

Advances in Stroke Research

Chapter 1

Recent Advances in Stroke Genetics

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1. Introduction

Stroke is a complex, polygenic, and multi-factorial neurological disorder caused by a combination of environmental, vascular and genetic factors [1]. Despite new developments in the treatment and prevention, stroke accounts for the primary cause of adult disability, the second leading cause of dementia and the third leading cause of mortality in most of the developed countries [2], [3]. In the last four decades, while it has been estimated a 42% decrease in stroke incidence in high-income countries, there is more than 100% increase in stroke incidence in low to middle income countries [4]. The National Commission on Macroeconomics and Health has projected that cases of stroke would increase from 1,081,480 in 2000 to 1,667,372 in 2015. According to WHO 80% of stroke cases in the world would occur in lower and middle income countries, mainly India and China by 2050 [5].

From pathological point of view, there are two main types of strokes: Ischemic stroke

(blockage of a blood vessel supplying the brain) and Hemorrhagic stroke (bleeding into or around the brain). Ischemic and Hemorrhagic strokes are further sub-classified which are tabulated in Table-1.

Table-1: Classification of Strokes

Ischemic stroke (IS)	Hemorrhagic Stroke (HS)
Large artery atherosclerosis (LVD)	Intracerebral hemorrhage (ICH) Subarachnoid hemorrhage (SAH)
Small-vessel occlusion (SVD)	
Cardio-embolism (CE)	
Stroke of other determined etiology	
Stroke of undetermined etiology	

2. Ischemic Stroke

A majority of strokes are ischemic (70–80%). As per Trial of Org 10172 in Acute Stroke Treatment (TOAST) classification, IS can be categorised into the main etiological subtypes (i) Large-artery atherosclerosis, (ii) Small-vessel occlusion, (iii) Cardio-embolic stroke, (iv) Stroke of other determined aetiology and (v) Stroke of undetermined aetiology based upon presumed pathophysiology.

(i) *Large-vessel disease (LVD)*: large-vessel occlusive disease denotes significant atherosclerotic narrowing (>50% measured by duplex imaging or arteriography) and usually due to atherosclerosis or occlusion of major brain artery or branch cortical artery. The proportion of LVD greatly varies as per age, sex and ethnicity e.g. LVD is 2-4 times more common in males as compared to females [6].

(ii) *Small-vessel disease (SVD)*: It is caused due to the involvement of small perforating end arteries within the brain. Twenty five percent of first ever stroke is caused due to SVD [7]. The most common pathology related to SVD are lipohyalonosis and atherosclerosis, restricted to small deep performing end-arteries providing basal ganglia, deep white matter, brain stem and thalamus [8]. In fact the brain stem or sub-cortical hemispheric region is usually small with a diameter of less than 1.5 cm and mostly asymptomatic.

(iii) *Cardioembolism (CE)*: It occurs when embolus initiating from thrombi in the heart occludes the cerebral arteries. Minimum one cardiac source for an embolus must be recognized for a potential diagnosis of CE subtype of IS.

(iv) *Stroke of other determined aetiology*: Less frequent causes of IS include arterial dissections [9], vasculitis [10], haematological diseases [11] the anti-phospholipids syndrome [12], and rare monogenic disorders [13].

(v) *Stroke of undetermined aetiology*: The underlying phenomenon of IS is still unidentified.

This may occur due to various reasons such as having two or more potential causes of stroke and the reason for the cryptogenic strokes or cause of superficial evaluation was unknown.

3. Hemorrhagic Stroke

Hemorrhagic stroke (HS) accounts for 10-20% of strokes and occurs when an artery leaks into the brain and subsequently blood spills out into the surrounding tissue of the brain, thereby causing disruption in the blood supply and subtle chemical balance that neurons require to function properly. HS can occur due to a number of reasons. One of the frequent causes is a bleeding aneurysm, a thin or weak mark on an artery wall. Over the time, these weak spots balloon or stretch out under the high arterial pressure and the thin walls of these ballooning aneurysms get ruptured causing bleeding into the space surrounding the brain cells. HS is categorized into two types: SAH and ICH. HS is associated with approximately four times higher mortality than IS [14] and only 38% of the first ever primary ICH patients have survived more than one year [15]. Primary ICH is caused due to spontaneous rupture of small vessels damaged by chronic hypertension or cerebral amyloid angiopathy [16].

4. Genetics and stroke

There are several known risk factors for stroke; however, these risk factors do not explain why some individuals are more susceptible to stroke when compared with other individuals in identical environmental determinants. Several case-control and cohort studies on family history of stroke supported the presence of a genetic component in both IS and HS [17-21]. Genetic factors could also act at various levels: by predisposing to conventional risk factors, by modelling the effects of such risk factors alternatively, and by a direct independent effect on stroke risk. Other factors such as lifestyle changes, migration from rural to urban regions, and an increase in the exposure to stroke risk factors contribute to the stroke burden in the future. The key risk factors for the development of stroke are age, hypertension, diabetes mellitus, obesity, cigarette smoking and cardiovascular diseases.

Genetics may contribute up to 50% of an individual's risk of developing a stroke in future (ISGC 2013; www.strokegenetics.org). Nevertheless, it is hard to interpret the stroke risk accurately with the help of these factors. Several epidemiological findings suggest that genetic risk factors are significant for common 'sporadic' stroke. Animal model studies, twin and family-based association studies have suggested a substantial genetic component of stroke [22]. There was a considerable increase in the prevalence of stroke among the monozygotic as compared to the dizygotic twin pairs suggesting involvement of genetics in determining stroke risk [23]. Numerous family-history studies have demonstrated that a family history of stroke leads to more stroke cases than in stroke-free controls, but such type of association may have occurred due to recall bias [24]. Furthermore, the findings for the association of family history of stroke with stroke risk has been confirmed from the data obtained from the

prospective Framingham heart study [25]. The relationship between family history and stroke risk may have occurred related to lifestyle environment and unraveling this from genetic risk is difficult. The data obtained from the family history studies have suggested that the genetic risk factors may differ by subtypes of stroke, with stronger associations accounted for the LVD and SVD subtypes based on TOAST classification. Genetic causes of stroke, range from classic Mendelian (a single gene leads to disease) to complex (multiple genes contribute to disease in combination with other genetic and/or environmental factors) [26].

