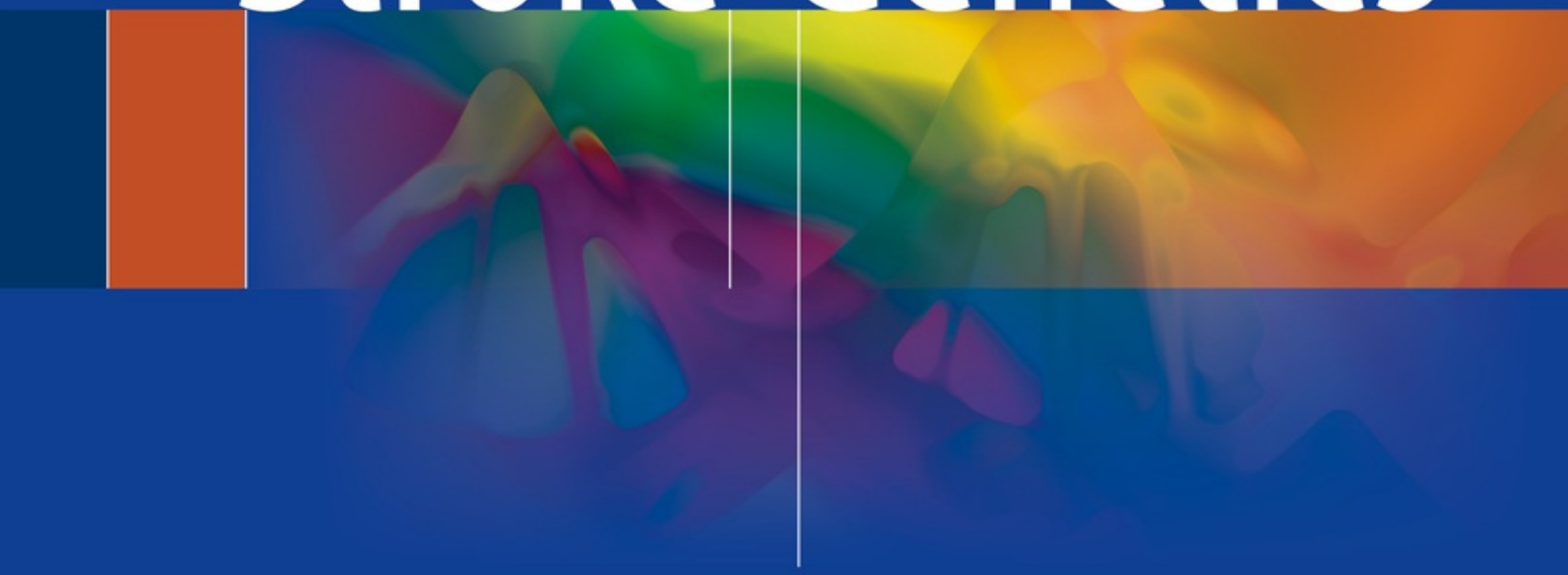
Pankaj Sharma
James F. Meschia
Editors

Stroke Genetics



Second Edition

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Editors
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Editors

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So they told us that writing a second edition is a whole lot easier than writing the first. Don’t believe them!

Without the support of our families this second edition would never have seen the light of day. For that, we dedicate it to Sapna, Aarti, Shyam, Diana, Catherine, Camille, and James.

Preface

We introduced our first edition of this book with the statement that ‘our understanding of the genetics of common diseases has come a long way in recent years’. That statement is as true now as it was then but even we could not have foretold the enormous strides being made in the genetics of all the common diseases, with stroke being an exemplar of the complexity and struggles of that science. The advances in our mathematical and statistical capabilities along with the strides in genetic laboratories and, just as importantly, the reduction in manufacturing cost of microarray chip technology have all helped in greatly improving our understanding of stroke genetics. Many thousands of willing patients have agreed to donate their DNA in the hopes of benefitting future generations. Researchers have meticulously and painstakingly compared and contrasted millions of human polymorphisms. The enormity of this task should not be underestimated.

The gratifying popularity of our first edition prompted the publishers to encourage us to produce a second edition. We approached our colleagues from across the continents, and despite their hectic schedules, not one hesitated in responding to our call.

The authors of each chapter in this book have been at the forefront of this research. Ongoing work means that our knowledge will change, perhaps on a daily basis. However, this book is tasked with not just providing a state-of-the-field overview but, just as importantly, principles upon which readers can critically assess future stroke genetics research. The international make-up of the contributors reflects the global alliance within which the stroke genetics community works and is a testament to the collaboration and purpose we all feel in tackling this disease that inflicts such a burden globally.

The book starts with an account of why we even thought that a late-age-related disorder could have a genetic basis. We then describe the genetic tools available in our armoury to discover its molecular aetiology. The book moves on to describe the major single-gene disorders in stroke and then some of its more common presentations. We add chapters on cerebral venous thrombosis and our state of knowledge of genetics of stroke in those of non-European descent. This is timely as by the middle of this century the vast majority of the burden of stroke is likely to lay with Asia. Our book provides a comprehensive review of the rapidly changing field of pharmacogenetics as it

applies to drugs used to prevent stroke and concludes with examples of the challenges that occur when genetics mix with the law.

When asked to undertake this second edition, the editors did not dither. We saw a continuing opportunity to bring clarity to the mass of conflicting data, and we recognise the need to educate and inspire a new generation of stroke researchers and clinicians. Most importantly, we were inspired by our need to provide a comprehensive yet still comprehensible reference for the practicing clinician when faced with a case of familial stroke in the clinic. However, we remain a long way from reaching our goal of a full understanding of stroke at the genetic level.

> The promised land always lies on the other side of the wilderness.
> Havelock Ellis (1859–1939)

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1. Introduction

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Introduction

Few conditions cause as much devastation to the human brain as stroke. The consequence of such damage ranges from being imperceptible to complete hemiparesis, a locked-in syndrome, reversal of personality, and, ultimately, death.

In itself such a disease would be undesirable, but the common frequency of stroke makes it all the more feared. Stroke is the fourth commonest cause of death in the West, yet this simple statement belies the heavy toll inflicted on so many patients, families, friends, and careers. In the USA alone, more than 700,000 people suffer from stroke, but by the middle of this century, around 80% of the world's burden will be in the emerging nations of India and China (WHO Non-communicable diseases report). The pathophysiologic nature of the disease means that atherosclerosis in cerebral arteries is merely a symptomatic representation of the total burden of disease in the systemic

arterial circulation. The result is that most stroke patients die of coronary artery disease and many also suffer from intermittent claudication from peripheral arterial disease.

