Three periods of one and a half decade of ischemic stroke susceptibility gene research: lessons we have learned

Anita Maasz and Bela Melegh*

Abstract
Candidate gene association studies, linkage studies and genome-wide association studies have highlighted the role of genetic factors in the development of ischemic stroke. This research started over a decade ago, and can be separated into three major periods of research. In the first wave classic susceptibility markers associated with other diseases (such as the Leiden mutation in Factor V and mutations in the prothrombin and 5,10-methylenetetrahydrofolate reductase (MTHFR) genes) were tested for their role in stroke. These first studies used just a couple of hundred samples or even less. The second and still ongoing period bridges the two other periods of research and has led to a rapid increase in the spectrum of functional variants of genes or genomic regions, discovered primarily in relation to other diseases, tested on larger stroke samples of clinically better stratified patients. Large numbers of these alleles were originally discovered by array-based genome-wide association studies. The third period of research involves the direct array screening of large samples; this approach represents significant progress for research in the field. Research into susceptibility genes for stroke has taught us that careful stratification of patients is critical, that susceptibility alleles are often shared between diseases, and that not all susceptibility factors that associate with clinical traits that are themselves risk factors for stroke (such as increase of triglycerides) necessarily represent susceptibility for stroke. Research so far has been mainly focused on large- and small-vessel associated stroke, and knowledge on other types of stroke, which represent much smaller population samples, is still very scarce. Although some susceptibility allele tests are on the palette of some direct-to-consumer companies, the clinical utility and clinical validity of these test results still do not support their use in clinical practice.
incidence rates, which show a decreasing gradient from eastern to western European countries [9]. The strict classification of ischemic stroke into subtypes is crucial if we are to understand its etiology. It can be divided into three major subgroups depending on the pathogenic mechanism: thrombotic, embolic and hemodynamic stroke. This classification is based on results provided by imaging studies, and although there can be overlap between the groups, the classification is still relevant for prevention and management purposes.

The first major clinical classification was introduced in 1991 with the Oxford Community Stroke Project [10], which was followed by a more widely accepted one in 1993. This was developed for the Trial of Org10172 in Acute Ischemic Stroke (TOAST) [11] and was based on the most common pathophysiological mechanisms involved in the development of stroke (Table 1). The system comprises five subgroups: (1) the large-vessel atherosclerotic type, in which patients have cortical or cerebellar lesions and/or brainstem infarcts or subcortical hemispheric infarcts greater than 1.5 cm in diameter on magnetic resonance imaging (MRI), with or without cerebral cortical impairment or brainstem or cerebellar dysfunction; (2) the small-vessel occlusion type, which includes patients with one or more subcortical hemispheric or brainstem infarcts with a diameter less than 1.5 cm on MRI, with one of the features of the traditional clinical lacunar syndrome (a constellation of clinical symptoms (pure motor stroke, pure sensory stroke, sensorimotor stroke and ataxic hemiparesis), and signs at the time of maximum deficit following a single cerebrovascular event) and without cerebral cortical dysfunction; (3) the cardioembolic subtype, which comprises patients with arterial occlusions presumably resulting from an embolus arising in the heart; (4) stroke with other determined etiology, which includes patients with rare causes of stroke, such as hematological disorders, non-atherosclerotic vasculopathies and hypercoagulability (increase in coagulability); and (5) stroke with unidentified etiology, in which the cause of stroke cannot be determined [11].

Stroke is a multifactorial disease. Environmental factors can have a crucial role in the development of the disease. Several risk factors have been identified, which were categorized as potentially modifiable and non-modifiable [12-14]. Age, gender and previous history of stroke are some of the non-modifiable risk factors, whereas hypertension, diabetes mellitus, obesity, elevated triglyceride and cholesterol levels, smoking, high alcohol consumption and presence of cardiovascular events are some modifiable risk factors.

Recently, the potential role for several naturally occurring genomic variants has come into the focus of epidemiological studies. Significant progress has already been made in the identification of genetic variants for stroke and other cerebral and cardiovascular disorders [15-19], and research into these variants provides new insights into the pathogenesis of these conditions.

