20]	Death/nonfatal MI/nonfatal stroke	ADD1 Gly460Trp	5,979 CAD patients/Verapamil SR, atenolol	ADD1 Trp460 black carriers had higher combined outcome risk (aHR 2.62, 95% CI 1.23–5.58), compared to whites (aHR 1.24 95% CI 0.90–1.71) and Hispanics (aHR 1.43 95% CI 0.86–2.39). No significant interaction between the ADD1 polymorphism and diuretic use for either primary outcome or secondary outcomes

Table 1: Continued.

Name	Outcome	Gene and variant	Sample size/drugs used	Effect estimates and significance levels
PROGRESS [21]	Fatal and nonfatal stroke (ischemic or hemorrhagic), nonfatal MI/coronary death, composite nonfatal stroke/nonfatal MI/vascular death, all-cause mortality, dementia, and cognitive decline	ACE I/D	5688 with stroke or TIA/perindopril	No associations between ACE genotypes and cerebrovascular disease history or cardiovascular risk factors was demonstrated. The ACE genotype was not associated with the long-term risks of stroke. The ACE genotype did not modify the relative benefits of perindopril over placebo

abnormal transaminase levels. Recent pharmacogenetics data has contributed to better understanding statin pharmacokinetics and pharmacodynamic variability [44]. Pharmacogenetic and dynamic properties have been extensively studied, but only few studies included stroke as outcome (Table 2) [22–25].

A population-based cohort study focused on apolipoprotein E, a protein involved in lipid clearance rate and conversion together with the production of triglycerides and very low-density lipoprotein. The Apo E gene encodes for three alleles: E2, E3, and E4 [45]. The results did not show gene-statin interaction with stroke; stroke risk was reduced independently of Apo E genotype in statin users [24]. The same author examined the effect of *ACE I/D* polymorphism on stroke using the Gen-HAT data. None of the outcomes evidenced significant ACE I/D-pravastatin interaction [25]. The randomised heart protection study focused on Kinesin family member 6 (*KIF*) gene, whose variant has been associated with reduced coronary events [46]. The authors did not find any significant interaction between the studied polymorphism *KIF Trp719Arg* and simvastatin use for any of the outcomes, including stroke [23]. The only study that yielded positive results was a case-control study that involved patient with MI and stroke. The authors focused on six genes that have been associated to statin treatment response: ATP-binding cassette subfamily B (*ABCB1*) gene that encodes for a drug transporter involved in statins metabolism; *CETP*, human hepatic lipase gene (*LIPC*) and low density lipoprotein receptor (*LDLR*), genes related to lipid metabolism; *HMGCR*, the target protein of statins; *NOS3*, a key gene implicated in maintaining the endothelium, which in turn mediates several effects of statins [22]. The authors found 5 polymorphisms (one in CETP and 1 in LIPC) that had significant interactions with statins on stroke outcome [22], the highest significance level was found in the CETP SNPs (rs5883), which was associated with stroke risk in simvastatin users. No gene level interactions were found for stroke [22].

3.3. Tissue Plasminogen Activator. Recombinant tissue plasminogen activator (rTPA) is the only licensed drug to treat ischemic stroke in the acute phase (within 3–4.5 hours from onset). This drug is administered to treat ischemic stroke and restore blood flow to the brain [47, 48]. The clinical benefit of rTPA is counterbalanced by a higher risk of hemorrhagic complications; 2–10% of patients develops symptomatic hemorrhagic transformations and 40% asymptomatic hemorrhagic events [49–51]. The functional role of rt-PA is to convert plasminogen into plasmin, which has fibrinolytic activity. The higher activity of the enzyme produces hyperfibrinolysis and consequently bleeding, whereas lower activity causes hypofibrinolysis and, as a consequence, thrombosis or embolism [52]. Genetic association studies have sought to investigate genetic profiles correlated with clinical and pathophysiological rt-PA response (Table 3). Broderick et al. [26, 53] examined the role of the *ApoE* phenotypes and reported that rt-PA efficacy was greater in acute stroke patients with an *ApoE E2* phenotype (OR: 6.4; 95%, CI: 2.7–15.3), whereas the outcome of placebo-treated patients with or without *Apo E E2* did not differ [26]. Conversely, a Spanish group did not report on any association with *Apo E* genotype and hemorrhagic risk and recanalisation rate after thrombolytic treatment [27]. The same group explored the hypothesis that matrix metalloproteinase-9 gene (*MMP-9*), which codes for proteins associated with blood-brain barrier disruption, was associated with hemorrhagic transformation in rTPA-treated patients. However, the authors did not find any association between a *MMP-9 C-1562T* common polymorphism and hemorrhagic risk [32]. On the other hand, the authors reported that thrombolytic intervention yielded middle cerebral artery (MCA) recanalisation associated with DD homozygosis of ACE I/D polymorphism; this has been linked to procoagulant factors including PAI-1, fibrinogen's levels as well as Factors VII and X activities [29]. Another study of the same group has identified *V34L factor XII* polymorphism as a predictor of outcome with rTPA treatment; good outcome was associated with *VV* genotype and low fibrinogen levels, while a higher risk of inefficacy of thrombolytic therapy and mortality was found with *L34* genotype and high fibrinogen levels [31]. In addition, Fernandez-Cadenas and colleagues studied the influence of two genes coding for fibrinolysis inhibitors, thrombin-activatable fibrinolysis inhibitor (*TAFI*), and plasminogen activator inhibitor-1 (*PAI-1*) genes. They demonstrated that *TAFI Thr325Ile* polymorphism predicted the absence of recanalisation with

Table 2: Statins.

Author's name/study name	Outcome	Gene (variant)	Sample size/drug	Effect estimates and findings
Hindorff et al. [22]	Nonfatal MI/nonfatal stroke	ABCB1, CETP, HMGCR, LDLR, LIPC, NOS3	865 with MI, 368 with stroke and 2686 controls/statins	No gene-statin interactions for stroke. 5 SNP-statin interactions on stroke (one CETP, four LIPC); no gene level association for stroke; SNP level association: two SNPs (one CETP, one LDLR) for stroke. The highest significance was found for stroke in CETP rs5883 carriers on simvastatin (OR 3.60 95% CI 1.22–7.70)
Heart protection study [23]	Major coronary event (coronary death or nonfatal MI), major vascular event (major coronary event plus revascularization or stroke)	KIF6 Trp719Arg polymorphism (rs20455) on vascular risk and response to statin therapy in from of the heart protection study	18,348 participants/simvastatin	No significant gene-statin interaction with any of the outcome, including stroke
Rotterdam study [24]	Death, MI, stroke	Apo E (E2, E3, E4)	7983 older than 55 yo/statins	No significant gene-statin interaction with any of the outcome. Statins reduce stroke risk (aOR 0.50 95% CI 0.28–0.91) independently of Apo E genotype
GenHAT [25]	Primary outcome: all-cause mortality, secondary outcomes (fatal CHD and nonfatal MI, CVD mortality, CHD, stroke, other CVD, non-CVD mortality, stroke, and heart failure)	ACE (I/D)	9467/pravastatin	No significant gene-statin interaction with any of the outcome

t-PA infusion. On the contrary, *PAI-1 4 G/5 G* polymorphism did not influence recanalisation rate. However, the combination of these two polymorphisms doubled the risk of negative response to therapy [30]. A recent study using a candidate gene approach has explored the association of 263 SNPs and recanalisation rate in TPA-treated patients; cluster of differentiation 40 (CD40) 1C > T and matrix Gla protein (MGP)-7*A* > *G* polymorphism were both associated with reocclusion although only the latter was associated with neurological worsening at 24 h [28]. This may be due to the role of CD40 in thrombosis and inflammation [54], while MGP gene might have a protective role in atherosclerosis [55]. To date, GWAs has not been performed on human subjects.

3.4. Anticoagulants. Anticoagulation is first-line treatment for cardioembolic stroke. Although these drugs are effective in almost 60% of cases, the hemorrhagic risk is double and even higher in the first period of therapy [1]. Recent acquisition on pharmacogenetics of warfarin has been suggested to be able to predict the optimal initial dosage of warfarin using a genotype-guided approach (Table 4). This approach promises to adequately prevent stroke and to minimize hemorrhagic risk. Several candidate gene studies have mainly focused on cytochrome P450 (CYP) and vitamin K epoxide reductase complex subunit 1 (VKORC1) [56, 57]. Cytocrome P 450 metabolises in the liver S-warfarin by CYP2C9 and R-warfarin by the CYP1A1, CYP1A2, and CYP3A4; these enzymes affect warfarin kinetics, and several SNPs of CYP450 have been correlated with its sensitivity [58]. The VKORC1 enzyme converts the epoxide into reduced vitamin K; however warfarin inhibits this reaction. As a consequence, the physiologic role of vitamin K, which produces γ-carboxylation of several coagulation factors (prothrombin, factor VII, IX, and X), is inhibited.

Several groups have studied the role of VKORC1 in warfarin/acenocoumarol dose finding, dose maintenance, and bleeding risk associated with these drugs [33, 34, 59–61]. Only two studies have focused on patients receiving vitamin

Table 3: Tissue plasminogen activator.

Author's name/study name	Outcome	Gene (Variant)	Sample Size/drug	Effect estimates and findings
Broderick et al. 2001 [26]	Favourable outcome (NIHSS of 0 or 1, Barthel Index of 95 or 100, Modified Rankin Scale of 0 or 1, and a Glasgow Outcome Scale of 1.)	ApoE (E2, E3, E4)	409 ischemic stroke/rTPA versus PB within 3 hours	ApoE E2 phenotype-rt-PA interaction was associated with good outcome at 3 months (OR: 6.4; 95% CI: 2.7–15.3). Apo E4 phenotype not related to favorable 3 month outcome, response to t-PA, 3-month mortality, or risk of intracerebral hemorrhage
Fernández-Cadenas et al. [27]	Recanalization rate, NIHSS at 48 hours and mRS score at 3 months, heamorrhagic transformation	ApoE (E2, E3, E4)	77 ischemic stroke/rTPA within 3 hours	No significant association of ApoE genotype and the studied outcome
Del Río Espínola et al. [28]	Reocclusion rate	236 SNPs form candidate genes for vascular risk factor	222 ischemic stroke/rTPA	rs1883832 SNP from CD40 gene (OR: 0.077; 95% CI: 0.009–0.66) and rs1800801 SNP from the MGP gene (OR 15.25; 95% CI: 2.23–104.46) were independently associated with reocclusion after adjustment for clinical predictors
Fernández-Cadenas et al. [29]	Recanalization	ACE (I/D)	96 ischemic stroke/rTPA within 3 hours	ACE DD homozygosis was significantly associated with recanalization rate following rTPA (OR: 4.3 95% CI: 1.35–13.49). No relation was found between ACE I/D polymorphism and symptomatic hemorrhagic complications. No association between ACE genotypes and Factor VII or Factor X activities
Fernández-Cadenas et al. [30]	Recanalization	PAI-1 4G/5G TAFI (Thr325Ile)	139 with ischemic stroke/TPA within 3 hours	PAI-1 4 *G*/5 *G* was not associated with recanalization. TAFI Thr325Ile polymorphism was associated with recanalization resistance (OR 5.6 95% CI 1.2–20). Combination of TAFI and PAI-1 polymorphisms double the risk of absence of recanalization (OR: 11.1; 95% CI: 1.4–89.8)
González-Conejero et al. [31]	Death, recanalization	Factor XIII (FXIII) V34L	200 with ischemic stroke/TPA within 3 hours	FXIII 34 L carriers had higher death risk than V/V (OR 2.50 95% CI 1.00–7.06); high fibrinogen levels higher risk than lower levels (OR 2.72 95% CI 1.01–7.44); FXIII 34L and high fibrinogen level higher risk than FXII V and low fibrinogen (OR 5.74 95% CI 1.51–11.56). No difference in recanalization rate
Montaner et al. [32]	Hemorrhagic transformation	MMP9 (C1562T)	61 with ischemic stroke/TPA within 3 hours	The polymorphism studied does not increase hemorrhagic risk

K antagonist following cardioembolic stroke. One found that the time and cumulative dosage of phenprocoumon needed to achieve target 2-3 INR ratio were associated with the presence of the *VKORC1 C283 + C837T* (*rs2359612*) polymorphism. Carriers of *TT* genotype needed shorter time to achieve target INR ratio (3.2 days) compared to *CC* carriers (6.5 days) [33]. The second paper evaluated the roles of *VKORC1*, gamma-glutamyl carboxylase (*GGCX*), calumenin (*CALU*), and cytochrome P450 2C9 (*CYP2C9*) in warfarin maintenance dose on Japanese stroke sufferers. Of the twelve

Table 4: Anticoagulants and antiplatelets.

Author's name/study name	Outcome	Gene (Variant)	Sample size/drug	Effect estimates and findings
Anticoagulants				
Arnold et al. [33]	Dose finding	VKORC1 C283 + 837C → T (rs2359612)	49 with cerebrovascular disease/phenprocoumon	VKORC1 TT carriers reached an INR of 2-3 after a mean time of 3.2 days ($n = 5$), CT carriers after 4.4 days ($n = 27$), and CC carriers after 6.5 days ($n = 15$)
Kimura et al. [34]	Warfarin maintenance dose	(VKORC1), gamma-glutamyl carboxylase (GGCX), calumenin (CALU), and cytochrome P450 2C9 (CYP2C9)	93 Japanese on stable anticoagulation therapy	1639 $G > A$ ($P = 0.004$) and 3730 $G > A$ genotypes ($P = 0.006$) in VKORC1, the 8016 $G > A$ genotype in GGCX ($P = 0.022$), and the 42613 $A > C$ genotype in CYP2C9 ($P = 0.015$) were associated with effective warfarin dose
Antiplatelets				
Meta-analysis of 9 different studies (CLARITY TIMI 28, EXCLESIOR, TRITION TIMI 38, AFIJI, FASSTS-MI, RECLOSE, ISAR, CLEAR PLATELETS, Intermountain) [35]	Composite outcome (cardiovascular death/MI/stroke) and stent thrombosis	CYP2C19/1 or 2 reduced function alleles (∗2, ∗3, ∗4, ∗5, ∗6, ∗7, and ∗8)	9685 patients (91% had PCI, 54% had ACS)/clopidogrel	Carriers of 1 (HR 1.55; 95% CI, 1.11–2.17) or 2 (HR 1.76; 95% CI, 1.24–2.50) reduced-function CYP2C19 alleles had higher risk of composite outcome events
TRITON-TIMI 38 [36]	Composite outcome (cardiovascular death/MI/ischemic stroke)	CYP2C19/1 or 2 reduced function alleles (∗2, ∗3, ∗4, ∗5, ∗6, ∗7, and ∗8) ABCB1/3435C → T	2932 patients with ACS undergoing PCI/clopidogrel versus prasugrel	TT homozygotes of ABCB1 genotype had increased risk of the composite outcome compared to CT or CC carriers (HR 1.72, 95% CI 1.22–2.44). Carriers of a CYP2C19 reduced-function allele only (Kaplan-Meier event rate 11.5%), ABCB1 3435 TT homozygotes only (Kaplan-Meier event rate 12.6%), or both (Kaplan-Meier event rate 13.6%) had increased risk of composite outcome (pooled HR 1.97, 95% CI 1.38–2.82). No significant genotype-prasugrel interaction was reported

Table 4: Continued.

Author's name/study name	Outcome	Gene (Variant)	Sample size/drug	Effect estimates and findings
PLATO [37]	Composite outcome (cardiovascular death/MI/stroke)	CYP2C19/1 or 2 reduced function alleles ABCB1/3435C → T	10285 patients with ACS undergoing non-CABG/clopidogrel versus ticagrelor	Either with (HR 0.77, 95% CI 0.60–0.99) and without (0.86, 0.74–1.01, $P = 0.0608$) any CYP2C19 reduced-function alleles patients on ticagrelor experienced lower risk of composite outcome compared to patients on clopidogrel (interaction $P = 0.46$). Independently of ABCB1 genotype, patients on ticagrelor had lower risk of the composite outcome compared to clopidogrel users (interaction $P = 0.39$; HR 0.71, 95% CI 0.55–0.92). No significant interaction was found on treatment and genotype regarding major bleeding
PAPI study and Mount Sinai study [38]	Composite outcome (cardiovascular death, MI, ischemic stroke, stent thrombosis, unplanned target vessel revascularization, unplanned nontarget vessel revascularization, hospitalization for coronary ischemia)	GWA	429 white healthy Amish individuals/clopidogrel; results replicated in 227 undergoing PCI	13 SNPs in 10q24 region, where CYP2C18–CYP2C19–CYP2C9–CYP2C8 gene cluster is found, were associated with reduced response to clopidogrel. CYP2C19 $*$ 2 allele carriers were at higher risk for composite outcome (adjusted HR 2.42 95% CI 1.18–4.99)
Clappers et al. [39]	Composite outcome (cardiovascular death/MI/stroke)	COX-1/C50T	496 admitted to Coronary Care Unit for different reasons/aspirin	No interaction was found on genotype and aspirin for the composite outcome
Hillarp et al. [40]	n.a.	COX-1/C116T, del 137–142, C144T, G6841A, G7331C, A7742A, C10427A, C10608A, del 10675A, G12254A, T12378C, G19187A, C19242T, G19255A	68 with recurrent stroke/ASA	14 variants of the Cox-1 gene were identified and 7 involved amino acid substitutions of the Cox-1 molecule. None of the mutations were located near the catalytic site

ABCB1: ATP-binding cassette subfamily B, ACEI: angiotensin convertin enzyme inhibitors, ACE I/D: angiotensin convertin enzyme insertion/deletion, ACS: acute coronary syndrome, ADD1: α-adducin, ADRB: β-adrenergic receptor, AGT: angiotensinogen, AGTR1: angiotensin receptor II type 1, APO E: apolipoprotein E, BP: blood pressure, CABG: coronary artery bypass graft, CAD: coronary heart disease, CD: cluster of differentiation, CEPT: cholesteryl ester transfer protein, CHD: coronary artery disease, COX: cyclooxygenase, CVD: cerebrovascular disease, CYP: cytochrome P, FGB: fibrinogen beta, GRK: G-protein-coupled receptor kinase, GWA: genome-wide association, HMG-CoR: hydroxyl-methylcoenzyme A reductase, HR: hazard ratio, HTN: hypertension, KCNMB: conductance calcium and voltage-dependent potassium channel, KIN 6: kinesin family member 6, LDLR: low-density lipoprotein receptor, LIPC: human hepatic lipase, MGP: matrix Gla protein, MI: myocardial infarction, MMP: matrix metalloproteinase, NIHSS: National Institute of Health stroke scale, NOS: nitric oxide synthase, NPPA: atrial natriuetic polypeptide precursor, OR: odds ratio, PAI: plasminogen activator inhibitor, PCI: percutaneous coronary intervention, SI: sinergy index, TAFI: thrombin-activable fibrinolysis inhibitor, verapamil SR: verapamil-sustained release, VKORC1: vitamin K epoxide reductase complex subunit 1.