Stroke does not respect social class, creed, race, or religion. From the old rickshaw man in the slums of India, from US Ambassador Joseph Kennedy and Sir Winston Churchill occupying the highest offices of the land to gangster Al Capone, stroke can inflict without notice. The disease has changed the course of human history when those occupying seats of power and about to make momentous decisions are struck down and replaced, famously as with Stalin and more recently with Israeli Prime Minister Ariel Sharon.

And yet, perhaps the cruelest affront of all is when stroke afflicts the young. Around a quarter of strokes affect those under 65 years of age. This brings additional challenges to be addressed such as employment issues and emotional difficulties, as well as those associated with caring for young children.

Being a vascular disease, a number of strategies are available to prevent first and recurrent ischemic stroke, ranging from lifestyle modifications (diet, exercise, and weight loss) to medications (antiplatelets, anticoagulants, statins, and antihypertensives).

Acute treatment options are increasingly available, all aimed at removing or reducing the size of the clot (thrombolytics to mechanical clot removal devices). After a long period within a therapeutic desert, stroke has taken center stage in just a few years.

Evidence for Heritability of Stroke from Twin and Family History Studies

Twin and family history studies suggest a genetic component to the risk of ischemic and hemorrhagic stroke. Twin studies in ischemic stroke have found concordance to be around 60% higher in monozygotic twins compared with dizygotic twins, while a positive family history of stroke seems to be associated with a one- to two-thirds relative increase in ischemic stroke risk.

The associations are probably stronger in younger subjects, but young stroke studies may be prone to inaccurate reporting of family history, potentially leading to an underestimation of the heritable component. Conversely, poor measurement of and other under-adjustment for

confounding may lead to an overestimation of the heritable component.

Various mechanisms have been proposed to explain these heritability patterns, including transmission of genetic risk factors for disease through mitochondrial DNA (passed only from mothers to offspring), with greater penetrance in females than males; genomic imprinting, involving differential expression of a disease susceptibility gene depending on the sex of the parent transmitting the gene; sex-specific fetal programming, whereby events in the maternal intrauterine environment predispose females to a disease in adult life; and environmental risk factors shared by female relatives, which are difficult to quantify and account for fully in analyses (e.g., stress, diet, and exercise). Misattributed paternity, thought to occur at a rate of at least 1%, may contribute to the heritability patterns observed by causing greater inaccuracy of family history data about fathers than mothers.

Available Genetic Strategies

The observation that stroke tends to run in families led researchers to postulate a genetic etiology. A number of genetic strategies have been used to dissect out that molecular etiology. Initially, single nucleotide polymorphisms (SNPs) were used in case-control allelic association studies. These led to a wide variety of favored candidate genes being investigated, mostly derived from our knowledge of thrombosis and atherosclerosis. Recent meta-analyses of these relatively small studies have begun to demonstrate reliable genetic associations for both hemorrhagic and ischemic stroke.

The Human Genome Project, the HapMap, and technological advances in genotyping pursuant to these have caused transition from testing isolated SNPs to hundreds of thousands of common SNPs covering the entire genome. This approach, which operates under the common disease-common variant hypothesis, is known as the genome-wide association study (GWAS) and does not require an *a priori* hypothesis. Having yielded several discoveries for disparate conditions like inflammatory bowel disease and age-related macular degeneration, multiple GWAS studies have been reported in stroke. However, unlike many of the other common diseases that have successfully used GWAS, stroke has additional challenges: principal among them is its heterogeneity. Not one condition but rather an umbrella term for potentially half-a-dozen conditions, stroke is best regarded as a syndrome; the

manifestation of large vessel, small vessel, atherothrombotic, cardioembolic, and hemorrhagic diseases—which conspire to produce a sudden focal neurological deficit. Appreciation of that heterogeneity has perhaps acted to constrain fast progress, but as larger biobanks of DNA accumulate, there is a real sense that unraveling the genetic architecture of stroke can seriously be tackled. Whole genome sequencing is becoming more practical, with the cost now around $1000.

A criticism leveled at genetic studies that utilize a case-control design is that, by themselves, they demonstrate association rather than causation. They are observational studies and thus cannot provide the same level of evidence supporting causation that properly controlled experimental studies can. When a single polymorphism is tested among cases and controls, there is often a concern over population stratification, a special case of confounding whereby there might be differences in underlying racial genetic structure between cases and controls. With GWAS, techniques like adjustment for principal components, mitigates risk of creating a spurious association by population stratification. Although there is no direct equivalent to Koch's postulates in human genetic association studies, generally speaking researchers want to see independent replication of an association along with validation through other approaches like the use of an experimental model.

Using a technique known as mendelian randomization, genetic studies can offer added proof for associations previously identified in classical epidemiological studies. Mendelian randomization refers to the random assortment of genes transferred from parent to offspring at the time of gamete formation. An association is less likely to be spurious due to residual confounding, ascertainment bias or reverse causation when it is supported by mendelian randomization. As powerful as the technique can be, there can still be confounding at the genetic level through population stratification and linkage disequilibrium and at the functional genomic level through canalization.

The Practicing Neurologist and Stroke Physician

This book seeks to bring together the explosion of knowledge that has taken place in stroke genetics in the past few years. The molecular aspects of this book will be of obvious interest to the academic community, and the factual content will appeal to the resident sitting exams; however, the question

arises: what bearing does this book have on the practicing stroke clinician?

The greatest advances in stroke genetics have been for monogenic disorders such as cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) and mitochondrial encephalopathy with lactic acidosis and stroke-like episodes (MELAS), and these disorders are of direct clinical relevance. We would argue that there are several important issues the physician should bear in mind. Firstly, patients with a family history of stroke may have inherited a predisposition to the same. Secondly, the younger patient is likely to have a greater genetic liability for the condition. Further, not recognizing a familial stroke syndrome like CADASIL puts patients and their relatives at risk of receiving unwarranted diagnostic testing and potentially unsafe and unnecessary therapeutic interventions. A molecular diagnosis also provides prognostic information that helps families plan for their futures and the future of succeeding generations.