5. Genetics of Ischemic Stroke (IS)

The role of genetic variation in stroke patients have emerged from the twin studies conducted in the early 1990s and it was reported that there is five times more higher risk in monozygotic twins as compared with dizygotic twins or siblings [23]. Findings from subsequent twin and family history studies corroborated these results and indicated that genetic predisposition to IS differs according to age and stroke subtype [27,28]. The genetic component is more prevalent in LVD than in SVD or cryptogenic IS [29,30]. Furthermore, genes have a stronger role in stroke patients younger than 70 years of age [31]. Researchers on a multicentric study concluded that siblings usually develop the same stroke subtype [32]. These findings have been confirmed and extended by studies in which the heritability of IS was calculated from genome-wide data, giving estimates of 40% for LVD, 33% for CE, 16% for SVD, and 38% for the combined endpoint of any IS [29].

6. Mendelian or Monogenic Inheritance

Monogenic disorders caused by rare or private (pedigree-specific) variants inherited with a clear Mendelian pattern include stroke as a phenotypic manifestations presented in Table-2. Common examples are cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), [31] mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS), [32] and Fabry's disease [33].

CADASIL is an autosomal-dominant disease that occurs by mutations in NOTCH3 gene, which encodes a cell-surface receptor expressed in pericytes as well as vascular smooth muscle cells and has a significant role in arterial development [34]. This syndrome is clinically characterised by recurrent strokes, psychiatric disturbances, migraine and progressive cognitive impairment. Imaging findings includes diffuse white matter hyperintensities, with preferential bilateral involvement of anterior temporal lobe and external capsule and lacunas [31]. A slightly different disorder than CADASIL is CARASIL which is a recessive small vessel disease caused by the mutations in HTRA1 gene [35]. The occurrence frequency of CARASIL is far less than CADASIL, with not more than 50 affected patients reported till date (predominantly in Asian populations and very rare cases reported in Caucasian populations).

MELAS is a heterogeneous disorder caused by mutations in various mitochondrial genes which is phenotypically characterised by clinical manifestations such as recurrent headaches, seizures, vomiting, muscle weakness and pain [32]. It is inherited from maternal mitochondrial DNA (Only the mother passes on the disorder but both male and female genders can be affected). Fabry's disease is caused by the mutations in GLA gene which encodes for α -galactosidase, a lysosomal enzyme resulting in systemic accumulation of glycosphingolipids various tissues. Initially Fabry's Disease was regarded as an X-linked recessive disorder, but research findings have reported that heterogeneous women with this disease can have life threatening clinical manifestations [36]. The clinically noticeable changes occurs early in childhood, typically through neuropathy-induced acroparaesthesias which also includes a variety of symptoms including hypertension, angiokeratomas, kidney dysfunction and stroke [37]. Numerous other mendelian disorders that can cause stroke includes Moyamoya Disease [38], homocystinuria [39], sickle cell disease [40] which is the most frequent cause of stroke in children, connective tissue diseases such as Ehlers-Danlos syndrome type IV [41], Marfan's syndrome [42], pseudoxanthoma elasticum [43] and osteogenesis imperfecta represented in Table-2.

7. Non-Mendelian or Polygenic Inheritance

During 2012-2014, several Single Nucleotide Polymorphisms (SNPs) have been related to common IS risk. Certain SNPs have been associated with risk of specific IS subtypes such as LVD and CE. Large international studies on stroke recovery and exome content are still ongoing. Advanced mathematical models have been used to study how several SNPs can act together and increase stroke risk burden. Such efforts require large numbers of patients and controls, which is achieved by co-operation in large international consortia such as the International Stroke Genetics Consortium (ISGC). Several other methods that could improve our knowledge of stroke genetics are being developed e.g.: Next-generation sequencing; Whole genome sequencing; and Epigenetics.

Table-2: Monogenetic form of ischemic stroke

Disorder	Gene	Chr	Gene wProduct	Mode of Inheritance	Stroke mechanism	Associated clinical features	Diagnostic test
CADASIL	NOTCH3	19p13.1	NOTCH3	AD	SVD	Migraine with aura, cognitive problems, depression, seizures, stroke	Mutational screening, skin biopsy
CARASIL	HTRA1	10q	Serine protease	AR	SVD	Premature baldness; severe low back pain; spondylosis deformans or disk herniation	Mutational analysis

Disorder	Gene	Chr	Gene wProduct	Mode of Inheritance	Stroke mechanism	Associated clinical features	Diagnostic test
Fabry's disease	GLA	Xq22	α -galactosidase A	X-linked	LVD and SVD	Angiokeratoma; neuropathic pain; acroparaesthesia; hypohydrosis; corneal opacities; cataract; renal and cardiac failure	α galactosidase activity, mutational screening
MELAS	MTTL1	mtDNA	tRNA ^{Leu} (UUR)	Maternal	Complex (microvascular & neuronal factors)	Developmental delay; sensorineural hearing loss; short stature; seizures; episodic vomiting; diabetes; migraine-like headache; cognitive decline	Muscle biopsy, mutational analysis of mtDNA
Sickle cell disease	HBB	11p15.4	Haemoglobin - β	AR	LVD, SVD, haemodynamic insufficiency	Pain crises; bacterial infection; vaso-occlusive crises; pulmonary and abdominal crises; anaemia; myelopathy; seizure	Peripheral blood smear, electrophoresis, mutational analysis
Marfan syndrome type 1	FBN1	15q21.1	Fibrillin 1	AD	CE, Arterial dissection	Pectus carinatum or excavatum; upper-to-lower segment ratio <0.86, or arm-span-to-height ratio >1.5; scoliosis >20%; ectopia lentis; dilation or dissection of the ascending aorta; lumbosacral dural ectasia	Clinical diagnosis (mutational screening)
Ehlers–Danlos syndrome type IV	COL3A1	2q31	Collagen, type III, α -1	AD	Arterial dissection	Easy bruising; thin skin with visible veins; characteristic facial features; rupture of arteries, uterus, or intestines	Biochemical studies, mutational screening
Homocystinuria due to MTHFR deficiency	MTHFR	1p36.22	Methylene tetrahydrofolate reductase	AR	Small and large artery vasculopathy	Cognitive problem, myopia, osteoporosis, thrombo-embolic events	Mutational screening and plasma level of homocysteine