SNPs analysed, the authors found that the *1639G > A, 3730G > A VKORC1* genotypes; the *8016G > A GGCX* genotype, and the *42613A > C CYP2C9* genotype were associated with dose maintenance. Thus, the variation in warfarin dose was explained for 33.3% by age, sex, weight, and three genetic polymorphisms (*VKORC1-1639G > A, CYP2C9 42613A > C, GGCX 8016G > A*). The importance of these loci has been recently confirmed using genome-wide association studies in acenocoumarol-treated patients [62, 63]. These studies found that the SNPs with the highest significance level were located in chromosome (cr.) 16 (rs10871454 and rs9923231) linked to VKORC1 and cr. 10 (rs4086116 and rs105791) linked with CYP2C9 gene. After adjusting for these two SNPs, two other polymorphisms reached significant association with acenocoumarol: rs2108622 within CYP4F2 gene on cr.19 and rs1995891 within CYP2C18 on cr. 10 [62, 63].

3.5. Antiplatelets. Antiplatelet drugs are commonly used treatment for ischemic noncardioembolic stroke [1].

3.5.1. Aspirin. Aspirin is the more commonly used drug of this class, and its efficacy ranges between 13% and 25%. Its physiological role is to acetylate serine residue 530 in the active site of cyclooxygenase-1 (COX-1), sterically inhibiting the metabolism of arachidonic acid and consequently reducing thromboxane A2 (TxB2), which activates platelets. Numerous studies have investigated the genetic basis associated with recurrence of ischemic event in aspirin-treated patients (e.g., aspirin failure) (Table 4).

COX-1 C50T allele has been correlated with a higher level of 11-dehydro-TxB2, both before and after aspirin; however, the haplotype studied did not confirm a genetic basis for aspirin failure [64, 65]. In addition, this polymorphism is not associated with a higher risk of stroke [39].

An interesting study compared the COX-gene sequence of patients with recurrent stroke (at least with two episodes) on aspirin and healthy subjects. The study found fourteen SNPs, and half of these lead to amino-acid substitutions; how-ever, none of these variations was located near the COX catalytic site, thus this genetic polymorphisms could not explain the failure to respond to aspirin in this population of stroke patients [40].

3.5.2. Clopidogrel. Clopidogrel is an oral, thienopyridine antiplatelet drug that irreversibly inhibits the P2Y12 subtype of ADP receptor, which has a major role in platelet aggregation. Clopidogrel has proven to be less effective in carriers of CYP2C19-reduced function allele [37, 38, 66]. These data have been confirmed in a recent meta-analysis that pooled 9 randomised trails for acute coronary syndrome or percutaneous coronary intervention; either homozygosis or heterozygosis carriers experience higher risk of stroke (Table 4) [35]. This could be caused by a relative reduction in the active metabolite of the drug, or by an insufficient inhibition of platelet aggregation. At a clinical level, CYP2C19 allele carriers have major adverse cardiovascular events, including stroke [35]. The TRITON TIMI 38 study on patients with acute coronary syndrome treated with PCI following clopidogrel versus another thienopyridine, "prasugrel," explored the role of ABCB1, a glycoprotein that might affect clopidog rel transport and metabolism. The polymorphism on ABCB1 3435C → T was correlated to a significant increase in adverse outcome including cardiovascular death, MI, or stroke ($P = 0.0064$). Specifically, TT homozygote patients had a 72% increased risk of the primary endpoint compared with CT/CC individuals [36]. This result might be a consequence of the absolute reduction in maximum platelet aggregation that has been evidenced in healthy subjects enrolled in the same study [36]. Furthermore, the PLATO study explored the same polymorphisms in noncoronary artery bypass graft patients on clopidogrel versus ticagrelor, a novel ADP receptor blocker that does not need hepatic activation, and so is not influenced by CYP2C19 alleles. Patients on ticagrelor were less likely to experience stroke independently of CYP2C19 or ABCB1 genotype. In addition, no specific genotype-drug interaction was associated with any major bleeding risk [37]. Finally, an important GWA study has been performed on a healthy Amish population and found a positive association with clopidogrel response measured by ADP platelet aggregation percentage and 10q24 region (Table 4). This region contains CYP2C19 $*$ 2 genotype, which accounts for approximately 12% of the variation in clopidogrel response [38]. In addition, this study found a relevant association between this CYP2C19 $*$ 2 variant and event-free survival of adverse cardiovascular outcome in an independent population of 227 patients that underwent percutaneous coronary intervention [38].

4. Conclusions

Pharmacogenetics of stroke is a promising approach for optimizing treatment strategies aimed at decreasing stroke incidence and recurrence. Many candidate gene studies have examined the roles of polymorphisms on stroke treatment, and some of these have been replicated in GWA studies. However, few studies have considered stroke as an independent outcome, probably due to the relatively small number of events in the trails.

Antihypertensive agents are the most extensively studied drug class. Some polymorphisms have been consistently identified but results remain controversial, probably due to differences in study designs and methods, small sample sizes, and short durations of follow-up [67].

Statins and stroke have failed to find any interaction with most of the studied polymorphism. In addition, GWAs that consider stroke as an outcome are not available.

Tissue plasminogen activator has been investigated only in small studies on acute intravenous thrombolysis; thus, the pharmacogenetic data need to be reproduced in larger trails, and GWAs should be planned on humans in order to move forward in this field. Interestingly, a GWA study on ischemic rats has shown the genes regulated by rTPA treatment where different from the ones involved in ischemic stroke. In addition, gene expression profiles differed when reperfusion was or was not achieved [68]. If these results

were to be replicated on humans, blood plasma could be used to monitor gene expression profiles, which are diversely associated with stroke and rtPA vessel recanalisation.

Anticoagulants dose variability has been consistently reported to be explained by CYP2C9 and VKORC1 for the 33% and up to the 58% when adding clinical information [69]. For this reason, the United States Food and Drug Administration suggests testing for these two polymorphisms in order to achieve stable dose and to avoid major hemorrhagic events. Although the pharmacogenetic approach on warfarin is feasible in clinical practice [70]; its use in improving outcome (i.e., shorter time to achieve range INR, more stable dosing, greater percentage of time in therapeutic range, and lesser major bleeding events) over the classical clinical approach has been proven in only small samples [71, 72]; for this reason, larger studies (GIFT, COAG, and EU-PACT) are ongoing to demonstrate the usefulness of the genetic approach and clinical algorithms (see www.clinicaltrials.gov/) on outcome improvement. Unfortunately, genotype-guided warfarin dosing has not been demonstrated to be cost effective [73]. Finally, in the near future, vitamin K antagonists could be gradually replaced in many indications with the newer anticoagulants (e.g., Dabigatran, Apixaban, and Rivaroxaban), which do not require monitoring and dose adjustment [74–76].

Antiaggregants: genetic studies on aspirin failure in recurrent stroke patients have been unsuccessful in finding their genetic determinant. Several polymorphisms have been linked to poor clinical response to clopidogrel, but to date, no study has proven the usefulness of the pharmacogenetic approach with clopidogrel in improving outcome. For this reason, the ACCF/AHA [77] disagreed with the FDA regarding their decision to add a warning on clopidogrel label recommending genetic testing when prescribing it for the first time. However, several studies, focusing on coronary disease, not on stroke, are ongoing: GeCCO, RAPID GENE, TARGET PCI, and GIANT (see http://www.clinicaltrials.gov/).

The future availability and low cost of technology will allow for the screening of a large number of genetic determinants. This will lead to the description of polymorphisms that affect drug pharmacokinetics and dynamics in each given patient. Furthermore, this information will optimize the efficacy/toxicity ratio. Although promising, the results of pharmacogenetic studies need to be confirmed in prospective randomised trials of comparative effectiveness, comparing the classical clinical and the genotype-guided approach, before being used in clinical settings. Furthermore, no study has explored yet the clinical usefulness of the genetic approach in reducing adverse events. For these reasons, although promising, the genotype-guided approach for drug prescriptions is not routinely recommended [56].

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Research Article

Genetics of Common Polygenic Ischaemic Stroke: Current Understanding and Future Challenges

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Stroke is the third commonest cause of death and the major cause of adult neurological disability worldwide. While much is known about conventional risk factors such as hypertension, diabetes and incidence of smoking, these environmental factors only account for a proportion of stroke risk. Up to 50% of stroke risk can be attributed to genetic risk factors, although to date no single risk allele has been convincingly identified as contributing to this risk. Advances in the field of genetics, most notably genome wide association studies (GWAS), have revealed genetic risks in other cardiovascular disease and these techniques are now being applied to ischaemic stroke. This review covers previous genetic studies in stroke including candidate gene studies, discusses the genome wide association approach, and future techniques such as next generation sequencing and the post-GWAS era. The review also considers the overlap from other cardiovascular diseases and whether findings from these may also be informative in ischaemic stroke.

1. Evidence for Genetic Factors in Stroke Risk

Stroke is the third commonest cause of death and the major cause of adult neurological disability, affecting both the developed world and increasingly having an impact in the developing world as well. It is also a major cause of dementia and the commonest cause of late onset epilepsy. Therefore, increasing our understanding of the risks, causes, and treatment of ischaemic stroke is of great importance.

Stroke is itself a syndrome cause by a number of different disease processes. About 80% of strokes are ischemic and 20% are due to primary hemorrhage. In this paper we will only address the genetics of ischaemic stroke. While much is known about conventional risk factors such as hypertension, diabetes, and incidence of smoking, studies suggest these only account for a proportion of ischaemic stroke risk. Considerable evidence suggests genetic predisposition may explain some of the remaining risk, including evidence from both twin and family studies [1]. Family studies have shown differential association with different subtypes of stroke, suggesting these may have different underlying genetic risk factors [2, 3].

Further evidence for a genetic contribution to ischaemic stroke risk comes from animal models [4] and from the study of intermediate phenotypes such as carotid artery intima-media thickness (IMT) as a marker for large artery disease and MRI white matter hyperintensities as a marker for small vessel stroke. Twin and family history studies have shown these both have significant heritability (the proportion of stroke risk attributable to genetic risk factors) with estimates ranging from 55–71% for IMT [5–7] and 30–68% for WMH [8–10]. The identification of genetic variants predisposing to known stroke risk factors such as atrial fibrillation (AF) [11] and myocardial infarction (MI) and coronary artery disease (CAD) [12] further highlights the role of genetic predisposition in stroke risk.

The clearest evidence that genetics can cause ischaemic stroke comes from monogenic forms of the disease, although these account for only a relatively small percentage of overall ischaemic stroke incidence [13] and appear to have limited relevance to common polygenic stroke. As such they will not be considered as part of this paper in detail, but are covered in reviews elsewhere [14]. Therefore, considerable evidence suggests genetic factors do play an important role

in ischaemic stroke, so why have so few genes been identified that contribute to this risk and why have other fields, including related cardiovascular disease phenotypes, been more successful?

2. Identification of Genetic Risk: Candidate Gene and Familial Linkage Studies

Until recently, identification of genetic variants contributing to disease has been attempted by 2 main techniques—candidate gene studies and familial linkage studies (See Box 1 for details of the different types of genetic investigation and their use). Of these, the candidate gene study has been the mainstay of genetic investigation into the vast majority of polygenic diseases thought to have a genetic component. Typically, a gene identified as a "candidate" is hypothesised to be involved in stroke risk, and then, genetic variants, usually single nucleotide polymorphisms (SNPs), are identified within that gene. The frequency of the SNPs is then determined in a series of cases and controls and the two compared.

The vast majority of candidate gene studies in ischaemic stroke have turned out to be disappointing. Reasons for this include insufficient sample size, a failure to replicate results initially reported as significant, poor stroke subtyping or phenotyping, and a failure to look for associations with specific subtypes of stroke [15]. Meta-analysis of published candidate gene studies has revealed some consistently positive findings however, such as Factor V Leiden Arg506Gln, MTHFR C677T and the ACE insertion/deletion polymorphism [16], although caution is required in interpretation due to the possible effect of publication bias meaning positive studies are more likely to be published. Although still useful when explaining specific hypotheses, candidate gene studies have now been largely superseded by the genome-wide association study (GWAS) technique.

Familial linkage studies examine genetic variants through multiple generations of families and correlate these with disease incidence. Associations with a specific gene are not sought using this approach, but rather one looks for variants anywhere in the entire genome, and they are therefore referred to as "nonhypothesis driven" experiments. The technique has had, and continues to have, great success in identifying genes underlying Mendelian disorders in monogenic conditions where a single gene contributes the entirety of genetic risk. Familial linkage studies rely on collection of families with the disease however, and this is a challenge in stroke where the late age of onset means parents are often not alive; this has hampered collection of cases in studies such as the siblings with ischaemic stroke study (SWISS) [17, 18].

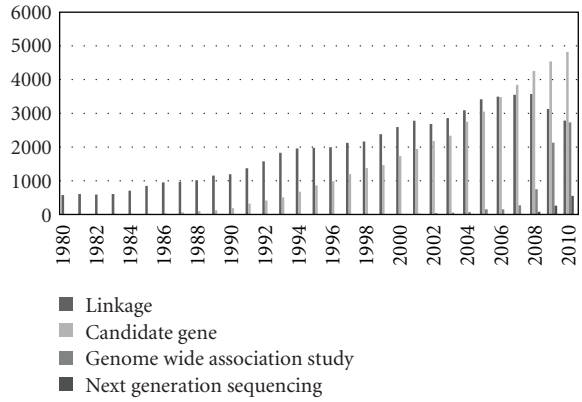
One notable exception to this has been in Iceland, where the DeCode group reported identification of the first genetic risk for common polygenic ischaemic stroke via such a familial linkage study, which they named *STRK1* [19]. This study used the unique national collection of genealogical samples and family structures tracked in the Icelandic population to retrospectively determine cause of death and provide material for genotyping. The *STRK1* locus was identified as overlying the gene phosphodiesterase4D (PDE4D), a cyclic AMP regulator which is a plausible biological candidate [19]. Subsequent replication in European cohorts failed to confirm these findings [20]. This study was undertaken as large-scale genome-wide experiments were being developed as a mainstream technique. By current standards the DeCode finding would today be considered underpowered as it failed to exceed the currently agreed statistical threshold for such studies.

3. Identification of Genetic Risk: The Genome Wide Association Study

The field of complex genetics has been revolutionized by the advent of the genome-wide association study (GWAS) [21]. This can be thought of as a large series of candidate gene studies performed in a single experiment on an array based format. As many as 1.2 million polymorphisms at a time can now be studied in this manner. Crucially, these are spread throughout the entire genome and such experiments are thus nonhypothesis driven, overcoming a major limitation of the candidate gene study. Such a large number of experiments in a single study requires a large sample sizes to allow sufficient power, even after statistical correction for multiple comparisons. Also crucial to progress has been the realisation that careful phenotyping is important, and that associations should be replicated in a second population before publication.

An early demonstration of the power of this technique was in age-related macular degeneration, a late onset eye disorder leading to blindness in which conventional cardiovascular risk factors play a part. Applying a GWAS approach to this condition revealed associations with the complement factor H gene, and identification of a single amino acid substitution which proved to be the causal variant in this condition [22]. Interestingly, the same locus had been identified by a familial linkage approach in previous studies, but refinement of the region and identification of the causal variant via familial linkage had been impossible.

As a consequence of this and other studies, the enormous potential of GWAS to identify common variants associated with common diseases became recognised, with perhaps the seminal GWAS publication by the Wellcome Trust Case Control Consortium 1 study making GWAS a mainstream technique in disease gene identification [23]. This study examined 14,000 cases of seven common diseases and 3,000 shared controls in an effort to identify genetic variants in human disease. Investigating bipolar disorder, coronary artery disease, Crohn's disease, hypertension, rheumatoid arthritis, and type I and type II diabetes, this single study identified over 58 novel loci as potentially contributing genetic risk in these conditions. To date, the GWAS technique has identified over 1212 new genetic loci predisposing to common polygenic disease (http://www.genome.gov/gwastudies). Novel genetic associations with a range of cardiovascular phenotypes including myocardial infarction, coronary artery disease, diabetes and hyperlipidaemia have been reported, but few variants have been confirmed for ischaemic stroke.



The figure shows number of publications per year identified with the search terms "genetic linkage", "candidate gene", "genome wide association study", and "next generation sequencing" according to PUBMED as of March 2011. The figure shows linkage studies appear to have peaked and are now in decline, while the term candidate gene is still in common usage, although it now typically refers to any gene identified as a plausible cause of disease however identified rather than as a type of study in its own right. The term "genome wide association study" has the steepest gradient indicating a rapid reporting of findings using this technique, and next generation sequencing is too new a term to have really made an impact on the literature yet.

Genetic Linkage. A technique which examines genetic variants through multiple generations of the same family and examines incidence with disease status. A powerful technique for Mendelian diseases where a single gene controls the phenotype, genetic linkage has had relatively little success in ischaemic stroke due to the late age of onset and lack of large pedigrees with multiple affected individuals available for meaningful study.

Candidate Gene Study. A gene hypothesised to be involved in or a risk factor for any disease with a genetic basis. Originally candidate gene study referred to a specific type of study whereby genes were typically identified on the basis of biological plausibility for the disease in question and then examined single for genetic variants at that one locus in a so called hypothesis driven manner. More recently the advent of candidate gene has evolved to include any gene identified from range of genetic investigations rather than referring to a particular type of study specifically. Its usage in the literature is commonplace and maintained for this reason.

Genome Wide Association Study. A technique which looks at multiple genetic variants, typically between 300,000 and 1 million at a time in a single experiment, in a nonhypothesis driven manner. The variants are spread throughout the genome at random and allows systematic investigation of a large number of regions in cases and controls. The technique relies on association of specific alleles with disease state. Unlike genetic linkage, individuals are not related, thus requiring large, typically several thousand, cases and controls for comparison. Although complex and expensive, this technique has identified more loci than any other for common polygenic disease under the Common Variant Common Disease (CVCD) hypothesis.

Next Generation Sequencing. Defined as next generation since it uses the same array based format as genome-wide association arrays rather than more traditional liquid based Sanger sequencing, NGS is currently the cutting edge technology available for direct sequencing of DNA. Allowing determination of the entire exome (coding portion) of the human genome in a single experiment, NGS is being used to examine the Rare Variant, Common Disease (RVCD) hypothesis. Although expensive, since it relies on the identification of rare variants far fewer individuals are required for studies. Current advances in multiplexing of samples by labeling with genetic tags before sequencing allows mixing of samples and a reduction in cost.