Table 1.1 Procedural considerations for young stroke patients

	Standard stroke clinic care	**Young stroke clinic—additional care required**	**Reason for additional care**
Constituent	Multidisciplinary team	Counseling service, psychology	Often a particular issue with young patients. Genetic testing requires specific counseling
History	Detailed history	Detailed family history of cardiovascular disease	May determine whether a monogenic disorder, mode of inheritance, or genetic liability
	Neuropsychometry	Assesses baseline cognitive function	Allows future comparison of function if repeated
Examination	AF, BP, carotid bruits, functional score; e.g., NIHSS, Bartel etc.	Hip:waist ratio, height	Specifically for collagen disorders; e.g., Marfan’s syndrome
	BMI		
		Renal bruit present?	Secondary causes of hypertension
Investigations	CT brain	MRI brain	Search for monogenic disorders; e.g., CADASIL
	CTA (extra/intracranial)	MRA (extra/intracranial)	Reduce radiation if multiple follow-up scans are required. Search for extra/intracranial stenosis, AV malformations, moyamoya disease

		Formal angiogram	Aneurysms. Beading for vasculitis
		Renal ultrasound	Secondary causes of hypertension, additional large vessel bruits
		Echocardiogram	Search for thrombus, search for PFO
	24-h ambulatory ECG	Longer term ambulatory ECG (or implantable detector)	Paroxysmal AF
	Full fasting lipid profile		
		ESR/CRP	Inflammatory causes; e.g., arteritidies
		α-galactosidase	Fabry's disease
		Anticardiolipin Ab, anti-beta2 glycoprotein 1 Ab, lupus anticoagulant	Autoantibody causes
		Factor V Leiden	Known genetic associations
		Protein C and S	Clotting cascade abnormalities
		Antithrombin III	
		Prothrombin	
		Antiphospholipid Ab	
		Homocysteine	Known associations
		Folate	

AF atrial fibrillation, *BP* blood pressure, *NIHSS* National Institutes of Health Stroke Scale, *BMI* body mass index, *CT* computed tomography, *CTA* computed tomography angiography, *MRI* magnetic resonance imaging, *MRA* magnetic resonance angiography, *ECG* electrocardiogram, *ESR* erythrocyte sedimentation rate, *CRP* C-reactive protein

Younger familial stroke patients are probably best seen outside the usual "stroke clinic," which can often be dominated by elderly disabled patients arriving in wheelchairs. Younger patients are likely to feel disheartened when attending these clinics.

Further, the prototypical adult stroke clinic often follows protocols for stroke management, like screening for hyperlipidemia that, while of importance, may not be of prime importance for those with potentially inherited disorders. Finally, the time required for young patients is greater than that usually available in a general stroke clinic. Time taken often includes that necessary for detailing a family history, while patients will have

questions about prognosis, recurrence, and likely transmission to offspring. Further, time needs to be set aside for providing counseling and obtaining consent for genetic testing.

The patients are therefore best managed in a “young patient” or “familial” specialized stroke clinic. It is wise to have established protocols, and the above Table 1.1 provides basic features that need to be considered that exceed the usual tests for a stroke patient. The opportunity of undertaking research in such a rich clinic will, of course, not be lost to those with an enquiring mind.

Undertaking a Genetic Stroke Clinic Constituent

- Fewer patients should be allocated compared to a standard clinic, with greater time devoted per patient.
- Encouragement for partner and other family members to attend to allow for collateral history and family history taking.
- Multidisciplinary, to include counseling, physio- and occupational therapists, speech and language experts, dietician. At the investigation stage, close collaborations with cardiology and haematology departments are important.

Specific Procedures

The only absolutely required element of a workup for a familial stroke syndrome is a detailed family history. Ideally, this family history would be structured to include not only inquiry about other family members with stroke, but also other family members with conditions that are seen in specific single-gene stroke syndromes, like renal disease and migraine headaches. A detailed family history may require multiple encounters with the proband/patient and with relatives of the patient. It is common that a family history initially dismissed as ‘negative,’ becomes informative with more effort.

The Table above is not an exhaustive list of diagnostic tests, and clearly not all tests may be appropriate for every patient. However, we place this

here to demonstrate the additional complexity associated with dealing with young stroke patients.

As the cause of the stroke may not be evident immediately, these patients need to be managed and have time invested much more aggressively in institutions with the necessary facilities and, more importantly, by those with an expertise and experience in dealing with them.

Once the possibility of an inherited cause of stroke is suspected and the appropriate investigations are undertaken, it would be wise to review the patient sooner than would normally be the case. Patients and their families are likely to be more concerned than the elderly stroke patient, who may (albeit erroneously) consider their stroke "expected for their age." Conversely, the younger patient is more fearful of recurrence and the consequences of a disability not to mention the sheer shock and surprise of the disease onset.

It may, of course, be the case that any specific etiological cause identified requires additional expert assistance. For example, abnormalities of the clotting cascade should trigger the involvement of the hematological department, while secondary causes of hypertension would require the help of the cardiologist, clinical pharmacologist, or the general medical team. These patients are therefore often under the shared care of several medical teams but tend to continue viewing their neurology/neurovascular stroke physician as being primarily responsible for their management.

Conclusion

As our understanding of stroke at the molecular level continues to improve, it is likely that an increasing number of stroke cases with a genetic etiology will present themselves to the stroke physician. An understanding of the patients' needs, concerns, and basic (at least initial) management will be necessary for those at the "front line" even if the ultimate long-term burden of responsibility eventually passes on to super-specialized centers.

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2. Familial Occurrence and Heritability of Stroke

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Introduction

Clustering of clinical strokes among related family members is an indication of the aggregate genetic, environmental, and lifestyle factors shared by these family members, all of which could increase susceptibility to stroke. A family history of stroke or of other atherosclerotic events, such as a myocardial infarction, is a moderate risk factor for stroke, as observed in twin, cohort, and case-control studies [1]. With the exception of some rare Mendelian disorders, most stroke occurring in a clinic or population sample has a complex inheritance pattern, but the relative importance of genetic factors can be estimated by studying the familial occurrence of stroke. Patterns of familial aggregation can also direct which subgroups to investigate further as more likely to share common underlying genetic variation; thus we could define subgroups by age, gender, ethnicity, presence of vascular risk factors, or stroke subtype [2, 3]. Focused investigations within these subgroups using

modern genotyping techniques (phenotyping ‘splitting’) can increase the yield in identifying gene variants associated with stroke [4, 5]. As a predisposing risk factor, family history can be used clinically for risk prediction and to encourage primary disease prevention [6].

Establishing the Heritability of Stroke

Heritability may be defined as the proportion of the observed phenotypic variation among individuals, in traits or in risk of disease, that is attributable to genetic variability. Family history and verified familial occurrence studies, particularly twin studies [7, 8], were instrumental in demonstrating that part of the variability recorded in the occurrence of stroke was attributable to genetic variation [9]. Familial aggregation of stroke risk factors, such as hypertension and smoking, could partially explain these associations. Studies that adjusted analyses for vascular risk factors showed an attenuated association. However, family history of stroke persists in these studies as an independent risk factor [1, 10].