Rare forms of ischemic stroke appear as prime manifestations of multisystemic disorders, such as cerebral arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), Fabry's disease, Marfan syndrome, and ‘mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes’ (MELAS syndrome). These conditions show typically Mendelian or maternal inheritance and result from defects in single genes, such as NOTCH3, α-galactosidase (GLA), fibrillin-1 (FBN1) and mitochondrial tRNA-encoding genes, respectively [20-24]. Numerous studies have revealed single-gene disorders [25-32]; the discussion of these conditions is outside the scope of this article.

Three periods in stroke susceptibility research

**Classic genetic risk factors for stroke**

The first studies into the genetic background of stroke involved the examination of single genetic variants that might be involved in pathogenic mechanisms of this condition. This period (approximately 1985 to 1995), which can be regarded as the first wave of stroke genetic research, was also the beginning of susceptibility allele research for numerous other multifactorial diseases, including ischemic heart disease, type 2 diabetes and metabolic syndrome. Several polymorphisms of genes influencing hemostasis and homocysteine metabolism and genes encoding enzymes involved in the renin-angiotensin system and nitric oxide (NO) production were identified as risk factors for ischemic stroke (Tables 2 and 3).

Disturbances in the balance of hemostasis can lead to increased aggregation of blood platelets and thrombus formation, which may result from defects in genes encoding components of the coagulation cascade, such as factors II, V, XIII and fibrinogen, promoting the

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### Table 1. Classification of stroke subtypes from TOAST [12]

| Subtype | Classification |
|---------|----------------|
| 1       | Large-artery atherosclerosis (embolus/thrombosis) |
| 2       | Small-vessel occlusion (lacune) |
| 3       | Cardioembolism (high-risk/medium-risk) |
| 4       | Stroke of other determined etiology |
| 5       | Stroke of undetermined etiology |

(a) Two or more causes identified
(b) Negative evaluation
(c) Incomplete evaluation

The classification of subtypes 1 to 4 includes possible or probable cases according to the results of ancillary studies as well as certain diagnoses.
development of several cardio- and cerebrovascular phenotypes. Activated factor V acts as a cofactor for the conversion of prothrombin to thrombin. Bertina and coworkers [33] first identified a c.1691G>A transition (p.Arg506Gln) in the \textit{F5} gene in a family with thrombophilia in 1994. The c.1691G>A alteration (called the Leiden variant) has been reported to be associated with resistance to degradation by activated protein C. Since then, numerous studies have examined this variant in several conditions that correlate with thromboembolism, but the results were inconsistent regarding the pathological role of this alteration [34-37]. According to the results of a large-scale epidemiological assessment, carriage of the 1691A allele was not associated with an increased risk of stroke [38]. However, a statistically significant association was found between the presence of the c.1691G>A transition and ischemic stroke in a meta-analysis [39]. We [40] found that the c.1691G>A alteration does not confer risk for stroke alone, but in combination with other unfavorable factors, such as hypertension or diabetes mellitus, it can increase the relative risk of ischemic stroke.

The most extensively studied alteration of the prothrombin (factor II) gene is c.20210G>A, which was first described in 1996 [41]. This sequence variation is associated with elevated prothrombin levels. Several studies found a statistically significant association between ischemic stroke and this substitution [42,43]; the data of a meta-analysis also suggested that the c.20210G>A variant is a risk factor for ischemic stroke [39].

The p.Val34Leu substitution in factor XIII has also been suggested as a risk factor, but its role remains ambiguous according to the findings of association studies [44,45]. A meta-analysis by Casas and colleagues [39] suggested that the p.Val34Leu variant does not confer an increased risk for development of stroke.

The c.455G>A alteration of the fibrinogen-encoding gene has been found to occur more frequently in homozygous form in patients with large-vessel stroke [46]. Carrying the rare allele causes elevated fibrinogen levels, which confers susceptibility to ischemic stroke by prothrombotic mechanisms. A similar relationship was found for other variants of the fibrinogen gene, but only in female patients [47].