Box 1: Types of analysis for genetic investigation.

It should be noted that while GWAS is a powerful technique, it requires very large, well phenotyped case series—typically in the thousands of samples, and even with these sample sizes is powered only to detect modest risks, typically with odds ratios in the region of 1.2–1.5. Thus the contribution of each risk locus to overall disease incidence is likely to be minor, although these risks are additive and as such identification of multiple loci may allow individual risk profiles to be determined. Identification of high risk individuals could be useful in early intervention to reduce conventional risk factors, more rigorous screening for early signs of disease and in investigating severity of disease at onset as well as associations with disease recurrence.

4. GWAS and Ischaemic Stroke

While GWAS has contributed greatly to identification of genetics of many complex diseases over the last 5 years, application of the technique to ischaemic stroke has been slower, with large-scale collaborative efforts only now beginning to emerge. An early study applied the GWAS technique to 249 ischaemic stroke cases and 268 controls, but we now

realize this was underpowered [24]. A more recent study in prospective population-based cohorts identified a region on Chromosome 12 overlying the NINJ2 gene in ischaemic stroke cases [25], although a subsequent large replication failed to confirm this finding [26].

The collection of large, well phenotyped sample cohorts for genetic analysis in stroke presents major challenges. In particular phenotyping, which we now realize is essential, requires detailed and expensive investigations. As in other complex diseases, collection of sufficiently large sample sizes depends on larges scale international collaborations, and to address this the International Stroke Genetics Consortium (ISGC—http://www.strokegentics.org/) was established. Currently an ischaemic stroke GWAS in 4000 cases is near completion as part of the Wellcome Trust Case Control Consortium 2 study (WTCCC2) in collaboration with the ISGC. GWAS studies in countries including the US and Australia are also ongoing with results expected in 2011. A lesson from other disease areas is that, even with sample sizes of several thousands, power is limited and meta-analysis of multiple GWAS studies has become standard practice. The Meta-stroke collaboration has been formed in ischaemic stroke to address this.

These collaborative efforts have achieved early success in ischaemic stroke via examination of genetic associations already identified in related cardiovascular diseases. The identification of a region on Chromosome 9p21 in myocardial infarction and coronary artery disease, which surrounds the CDKN2A and CDKN2B genes, has generated a large amount of interest [27]. An examination of this locus in a candidate gene study in ischaemic stroke cases revealed an association with large artery stroke, but not the other ischaemic stroke subtypes [28]. This association persisted across multiple populations and importantly emphasises the likely differing contributions of genetic risks to different ischaemic stroke subtypes.

Two genetic variants identified as contributing to the risk of atrial fibrillation (AF), in the genes PITX2 and ZFHX3, have also been shown to associate with cardioembolic stroke risk for which AF is an important risk factor [29, 30]. As new loci are identified for other cardiovascular diseases which themselves are associated with stroke, rapid testing these in stroke populations via large International collaborations is possible.

5. Recommendations for Future Genetic Studies in Stroke

Previous studies in stroke genetics have been disappointing. There are a number of reasons for this, most significant of which are poor phenotyping, small sample sizes, and failure to replicate initial findings in a second population. Any future genetic study, whether hypothesis driven or nonhypothesis driven, should address each of these issues prior to publication. Power calculations demonstrating the number of cases required for confirmation or refutation of a finding should be included to allow an estimate of the significance and robustness of the findings presented. Genetic risks in stroke are usually estimated to be between 1.1 and 1.5 for a single loci, and studies should be adequately powered (i.e., be comprised of sufficient cases) to detect risks of this size. Replication of positive associations prior to publication is important. This should be in a separate case series using a different control set.

Increasing evidence suggests genetic risks differ depending on ischaemic stroke subtype. Future genetic studies should therefore include reference to subtypes and subtype specific risks. Evidence of genetic risk in a homogenous population of ischaemic stroke without subtype investigation is likely to lead to spurious associations.

While these measures lead to increased cost and complexity of studies, it is only through such robust experimental procedures that we will truly begin to understand the genetic risks of stroke and how these are manifest.

6. The Post GWAS Era in Stroke Genetics

Genome-wide association studies have been specifically conceived to address the common variant, common disease (CVCD) hypothesis. This concept underlies the majority of genetic studies to date not just in stroke but in other common diseases. According to the CVCD hypothesis, multiple genetic risk factors contribute to disease, each with a small additional increase in risk. These risks are additive in nature and together provide an individual risk profile that allows for a significant genetic contribution. In order for this hypothesis to hold true however, variants have to be common in the population.

Despite the success of GWAS in identifying susceptibility loci, for the vast majority of diseases these account for only a fraction of the heritability initially attributed to genetic risk factors. Each risk identified carries a much smaller risk than originally thought under the CVCD hypothesis. For this reason the CVCD theory of genetic risk is now being questioned, and various mechanisms have been suggested to account for this "missing heritability" [31]. An alternative hypothesis is that rare variants are important in common disease risk (RVCD). This states that as well as common variants that each contribute very small risks, susceptible individuals may carry variants of higher risk which are rare and perhaps even private to themselves or closely related family members. Such risks would not be detectable via classical familial linkage since there would be multiple risk variants contributing to an individuals susceptibility to disease, but neither would they be detectable via GWAS since they would be specific to individuals or closely related family members and therefore not carried by the rest of the population. Under this hypothesis any one individual would be expected to carry many variants detectable by GWAS, and a handful of higher risk alleles in a private manner. Together these combine to produce an individuals overall risk profile of disease susceptibility, and may account for the so called "hidden heritability" conundrum which persists after GWAS. Identification of these rare variants requires a sequencing approach which provides information on every base pair across the region of interest, and this approach is provided by next generation sequencing (NGS).

NGS has arisen from both advances in technology, and from our ability to sequence the human genome as a consequence of the human genome project (http://www.hapmap.org/) and the 1000 genome (http://www.1000genomes.org/) project among others. It is now possible to obtain the entire coding sequence of the human genome in less than a week, and to make comparisons between genomes due to advances in computational methods and processing power. Sequencing of targeted regions at a previously unparalleled depth and fold coverage without the need for generation of vector libraries or bacterial culture is now routine and provided by a number of service providers using a variety of techniques [32]. While currently expensive, such techniques give access to perhaps the majority of the information that conventional genetics can be expected to provide, namely the entire coding sequence of the genome. Interpretation of that sequence is still in its relative infancy.

7. Beyond Genomics in Identifying Ischaemic Stroke Risk Factors

Understanding the genetic basis of disease risk requires an understanding of the way in which these genes have their effects in the body. Genes code for RNA, which is then translated into protein. These proteins can act alone, in multiples of themselves as homodimers or in conjunction with other proteins to form heterodimers. Similarly genes may interact with each other, so called epigenetics, or may interact with environmental factors in gene-environment interactions which only affect disease risk when both the environmental and genetic components of the interaction are present. Detecting such gene-gene or gene-environment interactions requires much larger sample sizes. Their importance has been shown in other cardiovascular diseases [33] and in association studies with the quantitative trait continuous carotid IMT [34].

Examination of RNA and proteins in a nonhypothesis driven manner, similar to GWAS for DNA, is also possible. Examination of the transcriptome is a relatively old technique via the use of microarrays, and it is this technology which actually led to the development of GWAS using DNA slides or cartridges rather than RNA and cDNA-based ones. More recently studies have been examining the possibility of ignoring the DNA level and trying to perform transcription profiling (examination of all the RNA's being produced at a specific time point). By examining changes in the level of transcription of a subset of RNAs and correlating these with changes in disease state, disease subtype or disease severity, we may be able to better understand how genetic differences influence disease processes [35].

There are reports that it is possible to differentiate between different stroke subtypes using this methodology, with expression levels of just 23 genes being able to differentiate between cardioembolic stroke and large vessel disease [36]. While such studies are not yet able to replace conventional investigative techniques for determining ischaemic stroke subtype, identification of a expression profiles may give novel insights into stroke pathogenesis, and perhaps identification of suitable biomarkers for monitoring risk reduction treatments. This technique has also been applied to associated phenotypes such as white matter hyperintensity in the brain, currently only detectable by MRI [37].

8. Conclusions

Considerable evidence suggests genetic factors are important in ischaemic stroke risk. The advent of new techniques such as GWAS has contributed enormously to the understanding of the genetics of other complex disease and progress is just beginning to be made in stroke. For success large, well phenotyped case cohorts are required, and international collaborations are essential. NGS technology and techniques such as transcription profiling and proteomics will allow us to look for rarer variants in stroke cases and attempt to identify how these exert their effects at the molecular level, but whether these will be important remains to be determined.

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Review Article

Genetics of Atrial Fibrillation and Possible Implications for Ischemic Stroke

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Atrial fibrillation is the most common cardiac arrhythmia mainly caused by valvular, ischemic, hypertensive, and myopathic heart disease. Atrial fibrillation can occur in families suggesting a genetic background especially in younger subjects. Additionally recent studies have identified common genetic variants to be associated with atrial fibrillation in the general population. This cardiac arrhythmia has important public health implications because of its main complications: congestive heart failure and ischemic stroke. Since atrial fibrillation can result in ischemic stroke, one might assume that genetic determinants of this cardiac arrhythmia are also implicated in cerebrovascular disease. Ischemic stroke is a multifactorial, complex disease where multiple environmental and genetic factors interact. Whether genetic variants associated with a risk factor for ischemic stroke also increase the risk of a particular vascular endpoint still needs to be confirmed in many cases. Here we review the current knowledge on the genetic background of atrial fibrillation and the consequences for cerebrovascular disease.

1. Introduction

Of all cardiac arrhythmias, atrial fibrillation (AF) is the most common, affecting approximately 1-2% of the population [1]. The prevalence is higher in men compared to women and increases with age, which is reflected by the finding that 25% of the population aged over 40 will develop AF [2]. Patients with AF frequently have other cardiovascular and noncardiovascular comorbidities, the most important condition being hypertension [3], which is an important risk factor for the development of AF [4]. AF is not a benign disease as it is associated with increased rates of death, stroke, ischemic heart disease, heart failure, and peripheral thrombo-embolic events. In patients with AF, various independent factors raise the risk of stroke such as the presence of hypertension, advancing age and diabetes and the previous occurrence of a stroke or transient ischemic attack (TIA) [5, 6]. Epidemiological studies have identified various risk factors for AF which include age, male sex, hypertension and the presence of structural heart abnormalities. However, it was suspected that the totality of the risk could not be explained exclusively by these factors, and a genetic risk component was suspected [7]. Although the vast majority of AF is sporadic and nonfamilial, familial (hereditary) forms of AF have been identified (Table 1). Also, the genetic background of AF in the general population has been studied through association studies (Table 1). Since stroke is a major complication of AF, genetic variants associated with this arrhythmia may be implicated in ischemic cerebrovascular disease. We present an overview of the current knowledge in the monogenic forms and complex genetics of AF and discuss the consequences for ischemic stroke.

2. Pathophysiology

Various structural cardiac abnormalities can result in a process of remodeling in the ventricles and atria. This remodeling is characterized by the proliferation of myofibroblasts

Table 1: Genetics of atrial fibrillation.

	Gene/Locus	Mechanism of action	Study design/Inheritance
Sodium channels	SCNA5 [8–17]	Cellular hyperexcitability (gain-of-function) as well as prolongation of the atrial action potential duration (loss-of-function)	Candidate gene/Familial and sporadic
	SCN1B/SCN2B [18]	Decreased peak sodium current amplitude	Candidate gene/Sporadic
Potassium channels	KCNQ1 [19–22]	Enhanced atrial action potential repolarization	Linkage/Familial
	KCNE2 [23]	Enhanced atrial action potential repolarization	Candidate gene/Familial
	KCNJ2 [24]	Enhanced atrial action potential repolarization	Candidate gene/Familial
	KCNE5 [25]	Enhanced atrial action potential repolarization	Candidate gene/Familial and sporadic
	KCNA5 [26–28]	Delayed atrial action potential repolarization	Candidate gene/Familial and sporadic
Other	NPPA [29]	Shortening of the atrial action potential duration	Linkage/Familial
	GJA5 [30–32]	Dispersion of conduction velocity	Candidate gene/Sporadic
	10q22 [33]	Unknown	Linkage/Familial
	6q14–16 [34]	Unknown	Linkage/Familial
	5p15 [35]	Unknown	Linkage/Familial
	4q25 (PITX2) [36, 37]	Unknown	Genome wide association/Sporadic
	16q22 (ZFHX3) [38]	Unknown	Genome wide association/Sporadic
	1q21 (KCNN3) [39]	Unknown	Genome wide association/Sporadic

and the development of fibrosis. Interstitial fibrosis leads to electrical uncoupling and conduction slowing, which promotes AF. If atrial fibrillation persists for several days, electrical and structural remodeling occurs promoting further maintenance of AF [40, 41]. Electrophysiologically several mechanisms have been identified which trigger and maintain the arrhythmia.

The mechanisms by which AF becomes thrombogenic are complex and have not been completely elucidated. It has become clear that the mere presence of blood stasis is not a sufficient explanation for thrombus formation. Various alterations related to AF and associated comorbidities as abnormal changes in flow, vessel wall, and blood components, probably drive a hypercoagulable state [42].

Considering the pathophysiology and taking into account the suspected genetic background in the disease it could be assumed that mutations or variants in genes of ion channels, which are of importance in atrial electrophysiology, are implicated in AF. This has been confirmed by linkage, candidate, and genome-wide genetic association studies.

3. Monogenetic Forms of AF

3.1. Familial AF. Studies aimed to identify the pathogenesis of AF have focused on the familial forms of the disease. In these rare familial forms different loci, as well as mutations in a range of genes have been linked to AF. Various mutations in genes encoding sodium channels (SCN5A, SCN1B, and SCN2B), potassium channels (KCNQ1, KCNE2, KCNJ2, KCNE5, and KCNA5), natriuretic peptide (ANP) and Connexin 40 have been identified. Additionally, the culprit genes at loci on chromosome 10q22 [33], 6q14-16 [34], and 5p15 [35] have yet to be found.

3.1.1. Sodium Channel Mutations. The human cardiac sodium channel (SCN5A) is responsible for fast depolarization of cardiomyocytes and has been a therapeutic target for antiarrhythmic drugs. Initially mutations in SCN5A were identified in families with long QT syndrome [8]. Over the years, more than 200 mutations have been reported in SCN5A which are associated with variable cardiac diseases like Brugada syndrome, progressive conduction defect, sick sinus node syndrome, dilated cardiomyopathy and AF [9]. Genotype-phenotype correlations revealed that most mutations are linked to specific clinical spectrums, but that clinical overlaps exist for the same genetic defects [10]. Both Brugada syndrome and long QT syndrome can be complicated with supraventricular arrhythmias which often include AF [11]. More evidence for the role of mutations in SCN5A in the pathophysiology of AF was provided by the identification of a family with a dilated cardiomyopathy and AF carrying a mutation in SCN5A [12]. Additionally, novel mutations in the same gene were reported in familial forms of AF with and without structural cardiac disease [13–16]. It was determined that rare variants in SCN5A are present in nearly 6% of AF probands [14]. In two studies, functional analysis of the mutation showed a depolarizing shift in steady-state inactivation resulting in cellular hyperexcitability (gain of

function) [15, 16]. A loss of function was suggested by the study of another variant which revealed a hyperpolarizing shift in steady-state inactivation resulting in prolongation of the atrial action potential duration [13]. This delayed atrial repolarization could induce atrial torsades resulting in AF. Different mechanisms, both loss of function as well as gain of function, have been suggested in various syndromes. Furthermore, there is a wide spectrum of mutations which are associated with overlapping syndromes, suggesting environmental or other genetic factors to be of importance in determining the phenotype.

Interestingly, nonsynonymous variants were identified in other sodium channel subunits, two in SCN1B and two in SCN2B, in patients with AF which were absent in controls [18].

3.1.2. Mutations in Potassium Channel Genes. The voltage-gated potassium current has a prominent role in the repolarization of the atrial action potential. Other potassium channels are of importance for the inward rectifier currents and the control of the resting potential. Multiple gain-of-function as well as loss-of-function mutations within potassium channel genes have been identified. A mutation in KCNQ1, a gene encoding the pore-forming α subunit of a cardiac voltage-gated potassium channel, was originally documented as the causative gene for long QT syndrome [19]. A mutation in the same gene was the first identified genetic defect linked to lone AF [20]. It was hypothesized that the pathogenic mechanism consisted of a gain of function by increasing the repolarizing current and shortening of the atrial action potential duration [20]. Various other gain of function mutations have subsequently been identified in isolated families in KCNQ1 [21, 22] as well as in other potassium channel genes, including KCNE2 [23], KCNJ2 [24], and KCNE5 [25]. Additionally, in another potassium channel gene, KCNA5, loss-of-function mutations were identified in a small number of families [26–28]. Functional analysis of this mutation revealed delayed action potential repolarization and prolongation of the atrial action potential duration (a mechanism similar as reported for a SCN5A mutation [13]).

3.1.3. Atrial Natriuretic Peptide (ANP). In a linkage study of a family with autosomal dominant AF, a mutation in the natriuretic peptide precursor gene (NPPA) was identified [29]. The mutation produced the loss of a stop codon resulting in the expression of a longer peptide. The concentration of the mutant peptide was 5 to 10 times increased in the plasma compared to the wild type suggesting a longer half-life potentially due to resistance to degradation. In an isolated whole-heart model, the mutant peptide caused shortening of the atrial action potential duration (analogous to what is reported for most of the potassium channel mutations).

Another possible pathophysiological mechanism of the mutation might involve structural changes resulting in atrial fibrosis due to exposure to high concentrations of (mutant) ANP [29].

3.1.4. Connexin 40. The initially discovered genes found in familial AF were a rare cause of lone AF [43, 44]. Therefore, additional research focused at other pathogenic pathways. Attractive genes included the connexins, which are proteins that form gap junctions and provide low-resistance electrical coupling between cells. Especially the gene GJA5, coding for connexin 40, gained interest since it is expressed at high levels in atria. Furthermore, conduction disturbances have been identified in GJA5-knockout mice resulting in atrial vulnerability and possibly arrhythmogenesis [45]. Sequencing of genomic DNA from cardiac tissue of 15 patients with idiopathic AF indeed identified four missense mutations, of which three were thought to be of somatic origin. The mutated protein was heterogeneously distributed in atrial tissue and a loss-of-function was hypothesized [30], resulting in exaggerated dispersion of conduction velocity predisposing to and sustaining AF. Moreover, a similar genetic mosaicism in atrial tissue linked to AF was recently reported in one patient with lone AF for the GJA1 gene, coding for the connexin 43 protein [46].