New methods have used data from genome-wide association studies (GWAS) to confirm the heritability of stoke. Genome-wide complex trait analysis, using known genetic associations with stroke, estimates the heritability of ischemic stroke and intracerebral hemorrhage. [11, 12] Heritability of endophenotypes, such as MRI markers of subclinical disease or intermediate vascular risk factors, have also been investigated. The degree of white matter hyperintensities measured on brain MRI, for example, is estimated to have a heritability as high as 55–80% [13, 14]. Heritability estimates for these phenotypes and endophenotypes are shown in Table 2.1.

Table 2.1 Heritability of ischemic stroke, stroke subtypes, and endophenotypes

Any ischemic stroke [11]	38%
Ischemic stroke subtype [11]	
Large vessel ischemic stroke	40%
Cardioembolic stroke	33%
Small vessel ischemic stroke	16%
Endophenotypes	
White matter hyperintensities [13, 14]	55–80%
Carotid intima media thickness [16, 17]	21–38%
Carotid artery plaque [17–19]	23–78%

Mediating risk factors [20]	
Hypertension	40–60%
High-density lipoprotein	25–50%
Intracerebral hemorrhage (ICH) [12]	44%
Deep ICH	34%
Lobar ICH	73%

Given the wide range of phenotypes and endophenotypes that characterize a clinical stroke, it is evident that many genes are involved in contributing to stroke risk. Genes may interact with other genes, as well, causing epistatic modification, which can impact true heritability. The majority of family and genetic studies have been performed using populations of European descent. The distribution and heritability of stroke may differ by ethnicity [15].

Twin Studies to Investigate the Genetic Epidemiology of Stroke

Twin studies are important for differentiating the role of genetic factors from that of shared environmental or lifestyle influences for stroke. Up to a doubling of risk is observed in monozygotic twins compared to dizygotic twins or siblings [1, 7]. Monozygotic twins are also more likely to have comparable increased volume of white matter hyperintensities on brain MRI than dizygotic twins [13]. A challenge for this type of study is the recruitment of sufficient twin pairs to analyze by subgroups, such as by ethnicity or stroke subtype. Stroke is common at older ages, and twins may be more likely to die of unrelated diseases as they age, so capturing sufficient cases may be difficult. Similar challenges are seen in recruiting non-twin sibling pairs [21].

Parental Stroke and Risk of Stroke in Offspring

The effects of familial relationship in family history of stroke have been investigated in prospective cohort and case-control studies [1, 10, 22–31]. In the Framingham Heart Study Original (parental) and Offspring cohorts, parental stroke by 65 years of age resulted in a nearly threefold increased risk of stroke in the offspring [10]. The risk of stroke was highest in those

offspring who also had a stroke before 65 years of age [10, 27]. A family history of stroke in both parents can more than double the risk of stroke [23, 32]. Parental history of stroke was associated with subclinical (silent) cerebral infarct, after adjustment for vascular risk factors [31]. Studies to date have not found a clear association between parental history of stroke and TIA or recurrent stroke [33–35].

Risk of Stroke Between Siblings

A history of ischemic stroke in a sibling is associated with an increased risk of ischemic stroke in a proband, and the same pattern is seen in concordance of hemorrhagic stroke between siblings [36]. Stroke in siblings may also be associated with risk of recurrent stroke and with stroke severity [37, 38]. Co-existence of stroke is higher in full, than in half siblings [39]. Environmental and genetic interactions may be even more pronounced among siblings, who share parents, common risk factor exposures, and lifestyle behaviors.

Siblings share similar cardiovascular risk factors [40, 41] but may not always develop similar stroke subtypes [36, 42–44]. Associations persist however, even after adjustment for known vascular risk factors, suggesting that genetic factors may have an independent mechanism and heritability apart from conventional risk factors [10, 37]. There may also be differences across ethnicities for how stroke and vascular risk factors aggregate between siblings [15].

Age at Onset with Familial Aggregation of Stroke

The extent to which genetic factors increase stroke risk appears to be age dependent. Typically, family history of stroke is a stronger stroke risk factor when either the affected first-degree relative or the individual stroke patient with positive family history are younger at the time of the stroke [1, 45–47]. In meta-analysis, family history of stroke was shown to be more frequent in studies that restricted the age of either the proband or the relatives to <70 years [1]. Genetic factors increasing risk for stroke may be enriched in families with early-onset of the disease. The proband may develop stroke at a younger age due to influences from the shared environment, adoption of similar lifestyle behaviors, as well as shared genetics [45, 48, 49].

Family History and Sex Differences

Differences in stroke risk associated with parental stroke are observed according to the sex of the proband and to whether there is a maternal or paternal history of stroke. Female stroke patients are more likely to have a first-degree relative with stroke, compared to male patients, and they are more likely to report a maternal history of stroke [10, 50–52]. An excess in affected sisters of female probands has also been observed [50]. In contrast, male probands are no more likely to have a paternal than a maternal history of stroke in many studies [53]. These observations suggest a sex-specific interaction [53, 54].

Sex differences, in female stroke patients overall and in younger cohorts, may be explained by genetic, developmental, and environmental pathways. Penetrance may be increased in females due to inheritance of mitochondrial DNA. Depending on the sex of the parent transmitting the gene, there may be differential imprinting of a disease susceptibility gene. Special imprinting may occur through sex chromosomes or mitochondrial inheritance [53]. In utero effects, stimulated by the immune system, tobacco, drugs, diet, or factors associated with socioeconomic status of the mother, for example, may cause specific epigenetic changes in the fetus. Fetal programming in certain environments may increase the risk of stroke as an adult. Potential differences between maternal and paternal transmission of stroke need further study [22, 28].

Familial Aggregation by Stroke Phenotype

A few studies have related family history of stroke to ischemic stroke subtypes of small artery occlusion and large-arty atherosclerosis [10, 26, 27]. Other subtypes, including cardioembolism, rare causes such as dissection, and undetermined causes, may share less significant or no association with family history of stroke [27, 51]. Data from the Framingham Heart Study showed that increased risk of stroke in offspring with a parental history of stroke was most associated with atherosclerotic brain infarction. This category includes small-vessel occlusion, ‘lacunar’ infarctions, and large-artery atherosclerosis [10].