Disorder	Gene	Chr	Gene wProduct	Mode of Inheritance	Stroke mechanism	Associated clinical features	Diagnostic test
Homocystinuria due to CBS deficiency	CBS	21q22.3	Cystathionine β -synthase	AR	LAA, SVD, CE, Arterial Dissection	Mental retardation; atraumatic dislocation of lenses; skeletal abnormalities (Marfan-like); premature atherosclerosis; thromboembolic events	Urine analysis, measurement of concentrations of homocysteine and methionine in plasma (mutational screening)
Neurofibromatosis type 1 (von ecklinghausen's disease)	NF1	17q11.2	Neurofibromin 1	AD	Small and large artery vasculopathy	Neurofibromas, optic glioma, cerebral ischemia	Mutational screening
HERNS	TREX1	3p21.31	TREX1	AD	Complex (microvascular and neuronal factors)	Visual loss, cognitive problems, stroke-like episodes, renal dysfunction	Mutational analysis

Abbreviations: Chr- chromosome; AD-Autosomal dominant; AR-Autosomal recessive; LVD- Large Vessel Disease; SVD- Small Vessel Disease; CE- Cardio Embolic; CADASIL- Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy; CARASIL- Cerebral Autosomal Recessive Arteriopathy with Subcortical Infarcts and Leukoencephalopathy; MELAS-Mitochondrial encephalomyopathy lactic acidosis and stroke-like episodes; HERNS- Hereditary endotheliopathy with retinopathy, nephropathy and stroke.

The types of studies to identify genetic risk factors for stroke include (i) Linkage study: this study maps a possible disease locus by studying how genetic markers have segregated in large multigenerational pedigrees with many affected family members in relation to the affection status of the pedigree members. (ii) Candidate gene association study: it includes a hypothesis-driven approach in which genes that may be involved in the pathogenesis of stroke are tested for association; these studies are focused on the selection of genes that have been related to disease previously in some way or other, thus coming with prior knowledge about gene function. This approach uses a case-control study design and is the most common approach for identifying genes for polygenic genetic disorders. (iii) Exome sequencing study: this includes efficient strategies to selectively sequence the coding regions of the genome. (iv) Genome-wide association study (GWAS): it is the study of genetic variation across the (entire) human genome.

(i) Linkage studies

Linkage studies involve identifying associations between chromosomal markers and

disease phenotype within families. These studies are generally ‘genome-wide’ or ‘chromosome-wide’ and only identify large regions of linkage, not specific genes or mutations. This method is most useful for variants that have a large effect. For numerous reasons the linkage approach is complex to apply in investigation of genes leading to stroke. Stroke is a late onset disease, due to which information collection from other family members becomes difficult. The polygenic nature of stroke, and shared environmental exposures, also contributes to the barriers in the linkage approach, because this approach is unable to detect genes with minimal or modest effect on risk of stroke [44,45]. Genome wide linkage studies on Icelandic individuals with stroke have indicated the presence of gene on chromosome 5, which may have significant role in the development of the stroke phenotype [46]. The same group reported that the likely candidate gene was PDE4D, especially in LVD stroke and CE stroke but not SVD stroke [47].

(ii) Candidate gene association studies in stroke

Candidate gene approach using case-control methodologies is the main method to find genes associated with stroke. In this approach gene that may be associated with pathogenesis of stroke are tested for association to determine frequency distribution of allele and allelic variant in comparison with controls. Candidate gene contribution to stroke is mild to moderate and to detect this small difference large sample size is required. Some of the important candidate gene association studies with stroke are summarized below:

Homocysteine Metabolising Gene

Methylene tetrahydrofolate reductase (MTHFR)

Numerous prospective and case-control studies confirmed that moderate increase of plasma Homocysteine (Hcy) is a potential risk factor for CVD, arterial and venous thrombosis including stroke [48]. Methylene tetrahydrofolate reductase (MTHFR) is an essential enzyme involved in the Hcy metabolism. A recent metaanalysis of thirty eight case-control studies comprising of 6310 IS patients and 8297 controls conducted by Kumar A et al. (2015) showed significant associations between -677C/T polymorphism of MTHFR gene and IS risk in dominant [OR= 1.09, 95% CI= 1.06 to 1.12, P-value<0.001] and recessive model [OR= 1.31, 95% CI= 1.19 to 1.44, P-value<0.001] [18].

Lipid Metabolising Genes

Apolipoprotein E (APOE)

Apolipoprotein E (ApoE) protein plays a major role in transport and metabolism of lipids and also extensively expressed in brain. It is one of the most common genes investigated in vascular and neurodegenerative disorders. The protein product of APOE are made of gly-

coprotein with 3 common isoforms, E2, E3, and E4, encoded by the particular alleles $\epsilon 2$, $\epsilon 3$, and $\epsilon 4$, giving rise to 6 genotypes. One study conducted in North India showed a significant association between ApoE gene polymorphism and risk of IS [49]. ApoE gene polymorphism may modify the associated modifiable risk factor, e.g.: the effect of smoking cigarettes on IS may be more in younger individuals who are detected with the morphic Apo $\epsilon 4$ allele [50]. A recent metaanalysis [51] of 24 studies involving 4778 IS cases and 14674 controls done by Kumar A et al. (2015) have showed a significant association between carrier of $\epsilon 4$ allele and risk of IS [OR= 1.55; 95% CI = 1.20 to 2.01].

Nitric oxide synthase metabolising gene

Endothelial Nitric Oxide Synthase (eNOS)

Endothelial Nitric Oxide Synthase (eNOS) gene is mapped on chromosome 7 (7q35-q36) and consist of 26 exons. It encodes for an enzyme that produces Nitric Oxide (NO) in the vascular endothelium [52]. eNOS gene polymorphism may lead to decrease in the action of vascular endothelial nitric oxide synthase which subsequently causes partly damaged endothelium-dependent vasodilatation (which is a general characteristic of atherosclerotic vessels), therefore, genetic variation could lead to the development of stroke [53]. A recent meta-analysis of 31 studies including 8,547 patients and 9,117 controls performed by Guo X et al. (2014) and Kumar A et al. (2017) suggests a significant association between eNOS gene 4b/a, T-786C, G894T polymorphism and risk of IS [54 55].