3.2. AF in Other Inherited Disorders. In several genetic cardiac syndromes, both with and without structural abnormalities, AF has been described as part of the clinical spectrum. Short and long QT and Brugada are associated with supraventricular arrhythmias, which often comprise AF. Various other inherited cardiac disorders are characterized by the occurrence of AF as hypertrophic cardiomyopathy, ventricular preexcitation and abnormal left ventricle hypertrophy linked to mutations in the PRKAG gene [11].

4. Complex Genetics of AF

Whereas genetic studies in familial forms of AF have implicated various genes in a limited number of cases, epidemiologic studies in the general population have documented a genetic component to AF in sporadic AF. Parental AF was shown to increase the risk for future AF, an association which was stronger at younger age and for lone AF [47–49]. Candidate gene studies in sporadic AF investigating common variants in the genome suggested a role for polymorphisms in regions implicated in familial AF [17, 31, 32, 50–54] as well as others [55–63]. An association for a common polymorphism in SCN5A with paroxysmal AF was reported, although replication of this finding is lacking [17]. Common variants in GJA5 have also been shown to associate with atrial fibrillation in sporadic AF [31, 32]. The sample sizes in most of these studies were relatively small and large replication studies were lacking. Meta-analyses of studies determining the role of polymorphisms in the renin-angiotensin system-related gene with AF suggested a possible association between angiotensin converting enzyme (ACE) insertion/deletion and AF risk [64, 65]. However a large replication study recently failed to replicate any of the reported associated polymorphisms at this locus or any other locus [66].

Since most of the candidate gene studies have not been successful, results of genome-wide studies were anxiously awaited as for many complex diseases [67]. Two sequence variants on chromosome 4q25 were identified in the first reported genome-wide association study in AF [36]. These

variants have been replicated extensively. Two additional variants in this region were found, and the possibility of multiple susceptibility signals at this locus was hypothesized [37]. The reported variants were adjacent to the PITX2 gene, a transcriptional factor critical for determining left-right asymmetry and for the differentiation of the left atrium [68]. Increasing the initial sample size revealed an additional sequence variant in the ZFHX3 gene in chromosome 16q22 as a risk factor for AF [38]. A third locus was identified on chromosome 1q21 in KCNN3, a gene which encodes a potassium channel protein involved in atrial repolarization [39]. The variant rs2200733 on 4q25 remained the variant with the largest effect size, with an estimated odds ratio in the range of 1.80 (95% CI 1.50–2.15) [37]/1.90 (95% CI 1.60–2.26) [69]. Increasing sample sizes in genetic association studies will potentially reveal yet unknown loci implicated in AF.

5. Implications for Ischemic Stroke

Ischemic stroke can be categorized into several etiological subgroups: cardioembolic stroke, large-artery atherosclerosis, small-vessel disease, undetermined (cryptogenic, multiple causes, or incomplete evaluation), or other causes [70]. Approximately 20% of ischemic strokes are due to cardiac embolism mainly caused by atrial fibrillation (AF), which can be either paroxysmal or persistent with both variants resulting in a similar stroke risk [71]. It is thought that some strokes of undetermined origin are actually due to undiagnosed paroxysmal AF [72]. Since stroke can be a clinical complication of sporadic AF, the risk of stroke is also increased in familial cases of AF. Other inherited cardiac disorders characterized by AF can also present with stroke. In hypertrophic cardiomyopathies, for instance, the stroke risk is increased and this is directly linked to the occurrence of AF [73].

Stroke is considered a complex disease where a genetic component in the etiology is suspected. Candidate gene studies in stroke have resulted in largely disappointing findings [74]. Findings of a large genome wide association study in stroke could not be replicated [75, 76]. An association was reported in a genome-wide association study in stroke for the initially reported risk variant rs2200733 on 4q25 with AF, OR 1.26 (95% CI 1.17–1.35) [77]. This association was most pronounced in the cardioembolic subgroup of stroke. We replicated these findings and identified this variant exclusively in cardioembolic stroke, suggesting that this variant is indirectly linked to stroke through AF [78]. The effect size of this genetic variant in cardioembolic stroke is somewhat smaller than what has been reported for AF, OR: 1.52 (95% CI 1.35–1.71) [77] and 1.47 (95% CI 1.28–1.71) [78]. This could be due the fact that in most selected controls, stroke-free status was ensured while AF-free status was not determined. Additionally, not all patients with cardioembolic stroke are diagnosed with AF (in our replication study this percentage was 61%). Similar findings were obtained for the variant on 16q22 which also confers risk for primarily cardioembolic stroke [38].

5.1. Influence of Cryptogenic Stroke?

The initial report on the association of the 4q25 locus with stroke stated that in subgroup analysis, the association of the 4q25 locus with stroke remained after all patients who suffered from cardioembolic stroke were excluded [77]. This study included five study populations totaling 29474 controls and 6222 patients. Our replication study was performed in six different populations with 3750 controls and 4199 patients. All studies included hospital-based populations (apart from the controls in one cohort in the discovery study). Phenotyping was performed according to similar protocols. Potentially the lack of replication after excluding stroke of cardioembolic embolism could be due to the somewhat smaller sample size. Additionally the findings of the initial report after excluding cardioembolic stroke might be caused by phenotypic misclassification or ascertainment bias: patients classified as having a stroke of undetermined etiology could in reality have a stroke of (unidentified) cardioembolic etiology because AF was not detected during the hospital stay or because the investigation into cardioembolic sources was not extensively performed. In our replication study, the variants on 4q25 lacked association with true cryptogenic stroke, arguing against a large percentage of these patients suffering from unrevealed cardioembolic stroke. This analysis was not performed in the initial report. Additionally in our replication study, we identified an interaction between cardioembolic stroke and the risk variant on 4q25 supporting the hypothesis that the relation is mainly caused by this subtype of stroke.

Since the genotyping of these variants has become commercially available, it might be tempting to offer patients with stroke of cryptogenic etiology genetic testing for AF inducing variants. Patients carrying the risk variant (of which the allele frequency is estimated to be 10% in the general population) could, for example, be more extensively studied for paroxysmal AF. Our results do not provide evidence for performing such individually based genotyping since no association was identified in cryptogenic stroke patients.

5.2. Stroke as a Phenotype or Stroke as a Conglomerate of Phenotypes. The findings from these genetic association studies underline that genetic risk factors may predispose to a particular subgroup of stroke. The fact that a variant on chromosome 9 associated with myocardial infarction and coronary artery disease also is associated with stroke due to large-vessel atherosclerotic disease emphasizes this assumption [79, 80]. Stroke is a heterogeneous disorder, and this heterogeneity might be the reason for the disappointing results in genetic association studies in the stroke field so far. It can be assumed that genetic risk factors are implicated only in subgroups of stroke, and, therefore, analyzing each subgroup is of importance. In order to obtain convincing results from GWAS, accurate stroke phenotyping and large sample sizes for each stroke subtype will be required.

5.3. Stroke as a Vascular Endpoint. Stroke can be regarded as the clinical outcome of other underlying diseases; this seems

applicable not only to AF and cardioembolic stroke but also for stroke due to large-vessel disease and atherosclerosis. One could argue that data of various disease traits that share underlying pathogenic mechanisms could be combined in meta-analyses of genome-wide association studies to increase power. Moreover, it could be considered to analyze cases of underlying diseases together with cases of the associated clinical outcome. For instance, data obtained in genome-wide association studies in AF can be merged with the data of all cardioembolic stroke cases. Similarly, coronary artery disease, peripheral artery disease and stroke due to large-vessel disease could be jointly examined. This does not entirely exclude the possibility that genetic variants will be identified for ischemic stroke in general. However, the other approaches (combining vascular diseases or merging risk factors and their outcomes) could increase power and could eventually lead to the identification of genetic polymorphisms conferring risk for (subtypes of) stroke. Although typically this will lead to findings of small effect size, this does not imply that the biological relevance cannot be greater than the genetic effect.

In complex diseases (as stroke), it is assumed that various environmental factors together with genetic variants can result in a certain disease phenotype. Interfering with several (but not necessarily all) of these factors might already result in reducing risk and, therefore, less clinical outcomes in the general population. Identifying the pathophysiological meaning of a genetic variant associated with AF could potentially lead to new therapeutic targets in AF and indirectly lead to a reduction in cardioembolic stroke.

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Research Article

Factor VII Activating Protease Polymorphism (G534E) Is Associated with Increased Risk for Stroke and Mortality

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Introduction. The FSAP-Marburg I polymorphism (1704G > A), which reduces FSAP activity, is associated with late complications of carotid stenosis in humans. Therefore, this study examines the influence of the Marburg I polymorphism and the closely linked Marburg II polymorphism (1280G > C) on various cardiovascular outcomes in two large independent study populations. *Methods*. The two Marburg polymorphisms in the HABP2 gene encoding FSAP were genotyped in a large population of elderly patients at risk for vascular disease (the PROSPER-study, $n = 5804$) and in a study population treated with a percutaneous coronary intervention (the GENDER-study, $n = 3104$). *Results*. In the PROSPER study, the Marburg I polymorphism was associated with an increased risk of clinical stroke (HR: 1.60, 95% CI: 1.13–2.28) and all-cause mortality (HR: 1.33, 95% CI: 1.04–1.71). In the GENDER study carriers of this variant seemed at lower risk of developing restenosis (HR: 0.59, 95% CI: 0.34–1.01). The Marburg II polymorphism showed similar but weaker results. *Conclusion*. The increase in stroke risk in Marburg I carriers could be due to differential effects on smooth muscle cells and on matrix metalloproteinases, thereby influencing plaque stability. The possible protective effect on restenosis could be the result of reduced activation of zymogens, which are involved in hemostasis and matrix remodeling.

1. Introduction

Factor seven activating protease (FSAP) is a plasma serine protease known to activate factor VII (FVII) [1] and prourokinase (pro-uPA) [2]. Despite these actions, it is unclear if endogenous FSAP has a relevant role in hemostasis. The Marburg I (1704G > A) polymorphism in the HABP2 gene encoding FSAP, which leads to an amino acid change in the protease domain of this protein, may lead to a prothrombotic phenotype when it is associated with reduced activation of pro-uPA, but unchanged activation of FVII [3]. Although its possible association with venous thrombosis remains controversial [4–6], the Marburg I variant is a risk factor for coronary heart disease [7] and late complications

of carotid stenosis [8]. The Marburg variant is associated with advanced atherogenesis (a lumen narrowing of >40%) but not with early atherogenesis (occurrence of new plaques) [8].

Furthermore, FSAP is identified as a potent inhibitor of smooth muscle cell proliferation and migration [9], specifically through its ability to cleave platelet derived growth factor-BB (PDGF-BB). The FSAP Marburg I variant, which also has reduced proteolytic activity towards PDGF-BB, is associated with a reduced capability to suppress neointima formation in an animal model [10]. This might be another mechanism by which the Marburg I polymorphism could play a role in carotid stenosis and many other aspects of cardiovascular disease [11].

Therefore, we investigated the influence of this polymorphism on clinical stroke, coronary events, vascular mortality, and all-cause mortality in a large population of elderly patients at risk for vascular disease (the PROSPER study, $n = 5804$) and on clinical restenosis after a percutaneous coronary intervention (PCI) (the GENDER study, $n = 3104$). Although not associated with altered enzymatic activity, we also investigated the Marburg II variant (1280G > C), a closely linked polymorphism that leads to an amino acid change in the protease domain of FSAP.

2. Methods

2.1. Study Design and Followup of the PROSPER Study. The protocol of PROSPER has been described in more detail elsewhere [12]. PROSPER is a prospective multicenter randomized placebo-controlled trial to assess whether treatment with pravastatin diminishes the risk of major vascular events in elderly individuals. Between December 1997 and May 1999, we screened and enrolled subjects in Scotland (Glasgow), Ireland (Cork), and the Netherlands (Leiden). Men and women aged 70–82 years were recruited if they had preexisting vascular disease or increased risk of such disease because of smoking, hypertension, or diabetes. A total of 5804 subjects were randomly assigned to pravastatin or placebo. In this genetic substudy, we evaluated the predefined endpoints all-cause mortality, vascular mortality, and the secondary endpoints fatal or nonfatal coronary events and fatal or nonfatal clinical stroke. Median followup was 3.3 (interquartile range 0.5) years and 604 (10.4%) patients died during the study [13].

2.2. Study Design and Followup of the GENDER Study. The present study sample has been described previously [14]. In brief, the GENetic DEterminants of Restenosis project (GENDER) was a multicenter followup study designed to study the association between various gene polymorphisms and clinical restenosis. The overall inclusion period of the GENDER study lasted from March 1999 until June 2001. A total of 3104 patients eligible for inclusion in the GENDER study were treated successfully for stable angina, non-ST-elevation acute coronary syndromes or silent ischemia by PCI in 4 of the 13 referral centers for interventional cardiology in the Netherlands. Patients treated for acute ST elevation myocardial infarction were excluded. Experienced operators, using a radial or femoral approach, performed standard angioplasty with and without stent placement. During the study, no drug-eluting stents were used. Followup lasted for at least 9 months, except when a coronary event occurred. The primary endpoint was clinical restenosis, defined as target vessel revascularization (TVR), either by PCI or coronary artery bypass grafting (CABG). Subjects with asymptomatic restenosis were not included and routine angiography was not obtained. Median followup duration was 9.6 (interquartile range 3.9) months and 304 (9.8%) patients underwent TVR during followup.

For both studies, all endpoints were adjudicated by independent clinical events committees. The protocols meet the criteria of the Declaration of Helsinki and were approved by the Medical Ethics Committees of each participating institution. Written informed consent was obtained from all participating patients.

2.3. Genotyping. Blood was collected in EDTA tubes at baseline and genomic DNA was extracted following standard procedures. The Marburg I (1704G > A) and II (1280G > C) polymorphisms were determined using the Sequenom Massarray genotyping platform (rs7080536 and rs11575688 resp.). A multiplex assay was designed using assay designer software (Sequenom Inc.). As quality controls, 5–10% of the samples were genotyped in duplicate; no inconsistencies were observed. Cluster plots were made of the signals from the low and the high mass allele. Two independent researchers carried out scoring. Disagreements or vaguely positioned dots produced by Genotyper 4.0 (Sequenom Inc.) were left out of the results.

2.4. Replication. We performed in silico replication in the genome-wide association study on stroke from the Rotterdam Study [15]. Genotyping was performed using the Illumina 550 K Beadchip in 5763 study participants with subsequent imputation to 2.5 million HapMap II SNPs. Marburg I polymorphism was not present nor were any proxies of the Marburg I polymorphism with $r^2 > 0.5$. Marburg II polymorphism was present in the imputed dataset with an imputation quality >0.8. The association between this SNP and incident stroke was assessed in 367 incident ischemic stroke cases.

2.5. Statistical Analysis. Allele frequencies were determined by gene counting. The Chi-squared test was used to test the consistency of the genotype frequencies at the SNP locus with Hardy-Weinberg equilibrium. Hazard ratios (HR) with 95% confidence intervals (CI) were calculated using a Cox proportional hazards model. All analyses were adjusted for sex and age. The analyses with PROSPER data were additionally adjusted for pravastatin use and country. In the GENDER study, polymorphisms were included in a multivariable model containing clinical and procedural risk factors for restenosis, such as diabetes, stenting, and total occlusion. The replication was assessed with a logistic regression model adjusted for sex and age. The SPSS software (version 17.0.1, SPSS Inc, Chicago, ILL) was used for all statistical analyses.

Table 1: Baseline characteristics of the PROSPER and the GENDER study in patients with Marburg I genotype.

	PROSPER $n = 5697$	GENDER $n = 2957$
Continuous variates (mean, SD)		
Age (years)	75.3 (3.4)	62.1 (10.7)
Body mass index (kg/m^2)	26.8 (4.2)	27.0 (3.9)
Categorical variates (n, %)		
Male sex	2752 (48)	2110 (71)
Current smoker	1529 (27)	730 (25)
History of diabetes	602 (11)	434 (15)
History of hypertension	3530 (62)	1189 (40)
History of myocardial infarction	764 (13)	1176 (40)
History of stable angina	1529 (27)	1977 (67)
Genotype, minor allele frequency (%)		
Marburg I G/A	5	4
Marburg II G/C*	2	2

All data are presented in number (%) unless otherwise stated.
*In PROSPER and GENDER measured in 5655 and 2959 participants, respectively.

3. Results

3.1. The PROSPER Study. Table 1 presents the patient characteristics and minor allele frequencies. Genotyping success rates were 98% and 97% for the Marburg I and II polymorphisms, respectively, and there were no significant deviations from Hardy-Weinberg equilibrium.

Using a Cox proportional hazards model, which included the variables sex, age, pravastatin use, and country, a significant association was found between the Marburg I polymorphism and clinical stroke. Figure 1 shows that the combined group of heterozygotes ($n = 518$) and homozygotes ($n = 17$) was at increased risk for clinical stroke (HR: 1.6, 95% CI: 1.13–2.28, $P = .009$) when compared to the wild type group ($n = 5162$). Also, all-cause mortality was significantly higher in patients carrying one or two copies of this variant (HR: 1.33, 95% CI: 1.04–1.71, $P = .025$). The increased mortality was mainly a result of an increase in vascular mortality (HR: 1.37, 95% CI: 0.96–1.97, $P = .082$), whereas vascular mortality was mainly determined by death from stroke. The Marburg I polymorphism did not seem to influence the risk for coronary events (HR: 0.98, 95% CI: 0.75–1.29). Additional adjustment for traditional risk factors (hypertension, diabetes, smoking, and cholesterol levels) did not change the results (HR: 1.63, 95% CI: 1.14–2.31, $P = .007$).

Due to its location in the same coding sequence and its proximity to the Marburg I polymorphism we also present data of the Marburg II polymorphism which show significant effects on mortality (Figure 2). The similar trends observed for this polymorphism are probably a result of the high linkage disequilibrium between the Marburg variants. The linkage disequilibrium coefficient was 0.79, and 62% of the Marburg II carriers were also genotyped with the Marburg I allele. Due to a lower allele frequency of the Marburg

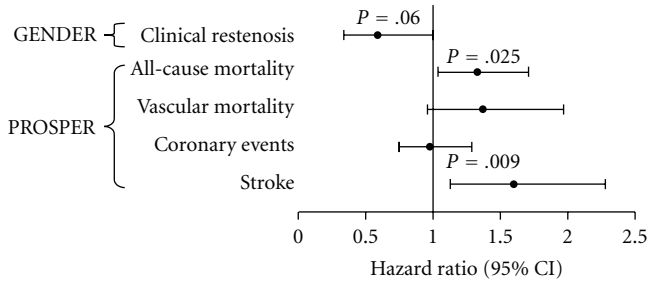

Figure 1: Marburg I hazard ratios for vascular endpoints in GENDER and PROSPER. The Marburg I (G534E) polymorphism is associated with an increased risk for stroke and mortality in the PROSPER study, whereas it tends to reduce the risk for clinical restenosis in the GENDER study.