Underlying disorders for cerebral small vessel disease may be more heterogeneous than for large-artery atherosclerosis. This may partly explain

why heritability of large vessel disease is observed to be higher [26, 27]. Heritability of carotid artery stenosis has been studied, showing a moderate association with family history of stroke [55]. Investigators were unable to correlate family history of stroke to stroke subtypes in an Asian population, specifically with risk of intracranial atherosclerotic disease [37]. Cardioembolic stroke may show less association with family history due to the heterogeneous etiology of the embolic sources, such as valve disease, myocardial infraction, patent foramen ovale, and cardiomyopathy [27]. There may be fewer overall cases of stroke due to cardioembolism, or these may be misclassified during hospitalization due to unrecognized factors, such as paroxysmal atrial fibrillation.

Intermediate phenotypes, such as neuroimaging biomarkers of cerebral small vessel disease, have also been studied in relation to family history of stroke. White matter hyperintensities seen on brain MRI are associated with stroke in first degree relatives [13, 56]. Familial aggregation is associated with asymptomatic lacunar infarcts in younger stroke patients age <65 years [46]. Further research is needed to confirm associations of family history of stroke with cerebral microbleeds, enlarged perivascular ('Virchow-Robin') spaces, and cortical cerebral micro-infarcts [57–59]. Methods for phenotyping stroke and subclinical disease are important for genetic studies, as the genetics of stroke may be subtype dependent [4, 5, 60].

Table 2.2 displays associations of family history of stroke in first degree relatives, parents, or siblings, with ischemic stroke subtype. Familial aggregation of intracerebral hemorrhage and cerebral amyloid angiopathy are discussed in a subsequent chapter. Intracerebral and subarachnoid hemorrhage risk are associated with a positive family history, as summarized in Table 2.3.

Table 2.2 Representative studies associating family history of stroke to increased stroke risk, adjusted for vascular risk factors

	All ischemic stroke [1, 10, 27, 39, 61]	**Stroke recurrence [33]**	**Small-artery occlusion [26, 27, 45, 62]**	**Carotid artery stenosis [55]**	**Large-artery atherosclerosis [26, 27, 45]**	**Cardioembolic stroke [26, 27, 45]**	**Cryptogenic or undetermined stroke [26, 27, 45]**
First degree relative	OR 1.3–1.8	HR NS-2.1[a]	OR NS[b]	OR 1.4	OR 1.7[b]	OR NS[b,c]	OR NS[b]
			OR 1.8[c]–2.8		OR 1.9[c]–2.1		OR 1.7[c]
			RR 2.9				

Parental stroke	OR 1.9–2.2[d]	HR NS	RR 4.5	OR NS	e	e	e
Sibling stroke	OR 1.7 RR 1.6	HR 1.7	RR 2.1	OR 1.5	e	e	e

OR odds ratio, *RR* relative risk, *HR* hazard ratio, *NS* non-significant association

[a]Relative's age at stroke onset <50 years

[b]First degree relative stroke by ≤65 years of age

[c]Family history of stroke in patients with stroke <70 years of age

[d]Parental stroke by ≤65 years of age

[e]Unknown or data unavailable

Table 2.3 Increased risk of intracranial hemorrhage in patients with family history of hemorrhagic stroke, adjusted for vascular risk factors

	Intracerebral hemorrhage [26, 47, 63, 64]	**Subarachnoid hemorrhage [65–67]**
Increased risk with positive family history of ICH/SAH	OR 2.1–6.3	OR 3.2[a]–4.3
	Lobar ICH: OR 3.9	
	Non-lobar ICH: OR 5.4	

[a]First degree relative with subarachnoid hemorrhage or intracranial aneurysm

Challenges in Family History Studies of Stroke

The variability of results from family history studies may be partially attributed to misclassification of stroke cases or insufficient data regarding associated vascular risk factors. Inaccurate reporting of family history may underestimate the heritable component of stroke. Insufficient adjustment for confounding factors may overestimate heritability.

Recall bias may occur in case-control studies because stroke patients are more likely to recall or know of a family history of stroke than controls, who have not suffered the disease or inquired with family members [68]. The accuracy of offspring reports of parental history of stroke is highest where no stroke has occurred in the parent [69]. Family history of subarachnoid hemorrhage, a rare outcome, is particularly difficult to ascertain [70]. Research with family history data need to consider not only whether or not

there is a positive family history but also the number of relatives affected [71].

Methods of ascertaining and recording the familial occurrence of stroke include recall by the proband in a structured interview or through use of a questionnaire. Concordance between reporting of stroke by parents and offspring is good when offspring are interviewed in a standardized manner by a health professional [72]. There are no differences between men and women in recall of family history when one examines data from community-based and hospital studies [69, 72]. Studies may also collect data on cerebrovascular disease within a family from their death certificates, although the accuracy of these data has been called into question [73]. Other sources include hospital and outpatient documentation, review of parental medical records, and data from medical registries. Hospital studies are susceptible to inclusion bias. Multigenerational cohort or clinic-based studies have the advantage of directly interviewing both parents and all offspring for information regarding whether or not they ever developed symptoms suggestive of a stroke and examining them for any residual signs of an old stroke. This is arguably the most accurate way to ascertain familial co-occurrence of stroke. A final factor that may introduce bias would be misattributed paternity. Rates of non-paternity are very low and this would have little effect overall.

Hemorrhagic and ischemic stroke types are frequently analyzed together in older studies. Ascertainment bias can occur where these types are not distinguished or if there is no information on stroke subtype. This may occur especially in the parents' generation, as older patients may have worse recall of distant events. Also, with strokes that occurred prior to 1970, the diagnosis of stroke subtypes was only reliable for fatal events that came to autopsy, as the available technologies such as a carotid arteriogram were more dangerous, less routinely undertaken, and less capable of correctly classifying the stroke type. CT scans were not widely available before the late 1970s and modern neuroimaging with MRI came into clinical use only in the 1990s.

Research Application in Genetic Studies

Family studies in stroke have provided strong evidence of familial aggregation, suggesting that research in stroke genetics can identify gene variants responsible for increasing disease risk. Candidate genes account for

stroke in many monogenic disorders, such as the NOTCH3 gene in CADASIL (see Chap. 6), explaining the grouping of stroke in certain families. GWAS and next-generation sequencing studies have identified over a dozen gene variants associated with stroke. These studies could be enriched by findings exploring stroke heritability patterns using various designs of family history studies. Future research would benefit from differentiation of ischemic and hemorrhagic stroke types, classification of ischemic stroke subtypes, subgroup analyses by age and gender, and adjustment for intermediate phenotypes or mediators of increased stroke risk. Well-defined subtypes allow investigators to identify specific pathogenic pathways that can be targeted for preventive strategies and treatments.