The renin-angiotensin system regulates blood pressure and water balance. Renin, the key protease of the cascade, is secreted from the kidneys and cleaves angiotensinogen to form angiotensin I. This intermediate form is then

\begin{table}[h]
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\caption{Genes and their polymorphisms associated with ischemic stroke in GWASs}
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System & Gene & Polymorphism & References \\
\hline
Hemostasis & Factor II (prothrombin) (+176930) & c.20210G>A & [39,41-43] \\
& Factor V (Leiden) (*612309) & p.Arg506Gln & [33-40] \\
& Factor XIII (+134570) & p.Val34Leu & [39,44,45] \\
Renin-angiotensin system & ACE (+106180) & Insertion/deletion & [40,48-51] \\
& AT1R (*106165) & c.1166A>C & [52-55] \\
Nitric-oxide-synthase system & eNOS (+163729) & p.Glu298Asp & [56-58] \\
Homocysteine metabolism & MTHFR (*607093) & c.677C>T and c.1298A>C & [43,59-61] \\
Lipid metabolism & APOE (+107741) & e4, e3, e2 & [40,46,62-66] \\
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\begin{table}[h]
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\caption{Functional SNPs associated with ischemic stroke}
\begin{tabular}{|l|l|l|l|}
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System & Gene & Polymorphism & References \\
\hline
Lipid metabolism & APOA5 (*606368) & g.-1131T>C, c.1259T>C, c.56C>G, c.4153+476G>A & [82-88] \\
& APOCIII (*107720) & g.-2854T>G, g.-4535C>T, g.-4823C>T, c.3238C>G & [73-76] \\
& LPL (*609708) & p.Asn291Ser & [92-95] \\
& GCKR (*600842) & c.1337C>T & [104] \\
& MLIPL (*605678) & rs17145738, rs3812316 & [105] \\
& GALT (*602274) & rs4846914 & [104] \\
Signal transduction & PDE4D (*600129) & Six SNPs, haplotypes & [96-100] \\
& ALOX5AP (*603700) & Seven SNPs, haplotypes & [97,100-103] \\
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\end{tabular}
\end{table}

\textit{ACE}, angiotensin converting enzyme; \textit{APOE}, apolipoprotein E; \textit{AT1R}, angiotensin-receptor; \textit{eNOS}, endothelial nitric oxide synthase; \textit{MTHFR}, 5,10-methylenetetrahydrofolate reductase.
converted to angiotensin II by angiotensin converting enzyme (ACE). The ACE gene, located on chromosome 17, is one of the most intensively studied candidate genes associated with ischemic stroke. Cambien and colleagues [48] first described the insertion/deletion (indel) polymorphism of the gene in myocardial infarction. Six years later, a meta-analysis reported a significant positive association between the deletion polymorphism of the ACE gene and ischemic stroke [49]. The results of a large, prospective study indicate that the ACE indel polymorphism is not associated with subsequent risk of stroke [50]. In a Hungarian population we have found the ACE D/D variants (homozygous carriers of the deletion) to be associated with risk for development of stroke [40,51]. Subsequent studies have mostly investigated variants of the gene encoding angiotensin II receptor type I (AT1R). AT1R has multiple effects, both cardiac and systemic, mediating vasoconstriction, cellular hypertrophy and catecholamine release. In a Caucasian population a weak association was found between carrying the AT1R 1166C allelic variant and stroke [52,53]. According to our results [54], the 1166C allelic variant is associated with susceptibility to the disease among hypertensive smokers. We found co-occurrence of ACE indel and AT1R c.1166A>C variants to confer risk for small-vessel occlusion type stroke in a Hungarian population [55]. Several studies have investigated ACE indel polymorphisms in association with numerous conditions, and they are believed to contribute to the development of atherosclerotic mechanisms, but their role remains controversial.

The NO synthase system is an important regulator of the integrity and growth of the vascular endothelium and is responsible for the maintenance of normal endothelial function. The endothelial nitric oxide synthase (eNOS) enzyme converts l-arginine to l-citrulline, generating NO in the arteries, and has a role in vasodilation. Under pathological conditions, cells produce superoxide instead of NO, which can initiate the atherosclerotic process. In 1996, Wang and colleagues [56] first identified a functional variant (eNOS4a/b) of the eNOS gene in association with coronary artery disease. Since then, several reports have studied variants in eNOS in relation to ischemic stroke. Howard and colleagues [57] found that a promoter region polymorphism (-786T>C) of eNOS is associated with ischemic stroke susceptibility in young black women. Another single-nucleotide polymorphism (SNP) in this gene (c.894G>T) has been shown not to be a susceptibility factor for ischemic stroke; however, co-occurrence with other SNPs, such as ACE D/D or the c.677C>T in the 5,10-methylenetetrahydrofolate reductase (MTHFR) gene, could increase stroke risk [58].