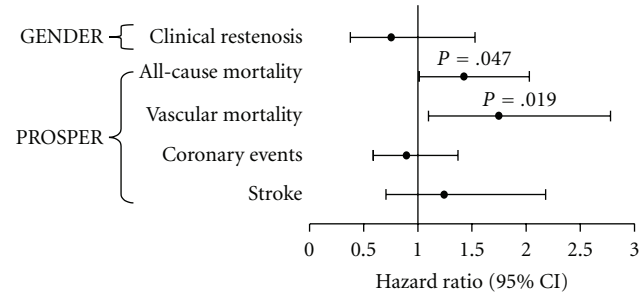

Figure 2: Marburg II hazard ratios for vascular endpoints in GENDER and PROSPER. As the Marburg II polymorphism does not lead to altered enzymatic activity, the similar trends that were observed for this variant are probably a result of the high linkage disequilibrium between the Marburg variants. Despite high confidence intervals, a significant effect was observed on mortality.

II polymorphism, there were only 234 heterozygotes and 4 homozygotes with the variant allele. The large confidence intervals indeed indicate that these results are less reliable than the Marburg I findings.

Finally, we performed an analysis comparing carriers of both alleles of Marburg I and Marburg II with carriers of the wild-type alleles in relation to clinical stroke. A hazard ratio of 1.60 (95% CI: 0.87–2.94) was found for the risk of clinical stroke for subjects carrying both variant alleles; however, this was not significant due to low numbers.

3.2. The GENDER Study. Table 1 presents the patient characteristics and minor allele frequencies. Genotyping success rates were 95% for both polymorphisms, and there were no significant deviations from Hardy-Weinberg equilibrium.

In contrast with the results from the PROSPER study, the Marburg I polymorphism (208 heterozygotes and 5 homozygotes for the variant allele) tended to reduce the risk for clinical restenosis in the GENDER study (Figure 1), (HR: 0.59, 95% CI: 0.34–1.02, $P = .061$). The protective effect was borderline significant. Carriers of the Marburg II variant had a slightly and nonsignificantly reduced risk of TVR (Figure 2). In the GENDER population, 67 patients were carriers of both Marburg I and II risk alleles, accounting

for 63% of all Marburg II carriers (105 heterozygotes and 1 homozygote).

3.3. Replication. The Marburg II polymorphism showed an increased risk for clinical stroke in 5763 subjects of the Rotterdam Study. Carriers of the variant allele have a 1.4 times increased risk of clinical stroke compared to carriers of the wild-type allele (β: 0.38, SE: 0.23, $P = .05$).

4. Discussion

Our data suggest that the Marburg I polymorphism, which leads to less FSAP activity, increases stroke risk and mortality. The Marburg I associated increase in mortality was mainly due to an increased risk of fatal stroke. Surprisingly, carriers of this variant seemed at lower risk of developing restenosis. The effect of the Marburg II polymorphism on clinical stroke was replicated in the Rotterdam Study cohort.

Although FSAP was originally identified as a potential activator of factor VII and prourokinase (pro-uPA), its role in hemostasis remains unclear. Endogenous FSAP, of which intravascular levels are low, has not been clearly shown to influence blood coagulation or fibrinolysis [11]. Moreover, the Marburg I polymorphism does not seem to influence the risk for venous thrombosis [4, 16]. However, FSAP cleaves PDGF-BB and has been shown to inhibit vascular smooth muscle cell proliferation and migration *in vitro* [9] and *in vivo* [10]. The activity of FSAP in Marburg carriers is low and could therefore be a risk factor for atherosclerosis and restenosis, processes which are known to be determined by vascular smooth muscle cell proliferation.

A possible role for FSAP in human atherosclerosis was suggested by a study showing an association of the Marburg I polymorphism with advanced atherogenesis in carotid arteries [8]. Despite the low number of carriers among cases ($n = 8$) and controls ($n = 2$), their findings suggest a role for Marburg I in carotid plaque formation. Another study, investigating the effect of Marburg I on coronary heart disease, found no significant effect in the whole population, but observed an interactive effect on risk between the Marburg I variant and elevated levels of cholesterol and triglyceride [7]. The primary endpoint in that study was a composite of myocardial infarction and the need for a PCI.

In agreement with these studies, we also found no association of Marburg I with myocardial infarction in the whole population of patients taking part in the PROSPER study, whereas we did find a significant association with stroke, which is known to be related to carotid plaque formation. The observed increase in stroke risk in Marburg I carriers could be a consequence of hyperproliferation of smooth muscle cells, due to a reduced proteolytic activity of Marburg I-FSAP towards PDGF-BB. Unfortunately, we have no measurements of carotid atherosclerosis within the PROSPER study on which to draw more definite conclusions.

The possible protective effect of Marburg I on clinical restenosis in patients treated for stable angina pectoris is difficult to explain. Despite some similarities between atherosclerosis and restenosis, such as the involvement of inflammation and smooth muscle cell proliferation, there are important mechanistic differences between these processes. In contrast with atherosclerosis, which develops partly in response to elevated lipoprotein levels and cigarette smoke, the restenotic process is not particularly sensitive to circulating lipids and smokers even seem to have a reduced risk for restenosis [14]. It is therefore not unlikely that a genetic risk factor would have opposite effects on stroke and clinical restenosis after PCI.

However, as opposed to WT-FSAP, Marburg I-FSAP was less effective in preventing neointima formation in an animal model for restenosis [10]. Based on these findings, the Marburg I polymorphism was expected to increase the risk for restenosis in humans. Although the protective effect observed in the GENDER study was (borderline) not significant, there was a strong trend towards protection. The contradicting results not only could relate to the subjects (mice versus humans) or the intervention site (femoral artery versus coronary arteries), but could also be the result of differences in the exact location and concentration of the Marburg I-FSAP-protein, which was locally applied in high concentrations to injured arteries in the mouse model. Furthermore, FSAP plays a role in many different processes known to be important in vascular remodeling. FSAP has recently been shown to activate the matrix metalloproteinases MMP2 and MMP9 (gelatinases) [17], which are important in matrix remodeling. Further research is needed to elucidate the precise role of FSAP in vascular remodeling and the pathogenesis of stroke.

The Marburg II polymorphism, also located in the protease domain of FSAP, has no known functional implications and is not associated with altered catalytic activity.

Due to its low allele frequency (2%), the Marburg II findings are less reliable. The similar, but much weaker, trends that were observed for this polymorphism could be a result of the linkage disequilibrium with the Marburg I variant. Large confidence intervals indicate that the observed significant association with vascular mortality, which is slightly stronger than the effect of Marburg I on vascular mortality, probably occurred by chance.

There were 263 stroke endpoints within the PROSPER population during the 3.3-year followup. Unfortunately, we do not have data on the separate ischemic and hemorrhagic strokes. In the present study, both types of stroke were combined into one clinical endpoint. Because we know from previous studies in elderly populations that approximately 80% of all strokes is attributable to ischemic events [18, 19], the association between the Marburg I polymorphism and clinical stroke is probably driven by an association between the polymorphism and ischemic stroke. If there is no association between the polymorphism and hemorrhagic stroke, then the association we found is an underestimation of the true relative risk for ischemic stroke.

In conclusion, we have demonstrated that carriers of the Marburg I polymorphism are at increased risk for clinical stroke and stroke-related mortality. Furthermore, a strong trend towards a reduced risk for clinical restenosis was

observed in Marburg I carriers. As Marburg I-FSAP has reduced proteolytic activity towards PDGF-BB, the increase in stroke risk could be a consequence of hyperproliferation of smooth muscle cells.

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Abbreviations

PROSPER:	Prospective Study of Pravastatin in the Elderly at Risk
GENDER project:	Genetic Determinants of Restenosis project
HR:	Hazard ratio
OR:	Odds ratio
CI:	Confidence interval
PCI:	Percutaneous coronary intervention
CABG:	Coronary artery bypass grafting
TVR:	Target vessel revascularization.

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Research Article

Lentiviral-Mediated shRNA Silencing of PDE4D Gene Inhibits Platelet-Derived Growth Factor-Induced Proliferation and Migration of Rat Aortic Smooth Muscle Cells

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Phosphodiesterase 4D (PDE4D) is a member of the large superfamily of phosphodiesterases. PDE4D polymorphisms have been found to associate with ischemic stroke. Proliferation and migration of vascular smooth muscle cells (VSMCs) play a critical role in the pathogenesis of atherosclerosis. In this study, infection of VSMCs with lentivrius particles carrying shRNA direct against PDE4D significantly inhibited platelet-derived growth factor-induced VSMC proliferation and migration, and the inhibitory effects were not associated with global intracellular cAMP level. Our results implicate that PDE4D has an important role in VSMC proliferation and migration which may explain its genetic susceptibility to ischemic stroke.

1. Introduction

Stroke, a complex disease, remains the third common cause of death behind heart disease and cancer and is the leading cause of disability in developed countries as well as in China. Ischemic stroke accounts for up to 80% of all strokes. Both environmental factors and genetic variants contribute to stroke. Although several genetic defects have been identified in rare forms of stroke, the genetic causes of common forms of ischemic stroke remain elusive. Recently, PDE4D has been identified as the first gene linked to common forms of stroke [1]. However, subsequent studies have produced inconsistent results for the association [2–9]. Due to the variable results, several meta-analyses on the gene have been accomplished, which also drew conflicting conclusions [10–13]. Racial differences might be responsible for the inconsistent results. Therefore, experimental studies on function of PDE4D will help to unveil the puzzle.

PDE4D belongs to a large superfamily of PDEs which are the only hydrolytic enzymes of cAMP and key signal transduction molecules in multiple cell types, including vascular smooth muscle cells. Eleven families (PDE1–PDE11) have been identified in the large superfamily of PDEs [14]. There are four major families found in VSMCs: PDE1, PDE5, PDE3, and PDE4. PDE1 and PDE5 are mainly responsible for cGMP-hydrolyzing activity, whereas PDE3 and PDE4 contribute to most of cAMP-hydrolyzing activity [15, 16]. PDE4 has four subfamilies: PDE4A, 4B, 4C, and 4D, which are differentially localized between cells [17]. PDE4D is expressed broadly in many kinds of cell [18, 19]. In arterial VSMCs, PDE4D is dominantly expressed and degrades second-messenger cAMP[20]. Recent studies suggest that PDE4D may have a critical role in atherosclerosis. For example, PDE4D is largely associated with atherosclerotic stroke such as cardiogenic and carotid strokes [1]. Furthermore, a decrease in cAMP level has been found to associate with increased proliferation and migration of vascular smooth-muscle cells, the central events in the development of atherosclerosis [21, 22]. More importantly, PDE4 antagonists have been shown to significantly inhibit smooth-muscle proliferation in a rat carotid balloon-injury model [23–25]. However, the reagents they used are not the specific inhibitor against PDE4D, which makes it hard to determinate the functional roles of PDE4D isoform in the process. Therefore,

genetic approach specifically targeting PDE4D gene such as use of shRNA to knockdown its expression would be necessary to address this issue.

The present study was conducted to investigate whether downregulation of PDE4D can inhibit VSMC proliferation and migration. Lentivirus particle carrying small hairpin RNA against PDE4D was applied to specifically reduce PDE4D expression in rat aortic smooth muscle cells. We found that PDE4D silence in rat aortic smooth muscle cells significantly inhibits their proliferation and migration induced by platelet-derived growth factor (PDGF), and the inhibition is not associated with global intracellular cAMP level.

2. Materials and Methods

2.1. Cell Culture. The human HEK-293T-cell line was obtained from American Type Culture Collection (ATCC) and grown in RPMI 1640 supplemented with 10% FBS (Sigma-Aldrich, St. Louis, MO, USA).

VSMCs were isolated from descending thoracic aorta of male Sprague-Dawley rats weighed about 150 g and characterized morphologically and immunohistochemically as described previously [26]. Cells were grown in Dulbecco's modified Eagle's medium (DMEM), with 10% fetal bovine serum (FBS), 1 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin, at 37°C/5% CO_2. Cells were trypsin passaged after 10 days and then at 95% confluence (Jinuo Biology and Medicine Company, China). All experiments were performed on cells at passage 4 through 6 and 60% to 90% confluence. In all experiments, the cells were made quiescent by incubating for 24 hours in serum-free medium prior to use.

2.2. PDE4D Gene Silence in Rat VMSCs. The lentivirus vector system used in the experiment was purchased from Shanghai Genchem Company (Shanghai, China), which was composed of three plasmids—pGCL-GFP, pHelper1.0, and pHelper2.0. The plasmid pGCL-GFP had an U6 promoter and a GFP reporter gene.

The oligonucleotide sequences of four different PDE4D-shRNAs and a control nonsense shRNA are as shown in Table 1. All shRNAs were synthesized by Shanghai Genchem Company. These four 21 nucleotide shRNA duplexes from four different parts of the PDE4D mRNA (Gen Bank Accession no. NM_017032) were designed using specific guidelines for designing shRNA hairpins encoded by shRNA expression vectors and shRNA expression cassettes of Ambion company. Of sequences tested for PDE4D knockdown using Western blot, the sequence locating at 181 achieved the best interfering effect and was subsequently cloned into the shRNA-pGCL-GFP lentiviral vector. Nonsense shRNA was served as a control. Infectious viruses were produced by cotransfecting the lentiviral vectors and packaging constructs into 293FT cells using Lipofectamine reagent (Invitrogen).

Recombinant lentviruses containing shRNA against PDE4D and nonsense shRNA were obtained from Genchem Company. VSMCs were infected with purified lentivirus at an MOI (multiplicities of infection) of 50 to obtain 95–100% efficiency, as determined by GFP expression 24 hours after infection.

Table 1: Information of four designed shRNA and nonsense sequences.

No.	Sequences	Locates	GC %
1	GCGATTATGACCTCTCTCC	181	52.60
2	CCAACCATCCATCAACAAA	368	42.10
3	CCCATGTGCAACCAACCAT	357	52.60
4	CCGGATAATGGAGGAGTTC	1499	52.60
nonsense	TTCTCCGAACGTGTCACGT	—	52.63

The no. 1 locating at 181 achieved the best interfering effect around 72%.

2.3. Western Blot. VSMCs were collected and sonicated by sonicator. Protein was extracted from the lysate and separated with SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrohoresis). Following electrophoresis, proteins were transferred to PVDF membranes, and the membranes were blocked by incubation with TBST (Tris Buffered Saline Tween) containing 5% skim milk for 1 hour. Blots were incubated with an appropriate dilution of primary antibodies against rat PDE4D (Abcam) and α-actin (Santacruz) for 2 hours and rinsed three times with TBST. Rinsed blots were incubated with second antibodies (Santacruz) for 2 hours and rinsed with TBST. Immunoreactivity was detected by chemiluminescence (Amersham ECL plus kit).

2.4. Cell Viability and Proliferation. Cell viability and proliferation was measured by assessing mitochondrial activity of VSMCs using an MTT kit (Sigma). Cells were growth-arrested in serum-free medium for 24 hours and then cultured in medium with 0.5% FBS and PDGF-BB (Pepro Tech, 20 ng/mL) for 24 hours before addition of MTT (5 mg/mL, Boli Company, China). The MTT solution (5 mg/mL MTT in medium without phenol red) was added to the culture solution to a volume equal to 10%. Further incubation at 37°C for four hours yielded purple MTT formazan crystals. Once solubilized with DMSO, the absorbance in each culture was measured at 570 nm with the background (650 nm) subtracted.

2.5. Migration Assay. VSMCs migration assays were performed using a modified Boyden's chamber fitted with 8-μm pore membrane (Corning). Briefly, VSMCs were resuspended in serum-free medium to a concentration of 1×10^5 cells/mL. A volume of 200 μL of cell suspension was added to the upper wells of the Boyden's chamber, and 0.5% FBS-medium with or without PDGF (20 ng/mL) was added to the lower chamber. Migration was allowed to proceed for 12 hours in a 37°C, 5% CO_2, humidified atmosphere. Then, cells remaining on the upper surface of the membrane were scraped off with cotton sticks. Cells that had migrated to the lower surface were fixed with paraformaldehyde (Guangzhou Reagent factory) and stained with DAPI (Jackson) and crystal violet (Weijia Technical Company, Guangzhou). Migration

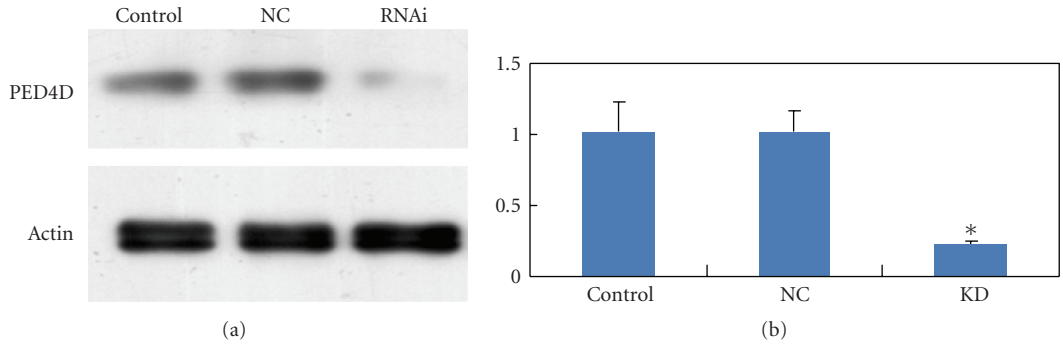

Figure 1: Knockdown of endogenous PDE4D expression by lentiviral vector-mediated shRNA in VSMCs. VSMCs were infected with the same amount (PDE4D-shRNA: 12.5 μL, nonsense shRNA: 6.25 μL) of lentivirus particles carrying either shRNA (titer 4×10^8 TU/mL) against PDE4D or nonsense shRNA (titer 8×10^8 TU/mL). PDE4D protein expression was determined at 48 hours postinfection. Values represent means ± SEM. E: inhibition of PDE4D expression in PDE4D shRNA-tranfected cells was calculated relative to the PDE4D expression in cells tranfected with the control nonsense shRNA. Sh-GFP: lentivirus particles carrying nonsense shRNA, Sh-PDE4D: lentivirus particles carrying shRNA against PDE4D.

capacity was identified by the average number of stained cells in six random fields of view (magnification ×400) per membrane. The experiment was repeated three times.