Clinical Application

One clinical application of family history studies is emphasizing the urgent need for, and benefits to, addressing environmental or lifestyle (modifiable) risk factors which predispose to disease in the person with a strong inherited (non-modifiable) risk profile. Data in Fig. 2.1, from the Framingham Heart Study, show that aggregation of vascular risk factors greatly amplifies the stroke risk associated with family history [10]. Thus, intense risk factor management can mitigate the increased risk from a positive family history. Separate research has shown associations between family history of stroke and specific modifiable risk factors, such as blood pressure [74].

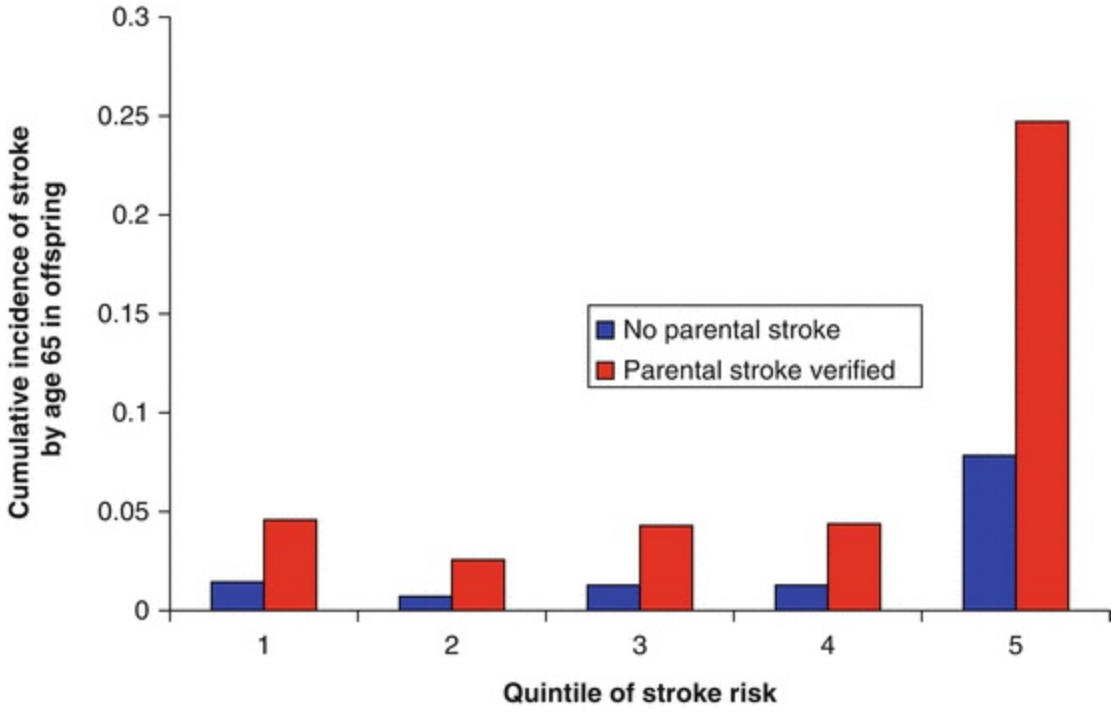

Fig. 2.1 Cumulative incidence of all stroke in offspring by quintile of baseline Framingham Stroke Risk Profile (FSRP) score* for offspring with and without parental occurrence of stroke before 65 years of age (Seshadri, et al. *Circulation*, 2010). *FSRP: covariates include age, sex, systolic blood pressure, antihypertensive therapy, diabetes mellitus, smoking status, history of cardiovascular disease other than stroke, and the presence of atrial fibrillation or left ventricular hypertrophy on electrocardiogram

In determining stroke risk, prediction algorithms may incorporate family history of stroke to improve clinical risk prediction. Used as a screening tool, practitioners can encourage behavioral modification or medications to prevent disease. Figure 2.2 is an example of a proforma that can be used in clinical practice to collect information about family history of stroke.

Born a twin? Yes (identical) Yes (fraternal) No		Were you adopted? Yes No	
First degree relative		Age at first diagnosis of stroke	Age at death
Biological mother			
Biological father			
Female siblings	Number:		
Male siblings	Number:		

Fig. 2.2 Proforma for family history of stroke

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3. Genetic Association Studies and Next Generation Sequencing in Stroke: Methods

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Introduction

Although some forms of stroke are inherited in a Mendelian manner, being caused by a single genetic locus of strong effect, most strokes are sporadic and, like other complex diseases such as asthma and diabetes mellitus, have a genetic component that is complex and involves multiple loci of modest effect. Controversy surrounds whether these genetic variants are likely to be common (>1–5% prevalence) across most populations, each with very small effect sizes (the so-called common disease-common variant hypothesis), or whether these variants are likely to be rare (<1%), perhaps even unique to families or individuals, with larger effect sizes (the so-called common disease-rare variant hypothesis). In either case, a common approach over the last 15 years has been to test for association between common genetic variants and stroke using traditional population-based designs such as case-control and cohort studies.

Genetic association studies are the most common study design used to test for a disease-associated variant and may use either a candidate or genome wide association study (GWAS) approach. A GWA study is defined by the National Institutes of Health as a study of common genetic variation across the entire human genome designed to identify genetic associations with observable traits. In practice, this means genotyping a sample at hundreds of thousands of SNPs and relating this to clinical conditions and measurable traits. In contrast, the candidate genetic association study (CGAS) typically investigates a smaller number of variants and thus is not hampered by multiple testing and requires a much less stringent p-value than the GWAS. These two methodological approaches are fundamentally different; the GWAS is a data-mining tool where no knowledge of the possible causative mechanisms is needed, and referred to as hypothesis generating, whereas the CGAS is hypothesis driven, where putative functional variants in genes are specified a priori and tested for association with disease risk.

More recently, techniques of automated sequencing piloted by Sanger, have been superseded by methods that are less expensive, and allow even larger throughput options. This has led to more attractive opportunities for sequencing compared to GWAs and the advent of the term Next Generation

Sequencing (NGS) [1]. While NGS offers faster and less expensive genotyping it also produces significantly larger amounts of data output and the emerging challenges include how to efficiently manage and interpret this data.