Fibrinolytic/thrombotic genes

Factor V Leiden (FVL)

The Factor V Leiden (FVL) mutation is the most prevalent prothrombotic genetic mutation and causes activated protein C resistance. It is associated with venous thromboembolism in IS patients [56]. A meta-analysis published in 2010 including 18 case-control studies conducted by Hamedani AG et al. involving 2,045 cases and 5,307 controls showed significant association of Factor V Leiden G894T gene polymorphism with the susceptibility of stroke [OR = 2.00;95% CI= 1.59 to 2.51] [57].

β -Fibrinogen Gene Polymorphism (C_{148} -T)

Fibrinogen levels are controlled by the both environmental and genetic factors. The concentration of fibrinogen increases with age, smoking, lack of exercise, trauma, use of contraceptives, hypertension, lipid abnormalities and diabetes mellitus [58]. Various SNPs in FGB gene that determine fibrinogen level have been identified and one of the most extensively studied is SNP -148 C/T (rs1800787) which is located in FGB promoter [59]. A meta-analysis done by Zhang LJ et al. (2014) including eighteen independent case-control studies involving

2159 IS patients and 3222 control subjects showed that -148C>T polymorphism in the FBG gene was associated with increased risk of IS (OR=1.40,95%CI;1.20-1.45,p<0.0001) respectively [60].

Phosphodiesterase 4D (PDE4D)

Phosphodiesterase 4D (PDE4D) gene is located on chromosome 5q12 with a spanning region of >1.5mb and known to have 24 exons. Through the use of alternate splicing and differential promoters, PDE4D gene expresses nine different isoforms [61]. PDE4D is the family of enzyme which breaks the phosphodiester bond of cyclic adenosine monophosphate (cAMP) which helps to maintain the duration of action and proper level of cAMP inside the cell. cAMP acts as secondary messenger which involves stimulating the genes to generate inflammatory mediators through different types of inflammatory cells and arteriosclerosis. The deCODE group published genome wide screen results for genetic susceptibility of stroke in Iceland in 2002 [47]. A recent meta-analysis of 25 studies comprising of 8,878 cases and 12,306 controls conducted by Yan Y et al. (2014) showed a significant association between PDE4D (SNP83) gene polymorphism and IS risk, especially in Asian and Chinese, but not in Caucasians populations (OR = 0.87, 95% CI = 0.69–1.11) [62]. Studies on different aspects of PDE4D gene and PDE4 pathway have provided a strong evidence for its role in stroke development. However, further advanced studies employing a new and comprehensive genomic, molecular and proteomic approach needs to be engaged that can revolutionize the understanding of PDE4 pathway/PDE4D gene in stroke pathophysiology in a better way.

Arachidonate 5-lipoxygenase-activating protein (ALOX5P)

Arachidonate 5-lipoxygenase-activating protein (ALOX5AP) is a protein-coding gene located on chromosome 13p12-13 which acts as a critical mediator in the biosynthesis of the leukotrienes, involved in the progress and pathogenesis of atherosclerosis [63]. A recent study published by Wang et al. (2014) showed a significant association of TT/TA genotype of ALOX5AP SG13S114 A/T with the increased risk of Acute cerebral infarct (ACI) in a Northern Han Chinese population (OR= 1.82, 95%, CI= 1.14–2.92, p = 0.012) [64]. A recent meta-analysis of 11 case-control studies comprised of 5,361 cases and 5,676 controls conducted by Ye F et al. (2014) showed that AA genotype of ALOX5AP SG13S114A/T are significantly associated with increased risk of incidence of IS as compared to the TT genotype in a Chinese population (OR= 1.47, 95% CI= 1.13-1.91, P=0.005) [65].

(iii) Exome Sequencing and Copy number variation

The genetic makeup of stroke is complex and likely includes non-SNP variations as disease causing factors. Current association studies test the ‘common disease-common variant’ hypothesis assuming that the risk variant is commonly found in >5% of the general

population. The variants tested are usually intronic or intragenic SNPs that do not provide any information on the role of protein coding part of the genome i.e. exome. However the lack of reliable associations in the recent stroke GWA studies points towards the possibility of rare or low frequency variants with high penetrance and large effect sizes. These non-SNP variations could be very informative about the genetic underpinnings of stroke, yet they remain under represented in most association studies.

The National Heart, Lung, and Blood Institute (NHLBI) Exome Sequencing Project recently identified rare genetic variations in HDL-associated paraoxonase-1 (PON1) gene to be associated with IS. In a sub group analysis of different ethnic populations, the study also showed that the variants had the strongest association with African American populations [66]. Another study recognized unusual exonic variants to be associated with stroke suggesting that coding variations in the human genome need to be closely examined [67]. The study was small, consisting of only 10 stroke cases (8 African-Americans and 2 Caucasians) and a non-standardized methodology. 48 genes that had at least one uncommon variant across all stroke cases were known. CSN3 gene, was found to contain an excess of rare variations as compared to other genes [67].

Copy number variations (CNVs) are large structural variations of the genome that include deletions, insertions, translocations, inversions and variable number repeats. CNVs alter the gene dosage without affecting function and are recognized to play a role in monogenic disorders although their role in complex character such as stroke is unclear [68]. GWA of CNVs associated with IS has not indicated any unique structural genomic variations that may contribute to stroke risk [69]. While smaller candidate gene based studies have provided slight evidence of a unique genomic structure in IS patients [70], large well-powered studies have failed to do the same [71]. The Human Genome Structural Variation initiative started by the National Human Genome Research Institute (NHGRI) to map structural variations within the human genome, is currently in the process of genotyping human CNVs and will add more information to the existing reference genome which may aid future stroke CNV analysis in identifying a disease-causing mutation.

(iv) Genome wide association studies (GWAS)

In the last decade, significant advances were made in sequencing and genotyping technologies. The field of complex genetics has been revolutionized by the GWAS approach, which uses microarray technology to genotype up to one million or more SNPs, spanning the whole genome, in an individual subject [72]. Such method provides the opportunity of genotyping thousands of SNPs together in GWAS to find genetic variations associated with specific disease. Such studies are mainly useful in investigating genetic variations that leads to common complex diseases.