Hyperhomocysteinemia caused by pathological homocysteine metabolism has been associated with increased risk for several cardio- and cerebrovascular events. Elevated homocysteine levels can result from reduction or complete absence of MTHFR enzyme activity, which catalyzes the methylation of homocysteine. Variants in the MTHFR gene (c.677C>T or c.1298A>C) can lead to deficiencies in enzyme activity. The c.677C>T alteration was found to be an important risk factor for cerebral events. Examination of these polymorphisms revealed no association between carriage of the 677T or 1298C alleles and development of stroke [43,59]. However, we [60] reported that co-occurrence of these two alleles confers susceptibility to ischemic stroke, in both heterozygous and homozygous carriers. A case-control study [61] demonstrated that the 677T allelic variant is more prevalent in ischemic stroke patients in co-occurrence with the AT1R c.1166A>C variant, and together they confer increased risk for stroke.

Apolipoprotein E, the main apoprotein in the lipoprotein-transporting chylomicron particles, serves as a ligand for cellular receptors on liver and peripheral cells and interacts with proteoglycans on the endothelium to guide lipoproteins to lipases. The APOE gene, located on chromosome 19, is polymorphic, with three co-dominant alleles, ε2, ε3 and ε4, defining six genotypes [62]. Carriers of the ε2 alleles have lower total and low-density lipoprotein (LDL)-cholesterol levels and elevated triglyceride levels. Carriers of the ε4 allele have higher levels of both total and LDL-cholesterol than ε3/ε3 homozygotes [63]. Multiple studies have shown that APOE is an important predicting factor for the development of ischemic stroke [46,64-66]. We [40] observed that the APOE ε4 allele alone represents a minor genetic risk factor for ischemic stroke. However, the co-occurrence of the APOE ε4 allele and traditional risk factors, such as hypertension, diabetes mellitus, smoking and alcohol consumption, results in a considerably elevated risk for ischemic stroke.

Further search for functional SNPs in stroke
In the second period of stroke research (approximately 1995 to 2007), the identification of functional variants as possible susceptibility genes increased rapidly. Increased concentrations of plasma lipids, triglyceride and cholesterol levels were found to be independent risk factors for cardio- and cerebrovascular disorders [67-69]. Plasma triglyceride levels are determined by numerous genes and their interactions with the environment. The search for genetic variations affecting plasma triglyceride concentrations is still ongoing; several loci identified so far include the apolipoprotein genes APOAI, APOCIII, APOAIV, and APOA5 and the lipoprotein lipase (LPL) gene [66,70].
Apolipoproteins are lipid binding proteins involved in the plasma transport of lipids. They can activate or inhibit enzymes involved in lipoprotein metabolism or serve as signals for receptors in the liver or other cells. Genetic variations influencing their protein structure or synthesis may affect lipid metabolism, resulting in progression to cardio- and cerebrovascular disorders. The currently known apolipoproteins are APOAI, APOAII, APOAIV, APOA5, APOB48, APOB100, APOCI–APOCIII and APOE. The APOAI, APOCIII and APOAIV genes on chromosome 11 are so far the most intensively studied genes [71]. APOCIII encodes a protein of 79 amino acids that is synthesized in the liver and the intestines and is an essential constituent of circulating particles, such as very low density lipoprotein (VLDL) and chylomicrons [72]. Several polymorphisms of APOCIII have been associated with hypertriglyceridemia [73]. Carriers of the G allele of the c.3238C>G polymorphism in the APOCIII 3′ untranslated region (UTR) were reported to have higher plasma triglyceride concentrations, as were individuals with two minor alleles (-482C>T and -455T>C) of the APOCIII promoter [74,75]. Because APOCIII inhibits the hydrolysis of triglyceride-rich particles by lipoprotein lipase, its overexpression leads to hypertriglyceridemia [72]. Only a few studies have explored the role of the APOCIII gene g.8017G>C (rs5128) polymorphism in the development of ischemic stroke. Aalto-Setälä and colleagues [76] found a negative association between this APOCIII polymorphism and ischemic stroke.