In this chapter, we cover the study designs of CGAS and GWAs, including sources of bias, advantages and disadvantages, challenges in genome wide association meta-analysis (GWAMA) and briefly, association analysis. The relationship between CGAS and GWAS will be addressed plus the advent and implications of NGS. In addition, this Chapter will touch on the emerging phenome wide association study (PheWAS) where large datasets derived from epidemiological, clinical and genomic sources are interrogated through association analysis to test for linkage and novel networks; and the role of CGAS in future GWAS by providing a targeted method to both elucidate causation using the method of Mendelian randomization and by providing genotype association replication in larger GWAs. The focus will be mainly methodological; for encyclopaedic reviews of actual genetic variants associated with stroke, readers are referred to other sources [2–6], as well as to the Human Genome Epidemiology (HuGE) Navigator, a continuously updated electronic tool that can help identify genetic association literature for specific diseases and genes [7].

Study Designs

Family Based Studies

Family-based designs using trios and sibling pairs have been used in the past [8] and more recently have found new applications especially in rare variant analysis [9]. The trio design is specific to genetic epidemiology; in a trio design, the affected case and both of his or her parents are genotyped. The frequency with which an allele is transmitted to an affected offspring from heterozygous parents is then estimated. Under the null hypothesis, the transmission frequency of a given allele is 50%; alleles associated with the disease will be transmitted more frequently from parents to affected offspring. It can be difficult to recruit complete trios especially in diseases with late onset such as stroke and as a result is rarely used. The advantage of this design is that it is not affected by genetic differences between cases and control participants that are not related to disease but are a result of sampling

from populations with different ancestry.

Candidate Genetic Association Studies

In CGAS studies, a particular genetic variant is tested in groups of ascertained cases and controls, and in CGAS cohort studies, a genetic variant is tested on blood drawn at baseline and the disease outcome determined at follow-up. Most commonly, allele frequencies in the cases are compared to the disease-free control group. The choice of genetic variant is most often a single nucleotide polymorphism (SNP) but can equally be a DNA repeat (which includes minisatellite, microsatellite, variable number tandem repeat, or trinucleotide repeat), an insertion/deletion polymorphism, or a copy number variant (a large genomic region, >1 kb, whose copy number varies from the expected diploid state due to deletion or duplication) (Fig. 3.1).

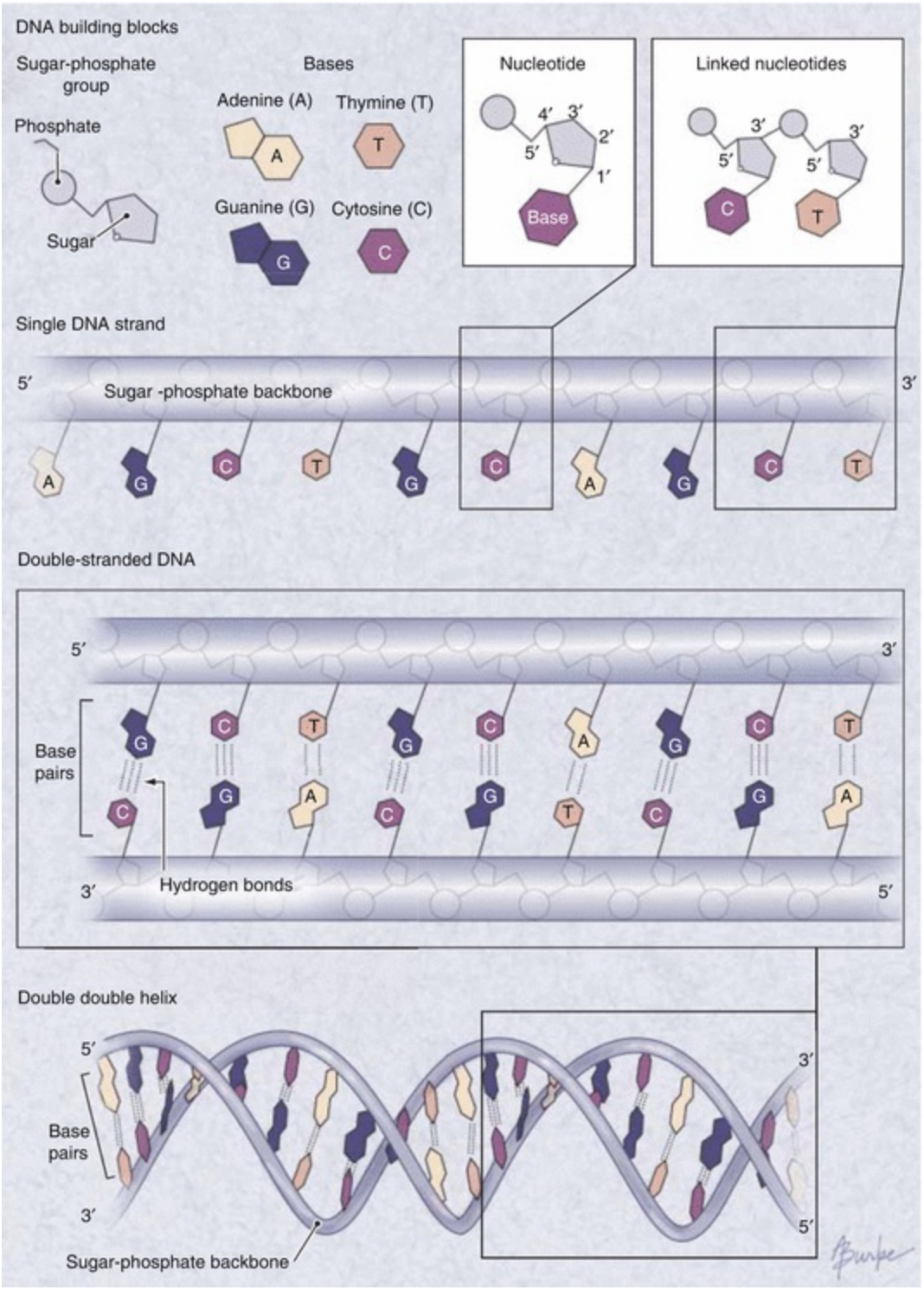


Fig. 3.1 The building block of DNA is the nucleotide—a sugar (deoxyribose) with a phosphate group at the 5′ carbon and a base (adenine, thymine, guanine, or cytosine) at the 1′ carbon. Nucleotides link together by a bond between the phosphate group of one nucleotide and the 3′ carbon of the previous nucleotide, to form a single DNA strand with a resulting directionality of 5′ to 3′. Two strands with opposite directionality combine to form a double helix that is held together by hydrogen bonds across

the bases. Adenine always binds to thymine and guanine always binds to cytosine. The sequence of base pairs encodes the genetic information (Reprinted with permission from Attia et al. [10])

Many poor-quality CGAS have been published and there is a perception that CGAS have a poor record of replication [11, 12]. Hence, they have fallen somewhat out of favor. This is unfortunate, given their tremendous efficiency in terms of power. Given the evidence that genetic effects are likely to be small, the candidate gene approach has an advantage over GWAS due to the relative lack of multiple comparisons. For example, imagine a SNP with minor allele frequency of 20% and an additive genetic model where each copy of the risk allele increases stroke risk by 20% (i.e., odds ratio of 1.2), and the baseline risk of stroke in a particular community is 1%. The sample size needed for 80% power at a significance level of $p < 0.05$ if only one genetic variant is tested is 1400 people in each group. However, if the genetic variant is one of about one million independent genetic variants tested (as is typical in GWAS), adjustment for multiple testing using a Bonferroni correction requires a significance level of $0.05/10^6$ or 5×10^{-8}. To detect the same association at this adjusted significance level with 80% power would require just over 7000 people/group, a very sizable increase.