GWAS on stroke were very few till 2003 when Gretarsdottir et al. (2003) identified PDE4D to be significantly associated with IS risk in an Icelandic population (DeCODE) [47]. However, numerous efforts to replicate these findings failed [73-75], while some studies reported conflicting results [76-78]. These inconsistencies were attributed to possible flaws in study design, i.e. pooling of LVD and CE strokes in order to investigate the risk association with PDE4D gene, which we now know are sub-type specific [79]. Lack of independent replication and limited experimental validation of results using B-cell lines was also suggested to be a major limitation of the original study [80]. Numerous other GWA studies followed, but no single locus was known at a genome wide level of significance (p value $\leq 5 \times 10^{-8}$).

Despite a number of genome-wide association studies reporting discoveries of novel genetic risk variants for stroke, these were rarely replicated implying that either these discoveries were fallacious or the effect is so minimal that much larger studies are required [81]. The largest and most recent GWA study published so far, the METASTROKE meta-analysis, (~12,000 cases and ~60,000 controls) validated previously reported genes PITX2, HDAC9 and ZFHX3 suggesting that these are true associations [82]. All loci exhibited heterogeneous effect across subtypes of IS, supporting different genetic make up for each subtype presented in Table-3.

Various other GWAS have been performed in stroke mostly are in European population, with very less comparative data available in other ethnic populations. A few studies have been conducted in populations of South East Asian ancestry [83-86]. The effect size is roughly similar across all GWAS's, ranging from 1.00 to 1.85, confirming that the effect sizes are small but the population attributable risks could be large given the general nature of this condition. Most of the studies however have failed to replicate their findings [87-89].

Several common genetic variants that affect common forms of IS without following a clear Mendelian pattern of inheritance are represented in Table-3. These variants have been identified by candidate-gene analyses and studies (GWAS), and they are likely to represent only the tip of the iceberg. Importantly, the most robustly replicated discoveries have been made by assessment of specific IS subtypes. In view of its wide acceptance within the research and clinical communities, studies addressing IS subtypes have used the TOAST classification system to categorise strokes on the basis of apparent mechanism. The first replicated genetic variants were identified through GWAS strategy

Moreover, because of large sample sizes accomplished by GWAS of cardiac disorders, it is not surprising that the primarily reproduced genetic variations were notable for CE stroke. Two loci situated on chromosomes 4q25 [90-93] and 16q22, [94] which were accounted for primarily to be related with AF, were along these lines recognized as risk factors for CE stroke. The major variation (minor allele frequency [MAF] 21%) inside chromosome 4q25 is related with a 36% expanded risk of CE stroke. This locus lies close PITX2, which encodes a

transcriptional activator that—in experimental models—has been appeared to have an influence being developed of the sino-atrial node (the natural pacemaker of the heart) and in management of ion channels involved in regulation of the cardiovascular activity potential [95]–[98]. The intermingle of a likely causative component for IS (AF), strong practical confirmation from experimental models, and hearty measurable outcomes in genetic-association studies (GAS) of IS makes this locus a case of a persuading hereditary risk factor for stroke. The main variant within chromosome 16q22 (MAF 19%) is associated with a 25% increased risk of CE stroke and a 5% higher risk of all stroke [94]. This locus encompasses ZFHX3, which encodes the transcription factor ATBF1; 74 however, the mechanism linking this locus to AF remains unidentified.

In the largest meta-analysis of GWAS of IS published to date, [82] the main SNP at the 9p21 locus (MAF 48%) was associated with a increase of 15% in LVD risk. Further support for the role of this genomic region in large-vessel atherosclerosis-related disorders comes from previous work in which this locus was associated with coronary artery disease (CAD) [99-102]. Shared genetic susceptibility to LVD stroke and CAD has been appeared for variations meeting a lower edge of significance, which could somewhat represent the known co-morbidity of these scatters [103]. Genetic studies have not yet yielded any variations in patients of white ethnic origin with SVD, potentially as a result of the high heterogeneity in phenotype ascertainment (i.e., numerous definitions of SVD can be utilized) and the genuinely small sample sizes. Research is progressing to enhance phenotyping in these cases. With a new classification system, the point is to standardise classification utilized as a part of SVD and related imaging discoveries and to reduce the number of diseases that mimic SVD and lead to misclassification [104]. Although common genetic variants affecting overall risk of IS (irrespective of stroke subtype) have not yet been recognized, findings of the EuroCLOT study showed that a locus known to affect coagulation function was associated with both LVD and SVD subtypes of IS [105]. Considering the well documented role of genetic variation in arterial thrombosis, [106] and the robust link described between ABO blood groups and risk of stroke, [107] the EuroCLOT researchers aimed to examine the role of genetic variants that may potentially play a part in end-stage coagulation and fibrin structure in people with IS. Mutations within the ABO locus (main variant had a MAF of 39%) were associated with a 13% increased risk of CE stroke and a 12% augmented risk of LVD. This finding lends support to the notion that genetic variation targeting biological pathways that affect all stroke mechanisms—the coagulation system in this case—will have a role in more than one stroke subtype.

Table 3: Genetic variants affecting risk of ischemic stroke in a non-mendelian fashion