The APOA5 gene is located near the APOAI-APOCIII-APOAIV cluster and encodes a 366 amino acid protein [77]. The mature APOA5 protein is synthesized in the liver and secreted into the plasma [78]. Evidence supports a central regulatory role for APOA5 in triglyceride metabolism through the inhibition of hepatic VLDL production or by guiding VLDL and chylomicrons to proteoglycan-bound lipoprotein lipase [79,80]. Furthermore, APOA5 has been described to independently influence plasma triglyceride levels in an opposing manner to APOCIII [81]. Around 40 polymorphisms of the APOA5 gene have been identified since the discovery of the gene [82-84]. These natural variants may alter the function of the protein. The most common variants, -1131T>C (rs662799) in the promoter region, g.1259T>C (rs2266788) in the 3’ UTR region, c.56C>G (rs3135506, resulting in a serine-to-tryptophan amino acid change in the signal peptide), and intronic g.IVS3+476G>A (rs2072560) have been repeatedly linked with elevated triglyceride levels [77,84,85]. Some of them have also been found to be independent risk factors for several conditions of triglyceride metabolism. The carriage of -1131C and g.IVS3+476A mutant alleles have been established to confer increased risk for all subtypes of ischemic stroke [86,87]. In Hungarian individuals, we found the 56G allele to be associated only with large-vessel stroke [88], but found the 1259C variant not to be associated with an increased risk for the disease [87]. The most common SNPs of APOA5 are in strong linkage disequilibrium and constitute haplotype variants designated by asterisks (APOA5*2, *3, *4 and *5). Among these, APOA5*2 was ascertained to be a risk factor for ischemic stroke. In any case, APOA5 is an example of shared susceptibility: we have found this naturally occurring variant and haplotypes to also confer susceptibility to other diseases, such as metabolic syndrome [89-91].

LPL is another important enzyme for triglyceride metabolism; it catalyzes the hydrolysis of chylomicron- and VLDL-associated triglycerides with the cofactor APOCII [92]. Approximately 100 polymorphisms have been described in the LPL gene. Most of these variants can lead to decreased LPL activity, which increases plasma triglyceride levels and the risk of atherosclerosis [93]. Huang et al. [94] found that the p.Asn291Ser alteration has a significant influence on serum levels of triglycerides, but this polymorphism does not contribute greatly to the overall risk of ischemic stroke. The results of a subsequent study [95] suggest that the p.Asn291Ser substitution is not associated with ischemic stroke in men but it possibly confers a twofold risk in women.

cAMP-specific 3′,5′-cyclic phosphodiesterase 4D (PDE4D) is an important regulator of proliferation of smooth muscle cells and macrophages, acting by degrading a key signaling molecule, cAMP. Defects in the protein result in the accumulation of cAMP in various cell types, such as monocytes and T lymphocytes. It can inhibit immune functions, such as proliferation and cytokine secretion, which can lead to atherosclerosis and plaque instability. The gene located on chromosome 5 encoding PDE4D was identified by Gretardottir et al. in 2003 [96]. Among the 260 SNPs examined by the authors [96], six have been found to be associated with stroke. Haplotype analyses have also shown significant correlation between certain haplotypes and ischemic stroke. Sixteen replication studies have since been published on the association between SNPs in PDE4D and ischemic stroke in different populations, with divergent results. Some of them confirmed the previously described positive association [97,98], whereas others dispute an association [99,100]. The inconsistent results may originate from differences between the population samples analyzed.

The gene encoding arachidonate 5-lipoxygenase-activating protein (ALOX5AP) has also been the focus of association studies. ALOX5AP is a proinflammatory mediator that is implicated in the pathogenesis and progression of atherosclerosis. Several polymorphisms and haplotypes were identified in ALOX5AP that have
been shown to confer increased risk for stroke in an Icelandic population [101]. This result was confirmed in German and Scottish populations [102,103]. However, some studies [97] found no association between the presence of polymorphisms in ALOX5AP and clinical phenotypes. Further investigation into the functional consequences of these variants is under way.

Before genome-wide association studies (GWASs) were used to study stroke, they were applied to other common diseases, such as Parkinson’s disease, myocardial infarction, inflammatory bowel disease, and even relatively rare conditions, such as macular degeneration. However, variants discovered in GWASs focusing on alleles involved in triglyceride metabolism, such as a variant (rs780094) of the glucokinase regulatory protein (GCKR), which is associated with increased triglyceride levels, were also investigated in relation to stroke [66, 70]. The GCKR gene, located on chromosome 2, encodes a protein of 625 amino acids that inhibits glucokinase in liver and pancreatic cells by binding the enzyme non-covalently to form an inactive complex. Järomi et al. [104] investigated the GCKR c.1337C>T (rs1260326) variant in a case-control study on a stratified stroke population; no association could be detected of GCKR alleles with triglyceride levels or with the susceptibility to stroke.