2.6. Measurement of cAMP. The intracellular cAMP levels in cultured VSMCs were determined by cAMP RIA test kit purchased from Shanghai Chinese Medicine University following the kit instruction (Shanghai, China). The detection limit of cAMP was 0.08 pmol/L, and the inter- and intra-assay coefficients of variation were <10%. Starved cells were incubated in 0.5% FBS-medium with or without PDGF (20 ng/mL) for another 24 hours. Cells were lysed in acid/alcohol (750 mL of ethanol : 248.5 mL of double-distilled H_2O : 1.5 mL of concentrated HCL) containing IBMX (500 μM). The lysate was acetylated with 10 μL volume of a 1 : 2 (v : v) mixture of acetic anhydride plus triethylamine. After acetylation, samples were preincubated for 1 hour at room temperature with 100 μL anti-cAMP before adding 100 μL ^{125}I-cAMP and incubating for overnight at 4°C. After separation with adsorbing materials, the samples were centrifuged at 3000 rpm for 15 min. The supernatant fluids were aspirated, the radioactivity of precipitates was counted by γ-counter, and cAMP levels were determined by comparison to a standard curve.

2.7. Statistical Analysis. All data were processed with SPSS 12.0 statistical software. All results are expressed as means ± SEM. Data were analyzed using a one-way ANOVA and Student-Newman-Keuls tests for multiple comparisons. In all cases, $P \leq .05$ was taken as statistically significant.

3. Results

3.1. Screening of shRNAs for Gene Silence of PDE4D after Infection with Lentivrius Particles Carrying shRNA against PDE4D in VSMCs. To determine PDE4D gene silencing effect, VSMCs were transiently transfected with lentivrius particles carrying shRNAs against PDE4D. Four different shRNA duplexes, named as no. 1, no. 2, no. 3, and no. 4, were designed to target different regions of rat PDE4D (NM_017032): start sites of 181, 368, 357, and 1499 (Table 1). All four different shRNAs could inhibit PDE4D at both transcription and protein levels as determined by real-time RT-PCR and western blotting (data not shown). These shRNA duplexes showed varying degrees of PDE4D gene silencing efficiency ranging from 53% to 72% (data not shown). The no. 1 with sequence locating at 181 achieved the best interfering effect around 72% and was subsequently cloned into the shRNA-pGCL-GFP lentiviral vector (Figure 1). The nonsense shRNA treatment did not significantly alter the levels of PDE4D transcript and protein and was subsequently cloned into the shRNA- pGCL-GFP lentiviral vector as a shRNA control (Figure 1).

3.2. PDE4D Gene Silence Inhibits PDGF-Induced VSMC Proliferation without Affecting Cell Viability. To determine whether PDE4D participates in PDGF-induced VSMC proliferation, we treated cells with PDGF and the proliferation was assayed using MTT at 24 hours after PDGF stimulation. As shown in Figure 2, noninfected control cells and cells infected with lentivirus particles carrying either shRNA direct against PDE4D or nonsense shRNA grew at similar rates. Cells treated with PDGF were increased by almost threefolds when compared to untreated cells. Infection of VSMCs with lentivius particles carrying shRNA direct against PDE4D significantly resulted in about 30% reduction of PDGF-induced proliferation. In contrast, infection of VSMCs with the lentivirus particle carrying nonsense shRNA did not significantly affect PDGF-induced proliferation. This result demonstrates an important role of PDE4D in PDGF directed VSMC proliferation.

3.3. PDE4D Gene Silence Suppresses Migration. To determine whether PDE4D gene contributes to VSMC migration, we silenced gene expression using lentivirus particle carrying

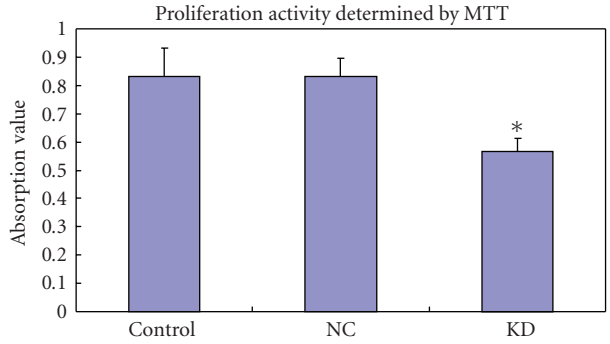

Figure 2: PDE4D regulates PDGF-induced VSMC proliferation. VSMC viability was determined using MTT assay at 48 hours postinfection. Data are mean ± SEM of 3 separate experiments performed in triplicate. $^{*}P$ less than .05 versus control. $^{\#}P$ less than .05 versus nonsense shRNA.

shRNA against PDE4D and evaluated migration using Boyden's chamber. Rat aortic VSMCs were made quiescent by 24 hour treatment in serum-free culture medium. As shown in Figure 3, noninfected control cells and cells infected with lentivirus encoding either shRNA direct against PDE4D or nonsense shRNA had similar migration rates. Treatment with PDGF significantly increased VSMC migration up to sixfolds when compared to untreated cells. Infection of VSMCs with lentivirus particle carrying shRNA direct against PDE4D significantly resulted in almost 39% reduction of PDGF-induced migration. In contrast, infection of VSMCs with the lentivirus carrying nonsense shRNA did not significantly affect PDGF-induced migration.

3.4. Intracellular cAMP Level. To examine whether the inhibitory effects of PDE4D gene silence on VSMC proliferation and migration were mediated through intracellular cAMP, we measured cAMP levels by enzyme immunoassay after infection of VSMCs with lentivrus carrying shRNA against PDE4D gene (Figure 4). Infection of VSMCs with lentivrus particle carrying either shRNA against PDE4D gene or nonsense shRNA did not result in any significant change in VSMC basal level of cAMP. The addition of PDGF (20 ng/mL) stimulated a statistically significant accumulation of cAMP in VSMCs compared with control. However, infection of VSMCs with lentivirus particle carrying shRNA against PDE4D gene or nonsense shRNA did not result in any significant change in PDGF-stimulated accumulation of cAMP.

4. Discussion

In the present study, we studied the functional consequence of PDE4D knockdown by RNA interference in PDGF-induced VSMC proliferation and migration. We found that knockdown of endogenous PDE4D significantly reduces rat aortic VSMC proliferation and migration. However, the inhibition does not lead to alteration of the global intracellular cAMP level, suggesting that the inhibition is less likely mediated through cAMP.

Atherosclerosis is a major cause of ischemic stroke [27]. Vascular endothelial cell injury and subsequent migration and proliferation of VSMCs are early key events of atherosclerosis. We here used cultured VSMCs after three passages as a model to study VSMC proliferation and migration because they represent VSMCs in a synthetic phenotype which is similar to migrating smooth muscle cells in vivo. Platelet-derived growth factor (PDGF), one of the most potent mitogens and chemoattractants for VSMCs produced by platelets, VSMCs, and endothelial cells in the injured vascular wall, plays the central role in the onset and development of vascular disorders [28, 29]. Therefore, VSMC proliferation and migration in this study were induced by the addition of the potent and pathophysiologically relevant chemotactic factor PDGF. As shown in Figure 2, we confirmed that PDGF caused proliferation in rat VSMCs. In addition, we observed about 30% reduction of PDGF-induced cell proliferation in PDE4D deficient VSMCs. We believe that the reduction of PDGF-induced cell proliferation in PDE4D deficient VSMCs is specific because knockdown of PDE4D had no effect on quiescent VSMCs where PDGF was absent. This finding is consistent with previous studies that Rolipram, a general PDE4 inhibitor, can inhibit proliferation of VSMCs from 26% to 37 ± 6% [30–32]. It is not surprised to note that knockdown of PDE4D did not achieve a full suppression of VSMC proliferation because multiple signals are involved in PDGF-induced VSMC proliferation. Furthermore, the members of PDE family in addition to PDE4D have been shown to closely associate with VSMC proliferation. For example, inhibition of PDE3 and PDE1A also reduce VSMC proliferation [33, 34]. Nevertheless, our data suggest that PDE4D is important in PDGF-induced VSMC proliferation.

Although VSMC migration plays a pivotal role in the pathogenesis of arteriosclerosis, the role of PDE4D in VSMC migration has received much less attention. Few investigators have studied the effects of PDE4D on VSMC migration. The results are inconsistent in those limited reports. For example, Goncharova and his colleagues found that cilomolast, a PDE4 inhibitor can inhibit basal (unstimulated) and PDGF-stimulated migration of airway smooth muscle and pulmonary vascular smooth muscle cells [35]. Palmer and his colleagues reported that Ro 20-1724, another PDE4 inhibitor with potent effect on PDE4D, potentiated antimigratory effect caused by forskolin [25]. However, neither cilomolast nor Ro 20-1724 is the specific inhibitor against PDE4D. As a result, lack of selective inhibitors against PDE4D makes it difficult to determinate the functional roles of PDE4D isoform. To overcome this problem, we used shRNA techniques to selectively knockdown PDE4D expression in VMSCs. We found that knockdown of PDE4D significantly inhibited PDGF-induced migration. This effect is in parallel to the inhibitory action of PDE4D on VMSC proliferation.

It has been well documented that cAMP and its signaling play a critical role in regulating VSMC proliferation and migration [21, 22, 36]. In addition, cAMP is the only substrate of PDE4D. Therefore, we hypothesized that the inhibitory effects of knockdown of PDE4D might be mediated through its regulation of cAMP. Although PDGF-induced robust production of cAMP in VMSC cells, we failed

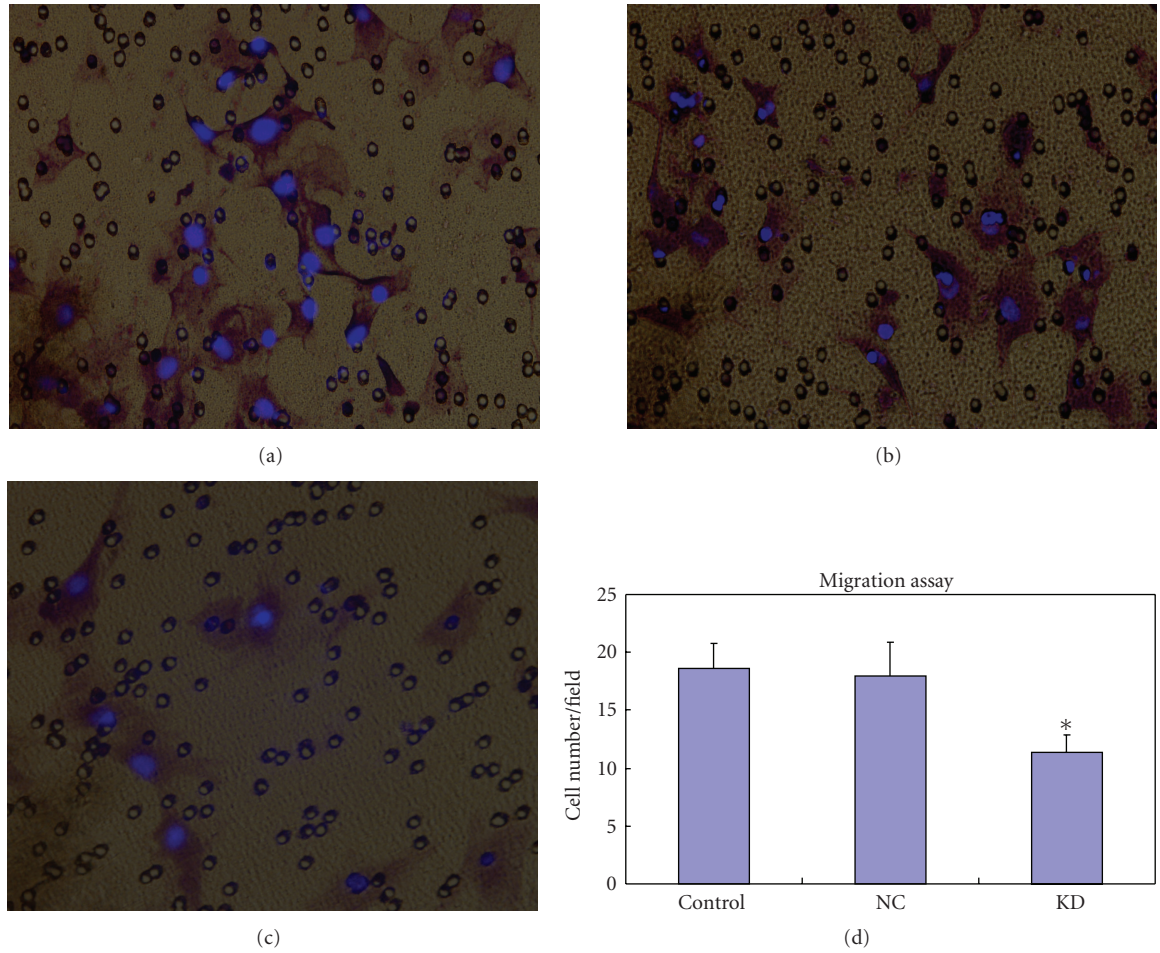

Figure 3: Knockdown of endogenous PDE4D inhibits PDGF-induced VSMC migration. PDGF-induced VSMC migration was assessed in a Boyden's chamber. Representative images (a, b, c) and group data (d) are shown. (a)–(c) Representative photomicrographs illustrating migration of VSMCs. (a) Migration of VSMCs stimulated by PDGF-BB. (b) Migration of VSMCs stimulated by PDGF-BB in cells infected with lentivirus particles carrying nonsense shRNA. (c) Migration of VSMCs stimulated by PDGF-BB in cells infected with lentivrius carrying shRNA against PDE4D. (d) Bar graph illustrating PDGF-BB-induced VSMC migration in noninfected cells and cells infected with lentivirus particles carrying either PDE4D or nonsense shRNA. Numbers of migrated VSMCs were much less in cells infected with lentivrius carrying PDE4D shRNA compared with noninfected cells or cells infected with lentivirus particles carrying nonsense shRNA. Bars are the mean plus minus SEM of six random fields of view from three separate experiments. Original magnification, ×400. $^{*}P$ less than .05 versus control. $^{\#}P$ less than .05 versus nonsense shRNA.

to detect any significant change of cAMP level in PDGF-induced PDE4D deficient VMSC which is consistent with several previous reports [28, 29]. Given that intracellular cAMP level is modulated by PDE family members, it is reasonable that inhibition of PDE4 or PDE4D alone may not be able to affect the global level of cAMP in cells. The other possible explanation is that the effect of PDE4 family on VSMC proliferation and migration may not be entirely mediated by change in intracellular cAMP concentration. In addition to its action on cAMP-signaling, PDE4D also has a cross-talk with extracellular-signal-regulated kinases (ERK) in response to both cAMP-dependent and non-cAMP-dependent agents. ERK has been reported to be important for PDGF- stimulated proliferation and migration of VSMC. In addition, cAMP are distributed with spatial gradient, not freely [37, 38]. Therefore, cAMP induced by PDE4 or other family member can be highly compartmentalized and insufficient to increase total cellular cAMP. This may also explain the failure to detect a significant change in cAMP after single inhibition of PDE4. Therefore, we speculate that knockdown of PDE4D may alter local concentration of cAMP and thus regulate its signaling locally to inhibit proliferation and migration of cells.

5. Conclusion

In this study, we infect VSMC with lentivrius particles carrying shRNA direct against PDE4D. Consequently, VSMC

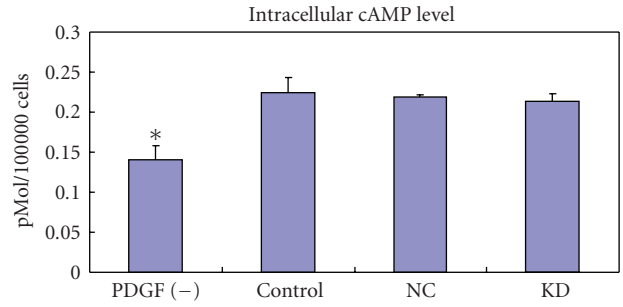

Figure 4: Knockdown of endogenous PDE4D does not affect PDGF-induced cAMP level in VSMC. Bar graph illustrates PDGF-BB induced cAMP level in noninfected cells and cells infected with lentivirus particles carrying either PDE4D or nonsense shRNA. There were no significant differences in cAMP level between cells infected with lentivrius carrying PDE4D shRNA and noninfected cells, cells infected with lentivirus particles carrying nonsense shRNA. Values represent means ± SEM from three separate experiments.* P less than .05 versus control.

proliferation and migration was significantly inhibited, and the inhibitory effects were not associated with global intracellular cAMP level. Our results implicate that PDE4D has an important role in VSMC proliferation and migration which may explain its genetic susceptibility to ischemic stroke.

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Research Article

Association of *TNF-857C>T*, *TNFRSF1A36A>G*, and *TNFRSF1B676T>G* Polymorphisms with Ischemic Stroke in a Greek Population

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Background. The role of genetic factors in the predisposition to develop ischemic stroke has been assessed by previous studies. The main goal of the current study was to determine any possible role of *TNF-857C>T, TNFRSF1A36A>G,* and *TNFRSF1B676T>G* polymorphisms in risk for stroke. *Materials and Methods*. One hundred seventy-three patients with first ever ischemic stroke of solely atherosclerotic etiology in Northwest Greece and a control group of 179 healthy unrelated subjects were evaluated. *Results*. *TNFα-857TT*, *TNFR136AA*, and *TNFR2676TT* genotypes were significantly increased in the patient group compared to controls ($P = .008$, OR = 2.47 (1.26–4.84), $P = .005$, OR = 1.97 (1.22–3.17), and $P = .003$, OR = 2.2 (1.43–3.37), resp.). In addition, the *TNFR136A* and the *TNFR2676T* alleles were found significantly increased in patients compared to controls ($P = .009$, OR = 1.48 (1.1–2) and $P = .001$, OR = 1.75 (1.25–2.46), resp.). *Conclusion*. The high incidence of these genotypes and alleles in patient group suggests that they are potentially predisposing factors for stroke in the Greek population studied. Large-scale multicenter controlled studies are needed to verify these polymorphisms effects on stroke susceptibility.