Author & year	Sample size cases/control	Gene	SNP	Chr	Pheno type	Ethnicity	RA	RAF (%)	OR (95% CI)	P value	Other studies
Traylor M 2012 [82]	12,389/62,004	HDAC9	rs2107595	7p21.1	LVD	Caucasian	A	16	1.39 (1.27 to 1.53)	2.03×10^{-16}	Blleguez C 2012; Kilarski LL 2014 [108], [109]
Traylor M 2012 [82]	12,389/62,004	PITX2	rs6843082	4q25	CE	Caucasian	G	21	1.36 (1.27 to 1.47)	2.8×10^{-16}	Gretarsdottir S 2008; bjartsson DF 2007; Lemmens R 2010; Lubitz SA 2010; Kilarski LL 2014 [90]–[93], [109]
Traylor M 2012 [82]	12,389/62,004	CDKN2A/B	rs2383207	9p21.3	LVD	Caucasian	G	52	1.15 (1.08 to 1.23)	3.32×10^{-5}	Matarin 2008; Smith JG 2009
Traylor M 2012 [82]	12,389/62,004	ZFH3	rs879324	16q22.3	CE	Caucasian	A	19	1.25 (1.15 to 1.35)	2.3×10^{-8}	Gudbjartsson DF 2009[69], [94], [110]
Holliday EG 2012 [79]	1162/1244	CDC5L/SUPT3H	rs556621	6p21.1	LVD	Caucasian	A	33	1.21 (1.12 to 1.28)	4.7×10^{-8}	Traylor M 2012 [82]
Williams FM 2013 [106]	8900/55,000	ABO	rs505922	9q34	CE, LVD	Caucasian	C	39	1.06 (1.06 to 1.14)	4.7×10^{-57}	Wu O 2008 [107]
Cheng YC 2011[111]	889/927	FMNL2	rs2304556	2q23.3	YS	Caucasian /African	G	-	0.69 (0.60 to 0.79)	1.20×10^{-7}	-
Cheng YC 2011 [111]	889/927	ARL6IP6	rs1986743	2q23.3	YS	Caucasian /African	A	-	0.69 (0.60 to 0.79)	2.70×10^{-7}	-

Author & year	Sample size cases/control	Gene	SNP	Chr	Pheno type	Ethnicity	RA	RAF (%)	OR (95% CI)	P value	Other studies
Meschia JF 2011[29]	248/84	ROBO1	rs1383407	3p12.2	IS	Caucasian	C	44	0.96	7.63×10^{-5}	-
Ikram MA 2009[87]	1164/3613	NINJ2	rs 11833579	12p13.33	IS	Caucasian	A	23	1.41 (1.27 to 1.56)	2.03×10^{-10}	Traylor M 2012 [82]
Yamada Y 2009 [84]	6341/3435	CELSR1	rs6007897	22	IS	Asian	G	-	1.85 (1.29 to 2.61)	6.0×10^{-4}	
Hata J 2007 [85]	1112/1112	AGTRL1	rs9943582	11q12	IS	Asian	G	-	1.30 (1.14 to 1.47)	6.5×10^{-5}	-
Kubo M 2007 [83]	188/188	PRKCH	rs1452	13q22-23	SVD	Asian	A	23	1.40 (1.23 to 1.59)	5.10×10^{-7}	-
Traylor M 2014 [112]	6778/12,095	MMP12	rs660599	11q22	LVD	Caucasian	T	19	1.18 (1.05–1.32)	2.5×10^{-7}	-
Kilariski LL 2014 [109]	3420/6821	NAA25/C12orf30	rs 17696736	12q24.12	IS	Caucasian	G	43	1.10 (1.08-1.12)	3.6×10^{-5}	

Abbreviations: RA-Risk allele; MAF-Minor Allele Frequency; OR-Odds Ratio; LVD- Large Vessel Disease; SVD- Small Vessel Disease; CE- Cardioembolic; IS-Ischemic stroke

8. Genetics of Intracerebral Hemorrhage (ICH)

Important genetic discoveries have been described for HS. Here, we focus on ICH, which is the most frequent type of HS. Similar to IS, recognition of the biological heterogeneity that underlies distinct subtypes of ICH—with implementation of phenotyping strategies reflecting these differences—has been a crucial element in the discovery of genetic susceptibility variants. ICH is categorised as lobar and non-lobar. On neuroimaging, the bleeding for lobar haemorrhages seems to originate in the cortico-subcortical junction of cerebral hemispheres, whereas in case of non-lobar ICHs the haematoma is located in deep supratentorial structures or in cerebellar or brain stem locations [113]. This classification reflects the differing small vessel pathological features that, in general, underlie the occurrence of ICH at each of these locations. Lipohyalinosis, which is associated most prominently with long standing HTN, is seen frequently in patients with non-lobar ICH,[114] whereas CAA is the most typical pathological feature in individuals with lobar ICH [115]. By combining imaging and clinical characteristics, the Boston criteria [116] enable reliable identification of CAA as a cause of

lobar ICH.

At the population level, findings of several studies show that familial aggregation is raised in ICH. In a population-based case-control analysis of participants in the Greater Cincinnati– Northern Kentucky study [117] who had a first-degree relative with ICH, a six fold increase in risk of ICH was seen, after adjustment for potential confounding factors. This effect of family history was equally strong in people with both non-lobar and lobar ICH [117]. Likewise, the analysis of a population-based hospital discharge register in Sweden indicated that sibling history of HS more than doubled the risk of ICH [118]. High heritability estimates have been reported for ICH [119]. Because of the relative rarity of this disorder, no such estimates from pedigree studies were available before the advent of GWAS. Similar to IS, the heritability of ICH—on the basis of genome-wide data—has been calculated by pooling data from across the International Stroke Genetics Consortium (ISGC). These analyses yielded heritability estimates of 29% for all ICH (lobar and non-lobar combined), 48% for lobar ICH, and 30% for non-lobar ICH. The high estimate for lobar ICH possibly reflects the strong effect that the *APOE* locus has on risk for this subtype[120].

9. Mendelian disorders

The most widely studied Mendelian disorder related to ICH is familial CAA which develops both as a familial and sporadic disorder presented in Table-4. In both forms of the disease, the underlying biological mechanism is deposition of amyloid- β peptide in small and medium brain vessels [121]. Familial forms follow a Mendelian (usually dominant) pattern of inheritance, present early in life, and have a severe clinical course and an early age of death. Most forms of familial CAA are produced by mutations in APP, which encodes amyloid- β precursor protein, and mutations cluster within the secretase processing region of the gene (exons 16 and 17) [115].

A second important mendelian disorder related to HS is COL4A1-related ICH [122]. Similar to CAA, familial and sporadic forms of ICH have been described for mutations in COL4A1. This gene encodes the α 1 chain of type IV collagen, a subtype of collagen that forms the basement membranes of all tissues, including the vasculature, and contributes to their strength. Rare mutations within this gene cause autosomal-dominant syndromes manifested in perinatal ICH and porencephaly, adult-onset ICH (all locations), small foci of chronic blood products in healthy brain tissue known as microbleeds, lacunar strokes, and leukoaraiosis [123-125]. Most disease causing mutations in COL4A1-related CVD are missense variants affecting a highly conserved hydrophobic glycine residue, which lead to inhibition of heterotrimer deposition into the vascular basement membrane, with a resultant alteration of its structural properties [126].