GWAS analysis has also identified SNPs localized at the GALNT2 and MLXIPL-TBL2 loci as being associated with increased triglyceride levels [66, 70]. The rs4846914 variant is an intronic variant of the UDP-N-acetyl-α-d-galactosamine:polypeptide N-acetylgalactosaminyltransferase 2 (GALNT2) gene, and its minor G allele was reported to associate with higher triglyceride concentrations. The GALNT2 gene is responsible for the transfer of an N-acetyl galactosamine to the hydroxyl group of a serine or threonine residue in the first step of O-linked oligosaccharide biosynthesis. Our Hungarian case-control study [104] evaluated the association of the GALNT2 rs4846914 variant with triglyceride levels and explored their possible contribution to the development of ischemic stroke. No positive association was detected either with triglyceride levels or with susceptibility to the disease [104].

The major alleles of the rs17145738 and rs3812316 variants within the MLXIPL-TBL2 region have been found to be associated with increased triglyceride levels [66, 70]. MLXIPL encodes a transcription factor, MXL interacting protein-like, which has a role in the activation of carbohydrate response element motifs in the promoter region of genes involved in lipogenesis and triglyceride synthesis and regulates lipogenesis and glucose utilization in the liver. TBL2 encodes transducin β-like 2 protein, which is thought to be involved in intracellular signaling. We [105] examined the effect of rs17145738 and rs3812316 variants on triglyceride levels and on stroke susceptibility. The results showed that the presence of these variants did not result in elevated triglyceride levels and did not confer risk for ischemic stroke.

**Genome-wide association studies in ischemic stroke**

The first GWAS directly focused on ischemic stroke patients was published in 2007 [106] (see Table 4 for a summary of GWASs reported so far in association with ischemic stroke). Matarín and colleagues [106] analyzed 278 ischemic stroke patients and 275 controls for more than 408,000 unique SNPs using Illumina Infinium Human-1 and HumanHap300 assays. The ischemic stroke subtypes were allocated according to the specified TOAST classification. The results showed hundreds of nominally statistically significant associated markers, among them the most notable candidate loci are inositol(myo)-1(4)-monophosphatase 2 (IMPA2) on chromosome 18, which is involved in the phosphatidyl-inositol signaling pathway, and Kv channel interacting protein 4 (KCNIP4) on chromosome 4 and potassium channel K17 (KCNK17) on chromosome 6, which are involved in potassium transport [106].

In 2008, Gretarsdottir and colleagues [107] described the results of a well powered GWAS with 1,661 ischemic stroke patients from the Icelandic population, classified according to TOAST, and 10,815 controls. The authors used Illumina Infinium Human-1 and HumanHap300 chips for genome-wide genotyping of more than 310,000 SNPs, from which 120 were selected for replication. Among those, rs2200733 and rs10033464 in the gene encoding the transcriptional activator PITX on chromosome 4q25 were found to be significantly associated with the development of ischemic stroke, especially for stroke due to cardioembolism (cardiogenic stroke) [107]. Some variants identified in the gene have previously been associated with Axenfeld-Rieger syndrome, which is a rare autosomal dominant, genetically heterogeneous disorder characterized by abnormal development of the anterior segment of the eye resulting in ocular changes and accompanied by systemic features, such as craniofacial dysmorphism, dental anomalies and cardiac malformations [108, 109].

A Japanese GWAS [110], which included 6,341 participants (992 ischemic stroke patients and 5,349 controls), analyzed more than 520,000 SNPs using the GeneChip Human Mapping 500K Array from Affymetrix and selected 100 SNPs for replication. This study [110] reported polymorphisms in the cadherin EGF LAG seven-pass G-type receptor 1 (CELSR1) gene on chromosome 22 as susceptibility factors for ischemic stroke. CELSR1 encodes a transmembrane G-protein-coupled receptor.