1. Introduction

Ischemic stroke is a multifactorial disease, caused by the interactions of genetic and environmental factors, based on atherosclerosis and arterial thrombogenesis [1]. Central nervous system (CNS) can mount a well-defined inflammatory response to a variety of insults including ischemia and atherosclerosis [2]. Atherosclerosis has many inflammatory mediators contributing to atheroma formation, atheromatous injury, rupture of the plaque, and hence to intraluminal thrombosis [2, 3]. Tumor necrosis factor-(TNF-) alpha is involved in every step of inflammation, from initiation to downregulation, and elevated levels of TNF and other cytokines have been demonstrated in the cerebrospinal fluid (CSF) and the plasma of acute stroke and subarachnoid hemorrhage patients [4–6]. TNF exerts its biological effects via the two cell surface receptors that act as physiological attenuators, TNFRSF1A and TNFRSF1B.

Functional polymorphisms inside the TNF gene promoter and polymorphisms that could influence the function or the expression of TNFRSF1 and TNFRSF1B have been investigated in various immune, inflammatory, and neurodegenerative conditions [7–14] but only few genetic studies have focused on the association of these polymorphisms with stroke [15, 16].

Under the rationale that individuals with genetic variants might possibly be at greater risk for ischemic stroke, we analyzed the role of the *TNF-857C>T, TNFRSF1A36A>G*, and *TNFRSF1B676T>G* polymorphisms in ischemic stroke patients in Northwestern Greece, a rather restricted area with limited recent immigration.

2. Materials and Methods

2.1. Study Population. Cases eligible for recruitment were 173 patients hospitalized with first ever ischemic stroke in the Stroke Reference Center of Northwestern Greece in a period of 18 months, from May 2008 to November 2009, defined according to the WHO definition, and confirmed by brain imaging showing a recent brain infarct corresponding to the clinical presentation. As a control group, 179 age-matched unrelated subjects were recruited from the same medical center, among nonfamily visitors. Controls had no history of previous ischemic cerebrovascular event, established by the Questionnaire for Verifying Stroke-Free Status (QVSFS) [17] and normal neurological examination. The study protocol was in compliance with the Helsinki Declaration, approved by the Institutional Ethics Committee. An informed consent was signed by all eligible patients and controls.

In all subjects a detailed medical history was obtained and a thorough physical examination was performed. Demographic data, biochemical profile, and established risk factors for stroke were recorded. Arterial hypertension was documented when systolic blood pressure (BP) was ≥140 mmHg or diastolic BP ≥ 90 mmHg or when individuals were receiving antihypertensive drugs for previously established hypertension. Smoking, active or ceased within the last 3 months, was considered as current. Diabetes mellitus was considered as present if fasting glucose was >126 mg/dl or the individual was treated with antidiabetic medication. Dyslipidemia was defined as fasting cholesterol >220 mg/dl or the individual was on current treatment with specific antilipidemic agents. Further evaluation of the patients included triplex ultrasound imaging or CT angiography of extracranial or/and intracranial arteries, electrocardiogram (ECG), echocardiography (transthoracic or transesophageal), and clinical/laboratory assessment to rule out systemic thrombotic and autoimmune disorders.

Recruitment in the study was performed according to TOAST definitions and included patients with lacunars (small, subcortical, hypodense lesions with a diameter less than 15 mm and corresponding to lesion clinical lacunar syndrome) and patients with large artery atherosclerosis (hypodense lesions with a diameter >15 mm and >50% stenosis in the appropriate intracranial artery) [18].

Cardioembolic strokes were excluded since they could result from different etiology (e.g., atrial fibrillation). Strokes occurred in the course of systemic conditions such as coagulopathies or immunological disorders or of undetermined etiology were also excluded in order to assure the solely atherosclerotic etiology of the stroke.

2.2. Genetic Analysis. Genomic DNA was extracted from peripheral blood lymphocytes according to the standard salt extraction procedure. Polymorphisms *TNF-857C>T (rs1799724), TNFRSF1A36A>G (rs767455), and TNFRSF1B-676T>G (rs1061622)* were amplified using the following primer pairs: 36F: 5′-GAG CCC AAA TGG GGG AGT -GAG AGG-3′ 36R: 5′-ACC AGG CCC GGG CAG GA-G AG-3′, 676F: 5′-ACT CTC CTA TCC TGC CTG CT-3′ 676R: 5′-TTC TGG AGT TGG CTG CGT GT-3′, and 857F: 5′-AAG TCG AGT ATG GGG ACC CCC CGT TA-A-3′ 857R: 5′-CCC CAG TGT GTG GCC ATA TCT TCT -T-3′. Subsequently, restriction assays were employed using the restriction endonucleases MspA1 I, Nla III, and Hinc II, respectively. All samples were run in duplicates with positive and negative for each genotype samples as controls and blanks.

2.3. Statistical Analysis. The Chi-square test was used to test the agreement of genotype frequencies with Hardy-Weinberg equilibrium expectations. Binary data were described as percentages, while continuous data were expressed as the mean ± standard deviation (SD). We calculated odds ratios and 95% confidence intervals (CIs) to compare allele and genotype distributions. All tests were two-sided with 95% significance level ($P < .05$). The statistical analysis was performed with the statistical package StatXact 3.0 (Cytel Inc., Cambridge, Mass, USA). Furthermore, a power analysis showed that at least 302 subjects should be recruited in each group (cases and controls) for *TNF-857C>T*, 272 subjects for *TNFRSF1A36A>G,* and 85 subjects in each group for *TNFRSF1B676T>G* polymorphism, if the study power required was 80 per cent with a significance level of 0.05.

3. Results

Of 173 patients, 113 (65%) were men and 60 (35%) were women with a mean age of 58.6 (SD ± 7.2 years). Of 179 controls, 123 (69%) were men and 56 (31%) were women and mean age was 57.1 (SD ± 5.9 years). Patients and controls demographics are shown in Table 1. In terms of stroke subtype, 84 patients had lacunar strokes, whereas 89 patients had large artery strokes.

The genotype frequencies for all three polymorphisms were in Hardy-Weinberg equilibrium in healthy controls. The genotype frequencies for TNFRSF1A and TNFRSF1B polymorphisms were in Hardy-Weinberg equilibrium in patients. Regarding the *TNF-857C>T* polymorphism distribution, a high prevalence of *TT* genotype in patients ($P = .008$, OR = 2.47 (1.26–4.84)) was revealed, implying a *TT* selection in patients causing Hardy-Weinberg disequilibrium, despite equilibrium in the controls. No statistically significant difference was observed in the allele frequencies, to further establish the hypothesis of the *TT* selection in patients.

The distribution and frequencies of genotypes and alleles for TNF, TNFRSF1A, and TNFRSF1B are summarized in Table 2. Regarding *TNFRSF1A36A>G*, a statistically significant difference was observed between patients carrying the *AA* genotype and controls ($P = .005$, OR = 1.97 (1.22–3.17)). Significant differences were also noted in the allele frequencies, namely, *A* and *G* with 58.1% in patients versus 48.3% in controls for *A* allele and 41.9% in patients versus 51.7% in controls for *G* allele ($P = .009$, OR = 1.48 (1.1–2)). For the *TNFRSF1B676T>G*, the *TT* genotype was more frequent in the stroke group than in controls, with 61.9% versus 42.5%, respectively ($P = .003$, OR = 2.2 (1.43–3.37)). Similarly, statistically significant differences between

Table 1: Clinical characteristics of patients and controls.

Data	Cases $N = 173$	Controls $N = 179$	P values
Male	113 (65)	123 (69)	.498
Female	60 (35)	56 (31)	.498
Age, mean	58.6 (7.2)	57.1 (5.9)	.167
Hypertension	137 (79.1)	129 (72.0)	.121
Smoking	74 (42.8)	70 (39.1)	.484
Diabetes mellitus	40 (23.1)	31 (17.3)	.176
Dyslipidemia	109 (63.0)	99 (55.3)	.142

Numbers in parentheses for nominal data indicate percentages and for continuous data SD.

Table 2: Genotypes and alleles in patients and control.

Polymorphisms	*TNF-857C>T*		*TNFRSF1A36A>G*		*TNFRSF1B676T>G*	
Genotypes	*TT*	*CT + CC*	*AA*	*AG + GG*	*TT*	*TG + GG*
Patients, *n* (%) *n* = 173	30 (17.3)	43 + 100 (24.9 + 57.8)	60 (34.7)	81 + 32(46.8 + 18.5)	107 (61.9)	57 + 9 (32.9 + 5.2)
Controls, *n* (%) *n* = 179	14 (7.8)	63 + 102 (35.2 + 57.0)	38 (21.2)	97 + 44 (54.2 + 24.6)	76 (42.5)	89 + 14(49.7 + 78)
P values, OR (95% CI)	$P = .008$, 2.47 (1.26–4.84)		$P = .005$, 1.97 (1.22–3.17)		$P = .003$, 2.2 (1.43–3.37)	
Alleles	*C*	*T*	*A*	*G*	*T*	*G*
Patients, *n* (%) *n* = 173	243 (70.2)	103 (29.8)	201 (58.1)	145 (41.9)	271 (78.3)	75 (21.7)
Controls, *n* (%) *n* = 179	267 (74.6)	91 (25.4)	173 (48.3)	185 (51.7)	241(67.3)	117 (32.7)
P values, OR (95% CI)	$P = .196$, 0.8 (0.57–1.12)		$P = .009$, 1.48 (1.1–2)		$P = .001$, 1.75 (1.25–2.46)	

the allele frequencies with 78.3% in patients versus 67.3% in controls for *T* allele and conversely 21.7% in patients versus 32.7% in controls for G allele ($P = .001$, OR = 1.75 (1.25–2.46)) (Table 2) were revealed.

In terms of stroke subtype, subgroups analysis did not disclose any statistically significant result when genotypes and alleles distribution in the lacunar stroke group was compared to the large artery atherosclerosis stroke group (data not shown).

4. Discussion

In the present study, we investigated the association *of TNF-857C>T, TNFRSF1A36A>G,* and *TNFRSF1B676T>G* polymorphisms with ischemic stroke, under the rationale that, as the proinflammatory cytokines play an important role in cerebral ischemia [4, 5, 19, 20], certain TNF and TNF receptors polymorphisms may be implicated in stroke occurrence.

A gene located on chromosome *6p21* encodes TNF-α. The *TNF-857C>T* polymorphism is a functional polymorphism through binding to the transcription factor octamer transcription factor-1 (OCT-1) [7]. No research group, working on stroke, has focused on this polymorphism. Most groups have worked on the *TNF-308G>A* polymorphism with Pereira et al. suggesting that the *TNF-308G>A* polymorphism may play a role in ischemic stroke [21] showing that young subjects of European ancestry carrying the *A* allele *(AA + GA* versus *GG)* were associated with a statistically significant increase in the risk of stroke compared with individuals homozygous for the *G* allele (OR 2.04, $P = .004$). In a cohort of young Italian stroke patients, the *A* allele of *−308G>A* polymorphism exerted an independent effect on predisposition to ischemic stroke in young stroke patients with *AA + GA* genotypes frequency being 25.2% + 1.7%, respectively, in the stroke group versus 15% + 0.6 5% in the control group [15]. Dissimilar findings suggest that this polymorphism is clinically important in stroke in sickle cell anemia children [22] but shows protective effects of *TNF(-308)A* allele (OR = 0.39, $P = .006$) with 18% carriers of the *A* allele in stroke group versus 38% in the control group.

In our healthy population in Northwest Greece, as recorded in a recent study conducted by our group [10], the *TNF-308G>A* polymorphism is not in Hardy-Weinberg equilibrium, and our study was designed on the specific characteristics of our population to exclude this polymorphism and focus on the *TNF-857C>T* polymorphism. Our study exerted that this other functional polymorphism plays a role in stroke development with the recessive genotype being more frequent in ischemic stroke patients.

TNFRSF1A is encoded by a gene located on chromosome *12p13.2*. The *TNFRSF1A36A>G* polymorphism is a silent mutation in codon 12, and it although has no obvious functional influence on protein structure, many studies have tried to find any association of this polymorphism with specific inflammatory disease manifestations and response to treatment, such as Multiple Sclerosis (MS), Crohn's disease, and Rheumatoid Arthritis (RA) [10, 11, 14].

TNFRSF1B is encoded by a gene located on chromosome *1 p36.3* with a higher affinity for TNF than TNFRSF1, and polymorphisms in this receptor affect the binding of TNF and the pathway involved in inflammation; associations of TNFRSF1B polymorphisms with chronic inflammatory diseases such as RA and systemic lupus erythematosus have been reported [10, 12].

Our results indicate that the most studied, for many inflammatory diseases, polymorphisms of TNFRSF1A and TNFRSF1B are also associated with stroke, with the *AA* genotype of TNFRSF1A and the *TT* of TNFRSF1B being more frequent in stroke patients. The same applies for the *A* allele of TNFSF1A and the *T* allele of TNFSF1B.

Limitations of our study include the relatively small number of patients and the *TNF-857TT* Hardy-Weinberg disequilibrium in our cases. As a consequence of the small number of the cases the power of our study is limited regarding the *TNF-857C>T* and the *TNFRSF1A36A>G* polymorphism. Regarding the *TNFRSF1B676T>G* polymorphism, power analysis confirmed a sound power of this result. The strengths of the study were the solely atherosclerotic etiology of subjects and the prospective design with all ischemic stroke patients under certain inclusion criteria and the significant result for *TNFRSF1B676T>G* polymorphism.

5. Conclusions

Conclusively, our results indicate a possible association of *−857C>T* polymorphism of TNF with stroke and are in favor of a direct, contributory effect of the most studied polymorphisms of TNFRSF1A and TNFRSF1B on ischemic stroke predisposition, with *TNFRSF1B676T>G* polymorphism showing the closest association. Our assumption is that specific polymorphisms are involved in the process of inflammation, and thus they promote extra- and intracranial atherosclerosis and subsequently strokes. The genetically homogenous population allows the extractions of assumptions concerning our population, but no further conclusions should be reached without large-scale multicenter studies and a wider range of results. Any role for TNF and TNF receptor genes on stroke is far from being established with these genes being excellent candidates for further research on the genetics of stroke.

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Research Article

Functional Inflammatory Genotypes in Ischemic Stroke: Could We Use Them to Predict Age of Onset and Long-Term Outcome?

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Functional single-nucleotide polymorphisms (SNPs) of inflammatory cytokines have been previously related to the occurrence of an ischemic stroke (IS). We investigated whether five functional SNPs (i.e., TNF-α -308G>A, IL6-174G>C, IL12B 1188A>C, IL4-589C>T, and IL10-1082G>A) might be associated with the age of onset and 6-month outcome of an acute IS. A probe-free real-time PCR methodology was used to genotype 145 consecutively admitted cases with a first-ever IS. Simple Kaplan-Mayer and adjusted Cox regression analyses showed no association between inflammatory genotypes and the age of IS onset. IL6-174G>C, IL12B 1188A>C, IL4-589C>T, and IL10-1082G>A were not found to significantly contribute to the long-term outcome of the disease. However, carriage of the TNF-α -308 GG genotype was significantly associated with reduced odds for an adverse outcome. Larger studies are needed to confirm our results.

1. Introduction

In the last decade, inflammatory mechanisms have been implicated both in the manifestation and evolution of brain ischemia [1]. Immune inflammatory processes may in time predispose to ischemic stroke (IS) through precipitation of atherosclerotic disease [2] and sustainment of atrial fibrillation [3]. Moreover, salvation of the ischemic penumbra and final size of the brain infarct, both of which strongly correlate to clinical outcome after an IS, seem to be massively regulated by networks of poststroke inflammatory responses [4, 5]. Thus, proinflammatory and anti-inflammatory cytokines are important mediators of the pathophysiological events which precede an acute IS [6] and have been further related to the clinical outcome of the disease (reviewed in [7]).

Among the various proinflammatory cytokines, tumor necrosis factor-alpha (TNF-α) and interleukin (IL) 6 seem to actively participate in the immune-mediated inflammation of stroke [5, 8]. IL12 is an important regulatory cytokine with proinflammatory inclination [9], which is also found upregulated during stroke [10]. IL4 and IL10 are cytokines of the anti-inflammatory arm which counteract the actions of proinflammatory molecules, and have been found suppressed prior to, as well as after a cerebral ischemic event [11–13].

On grounds of genetic regulation, the production of these inflammatory cytokines seems to be significantly influenced by the presence of point mutations located within regulatory gene regions, known as functional single-nucleotide polymorphisms (SNPs) [14]. SNP-association studies of inflammatory cytokines have been previously conducted in cases of IS, under the hypothesis that a genetic predisposition to enhanced (or suppressed) production of T-helper 1 (Th1) or Th2 cytokines could possibly modify the risk of the disease (reviewed in [15]). However, so far, none of these studies has addressed the question of whether such functional genetic variances could predispose to the premature onset of cerebrovascular disease or alter its clinical outcome.

In the present study, we investigated the possible impact of five common SNPs into the age of onset and long-term

Table 1: Allele-specific primers and real-time PCR conditions.

	Allele-specific primers	Real-time PCR conditions
IL4-589C>T		
FRW C primer	5′-*GCGGCGCGGCCC*ACTAAACTTGGGAGAACATTGA**C**-3′	
FRW T primer	5′-AGTAAACTTGGGAGAACATTGT**T**-3′	*Segment 1-Amplification (40 cycles)*
REV primer	5′-GGCAGAATAACAGGCAGAC-3′	
IL10-1082G>A		(i) Denaturation at 95°C for 30″
FRW G primer	5′-*GCGGCGCGGCGG*-TTAGTAAGGCTTCTTTGGGT**G** -3′	(ii) Annealing at 56°C for 30″
FRW A primer	5′-TACTACTAAGGCTTCTTTGGGA**A** -3′	
REV primer	5′-CTGGATAGGAGGTCCCTTAC-3′	(iii) Extension at 72°C for 20″
TNF-α-308G>A		
FRW G primer*	5′-*GCGGCGCGGCGG*-CTGGCTGAACCCCGTC**T**-3′	
FRW A primer*	5′-AGGCTGAACCCCGT**G**C**T**-3′	*Segment 2-Qualitative data collection (115 cycles)*:
REV primer*	5′-AAGGAAACAGACCACAGACCTG-3′	
IL6-174G>C		70–95°C for 18″
FRW G primer*	5′-ATTGTGACGTCCTTTAGCAT**C**-3′	
FRW C primer*	5′-*GCGGGCGGGCCG*-ATTGTGACGTCCTTTAGGTT**G**-3′	(Specifically for TNF-α-308G>A: annealing at 61°C for 25″)
REV primer*	5′-ATGACGACCTAAGCTGCAC-3′	
IL12B 1188A>C		
FRW A primer*	5′-TTTCAATGAGCATTTAGCAA**C**T-3′	
FRW C primer*	5′-*GCGGCGCGGCGG*-TACAATGAGCATTTAGCATG**G**-3′	
REV primer*	5′-TAGGATCACAATGATATCTTTGC-3′	

* Primer designed on the complementary sequence. FRW: forward, REV: reverse.
Underlined are sites of introduced mismatches. Bold are the polymorphic alleles. Italic is the intoduced GC-tail.

functional outcome of an acute IS. The studied mutations included three SNPs of proinflammatory cytokines (i.e., TNF-α-308G>A, IL6-174G>C, and IL12B 1188A>C), and two more SNPs concerning the major anti-inflammatory cytokines (i.e., IL4-589C>T, and IL10-1082G>A). These SNPs have been previously studied in various inflammatory diseases (e.g., rheumatic diseases, asthma, and inflammatory bowel disease) producing positive correlations [16–20]. Each of the above polymorphisms has been reported to exert functional properties in *in vitro* and *ex vivo* studies, following mitogenic stimulation [16, 21–24]. A more robust evidence for their functionality has been given for TNF-α-308G>A which has been found to affect a transcription-binding site and result in the formation of an altered transcription element [25] and also for IL10-1082G>A which contains a putative ETS-like transcription factor binding site [26].