Table 4: Monogenetic form of intracerebral hemorrhage

Disorder	Gene	Chr	Mode of Inheritance	Stroke mechanism	Associated clinical features	Diagnostic test
Familial cerebral amyloid angiopathy	APP	21q21.3	AD	Rupture of cortical cerebral small vessels	Cerebral lobar macrohaemorrhages and microhaemorrhages; white-matter lesions; cognitive impairment	Brain biopsy, mutational Screening
COL4A1-related intracerebral haemorrhage	COL4A1	13q34	AD	Rupture of cortical and subcortical cerebral small vessels	Infantile hemiparesis; congenital porencephaly; white matter lesions; cerebral macrohaemorrhages and microhaemorrhages (lobar and non-lobar); transient ischaemic attacks	Clinical diagnosis, mutational screening

Abbreviations: Chr- chromosome; AD-Autosomal dominant; AR-Autosomal recessive

10. Non-Mendelian inheritance

Several common and rare genetic variants with a non-mendelian pattern of inheritance have been identified that affect the risk of ICH which are mentioned in Table-5. Mutations have been reported within the APOE gene, which encodes apolipoprotein E; these variants are labelled €2, €3, and €4, with €3 being the most frequent allele in the general population. The APOE €2 and €4 alleles are related to the histopathological presence of CAA at autopsy or biopsy; both alleles are also associated with Alzheimer's disease but with different directions of effect: €4 increases risk and €2 is protective [127].

In the population-based Greater Cincinnati–Northern Kentucky study,[117] carriers of APOE €2 or €4 alleles accounted for a large proportion of cases of lobar ICH, yielding a population attributable risk of 30%. A sample of sufficient size was assembled to show definitively the relation between APOE variants (€2 and €4) and lobar ICH, with significance exceeding genome-wide levels (6.6×10^{-10} for €2 and 2.4×10^{-11} for €4) [128]. When cases of probable ICH associated with CAA (using Boston criteria) were analysed separately, it indicated strong correlation with both effect size and significance, providing further support to the role of the APOE € variants in CAA. By exploiting the overlapping biology between CAA and Alzheimer's disease, a recent study [129] assessed the role in ICH of the well-known susceptibility variant for Alzheimer's disease located within the CR1 gene, which encodes complement receptor 1. The main variant at this locus was associated with a 61% increase in risk of first lobar ICH related to CAA; moreover, a 35% augmented risk was noted for recurrent CAA-related lobar ICH [129].

Lately, ISGC finished a GWAS of ICH [130], recognizing a susceptibility locus on

chromosome 1q22, covering PMF1 and SLC25A44. The top SNP at this locus (rs2984613, MAF 32%) was related with a 33% expansion in risk of non-lobar ICH, however, it seemed to have no impact on lobar ICH. A GWAS of MRI-characterized white matter hyperintensities [131] recognized this same locus as one of its top outcomes. PMF1 codes for polyamine regulated variable 1, a protein required for typical chromosome arrangement and isolation and kinetochore development during mitosis; SLC25A44 codes for a mitochondrial carrier protein. This mitochondrial interface recommends a conceivable component, in light of the fact that both mitochondrial dysfunction and genetic variation in mitochondrial genes have been intended in ICH.

Uncommon variations with incomplete penetrance may likewise have an influence in sporadic ICH. The role of genetic variation inside COL4A1 in sporadic (non-familial) types of ICH was explored in a sequencing study that utilized the candidate gene approach. Two mutations in the COL4A1 protein were recognized that were available just in individuals with ICH: Pro352Leu and Arg538Gly. These variations brought about missense changes in amino acids that are profoundly highly conserved over species [132]. The result of COL4A1 is basically and practically identified with the collagen type IV $\alpha 2$ protein, which is encoded by COL4A2. Taking this functional relation into account, another research team surveyed the impact of COL4A2 variations on the risk of ICH and identified three uncommon, non-synonymous coding variations influencing evolutionary conserved amino acids that were present only in patients with ICH. Findings of the same study showed, using cellular assays, that these variants caused intracellular accumulation of COL4A1 and COL4A2 at the expense of their secretion, providing further support to their pathogenic role [133]. The aggregate burden of common genetic variation related to BP levels increased the risk of ICH. In GWAS of BP, researchers uncovered many common genetic variants that affect BP levels [134]. From this work, a polygenic risk score was built with 42 genetic variants known to affect BP, and the aggregate burden was associated with risk of non-lobar ICH [135]. Furthermore, the cumulative burden was related to ICH in patients without HTN, suggesting this genetic variation could play a part in assessment of individuals who might have been misclassified as having normal BP.

The cerebral small-vessel vasculopathy that leads to ICH also affects the severity of bleeding once a haemorrhage takes place. On the basis of observations at autopsy, Miller Fisher [136] proposed that ICH disrupts the surrounding vessels as it grows, suggesting that the final volume of the haematoma is affected by the severity of the SVD that initiated the ICH. This hypothesis is now supported further by genetic evidence, in which variants that increase the risk of ICH also affect haematoma volume and clinical outcome. Patients with lobar ICH who carry the APOE $\epsilon 2$ allele have higher haematoma volumes at presentation,[137] have an increased chance of contrast extravasation on CT angiography (i.e. the spot sign), and are

more likely to have ICH expansion [138]. In individuals with non-lobar ICH, the burden of HTN-related alleles not only boosts the risk of disease but also predicts who will have a large haematoma and, as a result, worse clinical outcome [139].