Ikram et al. [111] carried out a combined analysis of GWAS data generated from four large cohorts
(Framingham Heart Study, Rotterdam Study, ARIC and Cardiovascular Health Study). The cohorts include 19,602 participants, of whom 1,164 were patients having suffered ischemic stroke. The genotyping was performed with different platforms (Affymetrix GeneChip SNP Array 6.0, GeneChip Human Mapping 500K Array and 50K Human Gene Focused Panel; Illumina HumanCNV370-Duo and Infinium HumanHap550 3.0).

Two of the analyzed SNPs, rs11833579 and rs12415791 on chromosome 12 near the nerve injury-induced protein 2 (NINJ2) and WNK lysine deficient protein kinase 1 (WNK1) genes, were confirmed in the replication study, which revealed a positive association between carrying minor alleles and ischemic stroke, in particular the atherothrombotic stroke subtype [111].

Despite their huge power and impact, GWASs also have limitations. In a few cases [110], the studies involved a single population. Validation of the findings in different ethnic groups will be required. In certain replication studies [106], the number of participants was too small and the functional relevance of the identified polymorphisms remains unclear. Further genotyping as well as detailed functional and epidemiological studies in larger populations are therefore required to identify the exact role and associations of these genes and gene variants in the development of ischemic stroke.

**Concluding remarks**

Significant progress has been seen in the field of stroke susceptibility genetics over the past one and a half decades; however, research intensity has been moderate in comparison with other multifactorial diseases, such as diabetes, metabolic syndrome or ischemic heart diseases. It has become clear that stroke shows a complex phenotype and, as a result, a careful stratification of the patients is essential, as the genetic etiology can differ strongly between the subtypes. An imbalance in research intensity can also be observed for the different stroke groups: causative genes are still to be discovered for some Mendelian forms, whereas in the polygenic groups the majority of efforts are mainly restricted to the small- and large-vessel associated stroke variants, with less frequent rare variants receiving little attention. Thus, although the available biobanks have been valuable for moving small- and large-vessel associated stroke pathology research forwards, they should be strengthened with additional samples from the less frequent stroke types. This may not be a simple task, given the small incidence rates of these rare variants.

As has occurred for other diseases, the recent GWAS area has opened new perspectives for susceptibility gene research in relation to stroke. It is now clear that some susceptibility alleles can be shared: the same allele can confer susceptibility to stroke, ischemic heart diseases or even metabolic syndrome. Some susceptibility factors that are associated with clinical traits can themselves also be risk factors for stroke: thus, alleles associated with increased triglyceride levels can also be susceptibility factors for stroke. The possible association between triglyceride levels and stroke is an old question; future association studies will help us understand which naturally occurring genetic variants associate with increased triglycerides alone without stroke and which variants also associate with cerebral pathology. In a broader context, better knowledge about different metabolic and biochemical parameters in the healthy population will also help us to understand their potential role in the development of stroke [112].

Although susceptibility allele tests are offered on the palette of some direct-to-consumer companies, we have to keep in mind that current evidence does not yet support their use in clinical practice [113-118] given that...
the predictive value of these test results is still limited. The rapid spread of exome sequencing and whole-genome sequencing techniques will probably help us to identify novel genes in stroke types that have a Mendelian monogenic origin, even for rare stroke types, rather than helping to understand the complex pathology associated with polygenic types of stroke.

Abbreviations
ACE, angiotensin converting enzyme; ALOX5AP, arachidonate 5-lipoxygenase-activating protein; AP0C, apolipoprotein; AT1R, angiotensin receptor; CERS1, cadherin EGF-LAG seven-pass G-type receptor 1; eNOS, endothelial nitric oxide synthase; GCKR, glucokinase regulatory protein; GALNT2, UDP-N-acetyl-alpha-galactosamine polyepptide; N-acetylglactosaminyltransferase 2; GWAS, genome-wide association study; iNOS, nitrous oxide synthase; MTHFR, 5,10-methylenetetrahydrofolate reductase; INN2, nerve injury-induced protein 2; PDE4D, phosphodiesterase 4D; PITX, paired-like homeodomain 2; SNP, single-nucleotide polymorphism; TBL2, transducin (beta)-like 2; TOAST, Trial of Org 10172 in Acute Stroke Treatment; VKC2, vitamin-K-dependent protein C; VN1K, vitamin-K dependent protein kinase 1.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
Both authors contributed equally in the preparation of the review.

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