2. Methods

2.1. Study Population. We recruited 145 acute IS patients who presented consecutively at the Emergency Department of the University Hospital of Patras, Patras, Greece, with signs and symptoms of a first-ever cerebrovascular event, and were admitted in the Neurology and General Medicine wards between September 2006 and December 2007. Patients with intracerebral or subarachnoid haemorrhage, transient ischemic attacks (TIAs), chronic inflammatory/rheumatic diseases, comorbid malignancy, and strokes secondary to rare conditions (i.e., traumatic carotid dissection, endocarditis) were excluded from the study. Blood samples and baseline clinical data were collected upon admittance to the Emergency Department. Stroke severity on admission was assessed using the Scandinavian stroke scale. All patients had a brain computerized tomography (CT) on admission, and a second brain imaging (CT or magnetic resonance imaging) during hospitalization. Investigations such as carotid ultrasonography, carotid CT angiography, transesophageal heart ultrasonography, serology and coagulation studies, were performed according to patient-specific clinical criteria. Stroke subtype classification was based on TOAST definitions [27]. As confirmed at the baseline interview, participants were Caucasian in origin, and provided their informed consent at inclusion. The study protocol was approved by the Ethical Committee of the University Hospital of Patras.

2.2. Data and Clinical Assessments. Demographics, past medical history, and conventional stroke risk factors were collected at inclusion. Arterial hypertension was considered present if it was clearly documented (>140/90 mmHg) or when treated. Diabetes mellitus was defined as fasting glucose >126 mg/dL or current use of antidiabetic medication. Hypercholesterolemia was noted if fasting cholesterol levels were >220 mg/dL or when treated. History of angina, myocardial infarction and heart failure were assembled in "ischemic heart disease". Smoking, either active or ceased within the last 6 months, was recorded as current. By vascular death, we considered death in relation to vascular complications (i.e., stroke, myocardial infarction, pulmonary embolism, and deep vein thrombosis). Patients were

Table 2: Baseline characteristics and genotype frequencies of IS patients.

Characteristic	IS patients ($n = 145$)
Age, median (IQR)	68 (58–76)
Sex, females (%)	50 (34.5)
Arterial hypertension (%)	104 (72)
Diabetes mellitus (%)	40 (28)
Hypercholesterolemia (%)	102 (70)
Atrial fibrillation (%)	31 (21)
Ischemic heart disease (%)	37 (26)
Current smoking (%)	52 (36)
Stroke severity on admission (%)	
Mild (SSS = 45–58)	69 (48)
Moderate (SSS = 19–44)	44 (30)
Severe (SSS = 0–18)	32 (22)
TNF-α-308G>A	
GG (%)	115 (79)
GA (%)	30 (21)
IL6-174G>C	
GG (%)	85 (59)
GC (%)	50 (34)
CC (%)	10 (7)
IL12B 1188A>C	
AA (%)	77 (53)
AC (%)	57 (39)
CC (%)	11 (8)
IL4-589C>T	
CC (%)	123 (84.8)
CT (%)	22 (15.2)
IL10-1082G>A	
AA (%)	47 (32.4)
GA (%)	71 (49)
GG (%)	27 (18.6)

IQR: interquartile range, IS: ischemic stroke,
SSS: scandinavian stroke scale.

followed-up for 6 months registering medications, disease relapses, deaths, and functional outcome measured by the Barthel Index (BI), which is a reliable and widely used disability scale [28].

2.3. DNA Extraction and Genotyping Assays. DNA was extracted from whole blood in EDTA, according to standard purification protocols by Qiagen, QIAamp DNA blood Mini Kit, and stored at −80°C until use. For the genotyping, we applied a real-time polymerase chain reaction (RT-PCR) technique, which discriminates alleles by detecting differences in the melting temperatures of the products (T_m). Differences in T_m are produced by the introduction of a GC-tail at the end of one of the two allele-specific primers [29], and measured by SYBR Green fluorescence. For the studied SNPs, the method was used for the first time. Prototypically designed primers and conditions can be seen in Table 1. Primers were designed using the DNAman version 4.02 software, tested for specificity with BLAST (http://blast.ncbi.nlm.nih.gov/), and synthesized by Metabion International AG, Germany. All samples were processed in duplicates, and genotyping of 10% random samples for each SNP was confirmed by sequencing (VBC-BIOTECH Service GmbH, Sequencing Unit, Vienna, Austria). Analyses were employed in the Stratagene Mx3000P qPCR machine, using the Brilliant QPCR MasterMix by Stratagene, La Jolla, USA.

Table 3: Statistics of the disease-free survival analysis.

	Disease-free survival analysis		
	Median (SE)	KM log rank P	Cox regression* Odds ratio (95% CI)
TNF-α-308G>A			
GA	65 (4.93)	.70	Referent
GG	68 (1.97)		0.74 (0.49–1.12)
IL6-174G>C			
GG	65 (2.01)	.86	Referent
GC/CC	69 (1.66)		0.70 (0.51–1.22)
IL12B 1188A>C			
AA	67 (2.00)	.62	Referent
AC/CC	70 (2.58)		1.02 (0.72–1.44)
IL4-589C>T			
CC	67 (2.08)	.91	Referent
CT	68 (4.10)		1.07 (068–1.70)
IL10-1082G>A			
GA/AA	68 (2.09)	.55	Referent
GG	65 (6.06)		1.43 (0.92–2.22)

SE: standard error, KM: Kaplan-Mayer, CI: confidence intervals.
*Adjusted for sex, hypertension, diabetes, hyperlipidemia, atrial fibrillation, ischemic heart disease, and smoking.

2.4. Statistical Analysis. A Kaplan-Mayer disease-free survival analysis was used to test for possible differences in the age of IS onset, depending on the carriage of specific inflammatory genotype. Statistical significance was expressed with P log ranks. Cox regression analyses were further conducted for each SNP to include possible known confounders (i.e., sex, hypertension, diabetes, atrial fibrillation, hyperlipidemia, smoking) in the overall effect. To assess the potential genetic contribution in the odds of having a bad functional outcome, a binary logistic regression analysis was applied after adjustments for age, sex, vascular risk factors, stroke severity on admission and TOAST categories. Data of the regressions are given as Odds Ratios (OR) with 95% confidence intervals (95% CI).

3. Results

Baseline characteristics and genotypes of the studied polymorphisms in our IS cohort are seen on Table 2. All genotype frequencies were found in accordance to the Hardy-Weinberg

Table 4: Association of inflammatory genotypes with long-term functional outcome of an IS.

	Genotypes		Logistic regression		
	TNF-α-308G>A		Presence of GG		
6-month outcome	AG	GG	OR*	95% CI	P
(BI: 16–20) good (%)	15 (17)	73 (83)	Referent	0.04–0.86	.03
(BI: <16, or death) bad (%)	13 (25.5)	38 (74.5)	0.19		
	IL6-174G>C		Presence of GC/CC		
	GG	GC/CC	OR*	95% CI	P
(BI: 16–20) good (%)	54 (64.3)	30 (35.7)	Referent	0.37–3.54	.82
(BI: <16, or death) bad (%)	34 (62.8)	21 (38.2)	1.14		
	IL12B 1188A>C		Presence of AC		
	AA/CC	AC	OR*	95% CI	P
(BI: 16–20) good (%)	48 (54.5)	40 (45.5)	Referent	0.23–2.09	.52
(BI: <16, or death) bad (%)	37 (72.5)	14 (27.5)	0.69		
	IL4-589C>T		Presence of CT		
	CC	CT	OR*	95% CI	P
(BI: 16–20) good (%)	71 (84.1)	14 (15.9)	Referent	0.17–2.59	.56
(BI: <16, or death) bad (%)	43 (84.3)	8 (15.7)	0.67		
	IL10-1082G>A		Presence of GG		
	GG	AG/AA	OR*	95% CI	P
(BI: 16–20) good (%)	14 (15.9)	74 (84.1)	Referent	0.57–12.40	.22
(BI: <16, or death) bad (%)	12 (23.5)	39 (76.5)	2.65		

BI: barthel index, OR: odds ratio, CI: confidence intervals.
*ORs adjusted for age, sex, hypertension, diabetes, hyperlipidemia, ischemic heart disease, smoking, stroke severity on admission and TOAST categories.

equilibrium, that is, for TNF-α-308G>A $P = .17$, for IL6-174G>C $P = .48$, for IL12B 1188A>C $P = .92$, for IL4-589C>T $P = .32$, and for IL10-1082G>A $P = .98$.

Figure 1 shows the Kaplan-Mayer curves for the disease-free survival analysis depending on genotypes of each studied inflammatory SNP. As seen on the curves, carriers of the ancestral alleles did not significantly differ in the age of stroke onset compared to their homo- and heterozygous mutant counterparts. Estimates of median (standard error) age of IS onset for each inflammatory genotype, as well as the Kaplan-Mayer P log ranks can be seen on Table 3. A Cox regression analysis was further applied to correct the impact of studied genotypes on the age of disease onset, for other important confounders (i.e., sex, hypertension, diabetes, hyperlipidemia, atrial fibrillation, ischemic heart disease, and smoking). ORs with 95% CI of the adjusted genotype effects confirmed a lack of association between inflammatory SNPs and disease-free survival (Table 3). Interestingly, in all regressions, the most powerful predictors of the premature age of IS onset were smoking and hyperlipidemia (both $P < .001$, data not shown).

Because Kaplan-Mayer curves (Figure 1) involving TNF-α-308G>A, IL4-589C>T and IL10-1082G>A presented differentiating slopes approximately before the age of 65, we reapplied the above analysis separately for this younger age-group ($n = 76$). However, neither this subanalysis could point to significant differences in the age of IS onset between the different inflammatory genotypes. The subsequent Cox regression (OR (95% CI)) also showed no significant contribution of TNF-α-308GG (0.70 (0.37–1.35), IL4-589CT (0.53 (0.26–1.07)), or IL10-1082 GG (1.83 (0.97–3.47)) genotypes, after standard adjustments for sex and vascular risk factors.

Table 4 contains the genotypic distributions across categories of good and bad functional outcome, at 6 months after IS. Bad functional outcome included cases with BI<16, or death. Combinations of genotypes have been made so as to unite groups of "high" versus "low" cytokine producers. The binary logistic regression showed a statistical significant contribution of the TNF-α-308 GG ancestral genotype in the reduction of the odds for an adverse 6-month outcome (OR (95% CI) = 0.19 (0.04–0.86)), even after important corrections for age, sex, vascular risk factors, stroke severity on admission and TOAST categories. For the rest of the inflammatory SNPs, no similar association could be confirmed. As expected, stroke severity on admission was the most powerful predictor in the models ($P < .001$, data not shown).

4. Discussion

In the last decade, experimental as well as clinical studies have recognized both pro and anti-inflammatory cytokines as key mediators of the low-grade aseptic inflammation, which precedes an ischemic cerebrovascular event and follows its pathophysiological course [1]. SNPs that alter the production rate of inflammatory cytokines secondary to a given trigger, have been previously related to incident IS [15]. The present

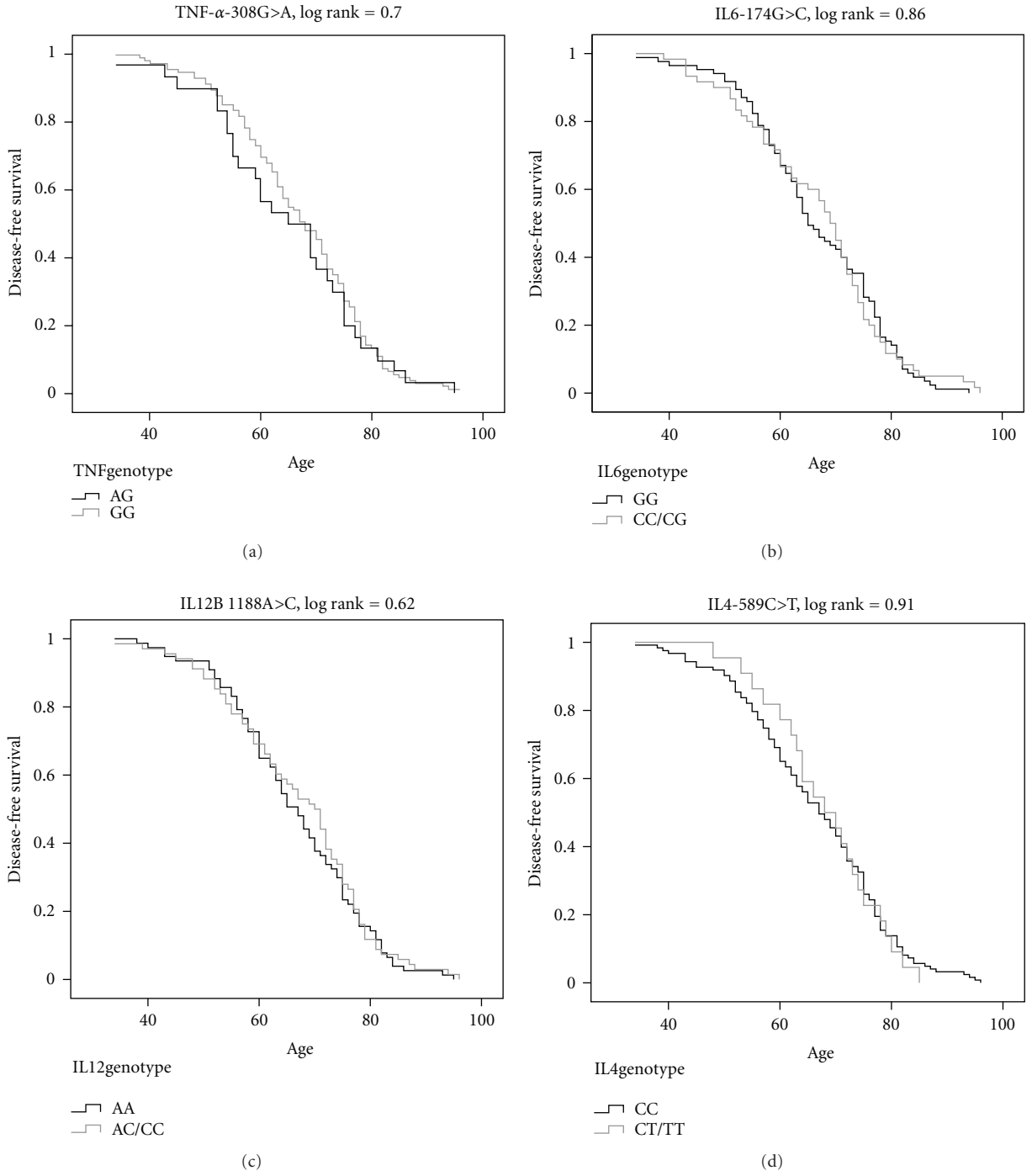

Figure 1: Kaplan-Mayer curves for the disease-free survival analysis, according to the studied inflammatory genotypes.

study investigated the possible genetic contribution of functional polymorphisms of major inflammatory molecules (i.e., TNF-α-308G>A, IL6-174G>C, IL12B 1188A>C, IL4-589C>T and IL10-1082G>A) in the age of onset and 6-month outcome of an acute IS. Genotyping was based on a PCR-allele discrimantion technique without oligonucleotide probes [29], which yielded comparable allele frequencies to those previously reported using different methods [30–34].

Despite previous promising data suggesting a significant association of the age of onset of Alzheimer's disease with genotypes of TNF-α-308G>A SNP [35], our results showed that the age of onset of ischemic cerebrovascular disease may not be predicted by inflammatory genotypes. Simple Kaplan-Mayer disease-free survival curves, as well as Cox regressions adjusted for sex and vascular risk factors, confirmed that IS is a complex, multifactorial clinical phenotype, which

can manifest prematurely or delayed, irrespectively of the genetic inflammatory burden. Furthermore, it is possible that the gross effect of conventional vascular risk factors in the incidence of IS may not be easily overwhelmed. Under this rationale, it would be interesting to know whether the reported genotype frequencies differ from those of a healthy population. This investigation could be the aim of a future case-control association study.

Although genome-wide association studies are reasonably more relevant to detect combinations of genetic traits in relation to a specific clinical phenotype over a large number of patients, they tend to miss important clinical aspects of the disease. This is why, smaller-scale SNP association studies of molecules which have some a priori evidence for a pathogenic role in the disease of interest, may still be useful in implying possible relations with meaningful clinical impact. Under this scope, we set to investigate whether our selected inflammatory functional SNPs could predict the long-term outcome of an IS. After applying adjusted analyses, we found a lack of association between IL6-174G>C, IL12B 1188A>C, IL4-589C>T and IL10-1082G>A and 6-month outcome after stroke. The relatively limited number of IS cases included, may partially explain the above lack of associations. However, we were able to identify a significant contribution of the ancestral TNF-α-308 GG genotype in about 80% reduced odds for having an adverse outcome (OR (95% CI) = 0.19 (0.04–0.86)). This long-term association of TNF-α-308 G-allele double carriage is in line with its described low TNF-secreting properties [36]. High TNF-α levels at 3 months after an IS, have been previously found to relate with undesired functional dependency [37].

To conclude, our results show that functional genotypes of important inflammatory cytokines cannot either delay or precipitate the age of IS onset, despite the widely accepted role of such molecules in the pathophysiology of the disease. We provide implications for a possible predictive effect of TNF-α-308 GG ancestral genotype into better clinical outcome 6 months after stroke. It is possible that the small size of our study may have not allowed for identification of other statistical significant associations. Therefore, further studies in larger IS cohorts, are needed to confirm our results.

Conflict of Interests

The authors declare that there is no conflict of interests.

Aknowledgment

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