Table 5: Genetic variants affecting risk of intracerebral hemorrhage in a non-mendelian fashion

Author & year	Sample size cases/ controls	Gene	SNP	Chr	Pheno type	Ethnicity	RA	RAF (%)	OR (95% CI)	P value
Woo D 2014	1545/1481	PMF1/ SLC25A44	rs2984613	1q22	Non- lobar ICH	Caucasian	C	32	1.33 (1.22 to 1.46)	2.2×10^{-10}
Biffi A 2010	2189/4041	APO E	rs429358/ rs7412	19q13	Lobar ICH	Caucasian	€2	7	1.82 (1.50 to 2.23)	6.66×10^{-10}
Biffi A 2010	2189/4041	APO E	rs429358/ rs7412	19q13	Lobar ICH	Caucasian	€4	12	2.20 (1.85 to 2.63)	2.4×10^{-11}
Weng YC 2012	96/145	COL4A1	1055C/T; 1612C/T	13q34	Lobar & non-lobar ICH	Caucasian	T;G	<1	-	-
Jeanne M 2012	96/144	COL4A2	3448C/A; 5068G/A; 3368A/G	13q34	Lobar & non-lobar	Caucasian	A; A; G	<1	-	-
Ma Q 2013	1364/1293	KCNK17	rs10947803	6p21	ICH	Asian	A	39	1.70 (1.08– 2.69)	2×10^{-2}

Abbreviations: Chr-chromosome; SNP-Single nucleotide polymorphisms; RA-Risk allele; MAF-Minor Allele Frequency; OR-Odds Ratio

11. Future advances

Meta-analysis of GWAS data from tens of thousands of patients with stroke is likely to identify further variants, similar to other complex diseases. Larger sample sizes may be required if the variants are predisposed to specific stroke subtypes. For example, even though a sample size of 10,000 sounds large, it will only include approximately 2,000 individuals with large artery stroke. The GWAS approach is suited to identify common variants, each of which contributes a small amount to disease risk. It is less effective at detecting rare variants, which might still be important in disease risk. Whole-genome sequencing enables these rare variants to be identified, and the cost of this technique is rapidly falling [140]. Although studies are underway, but results for stroke are unavailable from this approach as for now. Many current sequencing studies limit coverage to sequencing of the exome, or protein coding part, of the genome. Exome sequencing has been successful in many rare, primarily monogenic, diseases and may offer a cost-effective way to screen multiple single-gene causes of stroke in one assay. Studies are underway using exome sequencing to identify rare variants that may contribute to

more common polygenic diseases [141]. Such studies may particularly benefit from the use of family-based approaches, to help differentiate causal from non-causal variants.

Another emerging area is epigenetics; though there have been few studies for stroke as of now. Epigenetics describes the study of heritable changes in gene expression or cellular phenotype, which are caused by mechanisms other than changes in the underlying DNA sequence [142]. It therefore refers to functionally relevant modifications in the genome that do not involve the changed nucleotide sequence. Examples of such changes are DNA methylation and histone modification, both of which serve to regulate gene expression without altering the DNA structure. Methods are being made available to assess epigenetic changes.

The genetic variants for stroke described till date account for only a small proportion of overall stroke risk. Therefore, their predictive value is low even when combined. For instance, individuals possessing these variants may not develop stroke during their lifetime, contrary to those without these variants could be at risk of stroke. Until we have a comprehensive understanding of the molecular basis of genetic variation, such predictive testing is likely to provide limited information. There are also questions about the usefulness of such personalized testing in patients with complex diseases such as stroke. We already know many risk factors for stroke, such as hypertension and smoking, but despite their importance, patient compliance is often suboptimal. Unless there are specific novel treatments for individual genetic variants, it is likely that the advice given to a patient identified as having a high genetic risk of stroke would merely be able to adhere more closely to cardiovascular risk-factor prevention, yet it is unclear whether such high-risk patients would indeed do so. Besides, patients deemed to have low genetic risk might pay less attention to general risk-factor prevention and therefore get exposed to an increased risk. Also, there are concerns over the psychological consequences of testing. Therefore, a correct genetic diagnosis might improve clinical care once specific preventive measures are implemented on the basis of the known natural evolution of stroke.

12. Pharmacogenetics in stroke

The variation in the genetic sequences not only influence susceptibility to stroke but also been indicated to modify the reaction to pharmacological agents and manipulate the clinical outcome of the disease. The consequence of drugs prescribed to a group of patients with same age and similar disease may vary from individual to individual. The Inter-individual variability in drug toxicity and efficacy has been reported to be associated to numerous factors such as race, age, gender, related medicines and co-morbid factors [143]. In addition to this inherited difference in the genes that control drug disposition and effects in humans have also been found to affect the drug response. The heterogeneous mechanisms involved in the pathogenesis of stroke and variability in drug response create opportunities for the development of novel and targeted therapeutic agents using pharmacogenetics.

SNPs are the most frequent form of sequence variations in the human genome affecting the therapeutic response of drugs used in the treatment of stroke. Pharmacogenetics is the science which identifies the influence of genetic variation on the efficacy and tolerability of various therapeutic agents. Genetic variants of genes encoding drug metabolizing enzymes (e.g. Cytochrome P450 family), transporter proteins [ATP cassette binding protein (ABCB1), Selective cation transporter (SLCO1B1) and Organic anion transporter polypeptide (OATP1B1) and target receptor proteins [Cholesterol Ester transfer protein (CETP), Cyclooxygenase (COX), Apolipoprotein (ApoE), Angiotension Converting enzyme (ACE) and Low density lipoprotein (LDL) have been reported to influence the functional activity of respective enzymes/proteins, thus significantly altering the drug response [144]. Pharmacogenetics promises to personalize the treatment strategies in stroke with maximum therapeutic benefits and minimum side effects[145].

Genetic variants affecting clopidogrel and warfarin metabolism may identify non-responders and reduce side-effects, but their use is not widely accepted in clinical practice [146,147]. An excellent field for pharmacogenetics in stroke treatment might be the identification of genes related to safety and efficacy of thrombolytic (if technical advances are done in order to rapidly genotype patients), and the predicting the response of secondary prevention strategies for anticoagulant or antiplatelet agents to avoid a new cardiovascular event. In this regard many ongoing clinical trials hold great promise in applying the knowledge of Pharmacogenomics into stroke treatment in future. However, currently there is little information available for development of drugs targeted at the genetic influences. The genetic information has the potential to go a long way in improving therapeutic outcome.

Although pharmacogenetic testing is not widely conducted at present, it is likely to become an integral part of standard patient management in selecting and monitoring the therapeutic strategy of stroke.

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