As the number of variants tested in a CGAS is much smaller than for GWAS, there is considerably more scope to fit complex models and tease out the nature of effects in a reasonable time frame. For instance, if there are K SNPs, then if we do not consider interactions, there are effectively 2^K possibilities of models. There are a variety of computational techniques capable of exploring this large model space for CGAS [13], some that can determine possible Gene × Gene and Gene × Environment interactions.

For smaller numbers of variants, it is also possible to consider the joint effects of markers via haplotype association tests. Haplotypes are a set of alleles on the same strand of DNA that are inherited as a unit, and are thus in strong linkage disequilibrium (LD). By testing haplotypes rather than single markers for association, an increase in power may be gained by reducing the number of comparisons. It is also possible that the effect due to the haplotype will be stronger than the effect observed at constitutive alleles. When investigating the statistical significance of an association due to a haplotype, special algorithms should be employed that account for the LD structure of the haplotype (e.g., permutation procedures).

The major drawback, however, to the candidate gene approach is the limited knowledge of biochemical pathways and disease etiology on which to

base the choice of candidate. Thus, these studies depend on the ability to predict functional candidate genes and polymorphisms. The GWAS approach is often touted positively as “agnostic” or “hypothesis neutral,” in that no prior biological knowledge is needed. Nevertheless, the choice of a candidate gene is increasingly being refined. With the Human Genome Map complete, and the catalogue of variants continually increasing—i.e., HapMap [14] and 1000 Genomes [12]—there is more information on which to base the choice of candidate. SNPs can be chosen because they are:

- Nonsynonymous (i.e., lead to an amino acid change, and presumably to a functional effect).
- In a splice site (i.e., they may interfere with RNA splicing of the transcript).
- In a promoter site, upstream of the gene of interest.
- In the same biological pathway as another implicated gene. There are increasingly complete catalogues of biological pathways that can be searched, e.g., KEGG [15].
- In genes with SNPs that have shown evidence of association in a GWAS.
- In genes within loci identified through a linkage study.
- In genes that are differentially expressed in a microarray study.
- In genes shown to have functional effects in in vitro models or in animal models.
- In genes that are good candidates for an intermediate in the disease pathway.

However, it is still difficult to anticipate what SNPs will have a functional effect and hence be “good” candidates; for example, some synonymous SNPs (i.e., those that do not change the amino acid sequence) have been shown to have functional effects via an effect on mRNA stability [16].

Genome Wide Association

Compared to the CGAS, the GWAS is the more powerful technique for identification of single nucleotide polymorphisms (SNPs) associated with

complex diseases. Until recently, analytically robust genotyping of more than 100,000 SNPs per sample was not possible. The typical GWA study has four parts: (1) selection of a large number of individuals with the disease or trait of interest and a suitable comparison group; (2) DNA isolation, genotyping, and data review to ensure high genotyping quality; (3) statistical tests for associations between the SNPs passing quality thresholds and the disease/trait; and (4) replication of identified associations in an independent population sample or examination of functional implications experimentally. Most published GWA studies were designed to identify SNPs associated with common diseases. However, the technique can also be used to identify genetic variants related to quantitative traits such as body weight [17] or longevity [18].

GWA studies can also demonstrate gene-gene interactions, or modification of the association of one genetic variant by another, as with GAB2 and APOE in Alzheimer disease [19, 20], and can detect high-risk haplotypes or combinations of multiple SNPs within a single gene, as in exfoliation glaucoma [21] or atrial fibrillation [22]. These studies have also been used to identify SNPs associated with gene expression, either as confirmation of a phenotypic association, such as for blood pressure and hypertension [23] or more globally [24]. Thus, GWA studies have a broader application than discovery of individual SNPs associated with discrete disease endpoints.

The GWA study design has become an established and important method in genomics and resulted in an explosion of positive whole-genome association studies and the identification of many new genes for common diseases. GWA studies have successfully identified common genetic determinants of coronary artery disease [25], atrial fibrillation [22, 26], blood pressure [27–29], blood lipid concentration [30], diabetes [31], obesity [4], smoking behavior [32], plasma homocysteine [33], and intracranial aneurysm [34]. Indeed, for ischaemic and hemorrhagic stroke, GWA studies have now identified and replicated several risk variants which include HDAC9 for large artery atherosclerosis stroke, PITX2 and ZFHX3 for cardioembolic stroke [35] and 12q21.1 and 1q22 for lobar and non-lobar ICH, respectively [36].

Discovery of novel stroke-specific genetic associations provides a unique opportunity to generate and test new hypotheses relating to stroke pathophysiology. Genetic associations might aid to identify patients at high risk of poor recovery and therefore are possible good candidates for

aggressive therapy in the acute setting, as suggested by association of APOE variants with susceptibility to cerebral injury [37–39]. Results from GWA studies can also inform diagnostic accuracy, as indicated by the fact that a SNP associated with atrial fibrillation [40] (rs2200733; OR, 1.90; 95% CI = 1.60–2.26) was associated with risk of cardioembolic (OR, 1.52; 95% CI = 1.35–1.71) and non-cardioembolic ischemic stroke (OR, 1.18; 95% CI = 1.09–1.28) [27], suggesting atrial fibrillation might be underdiagnosed in patients presenting with stroke. Of interest, this variant was also found to be a susceptibility locus for additional AF signals [41].

Association of specific polymorphisms with different stroke subtypes is relevant to stroke prevention since it provides proof of overlapping diathesis. Other examples include a coding variant of glucokinase regulatory protein associated with C-reactive protein levels, triglycerides, fasting glucose, diabetes and Crohn's disease, and a SH3B2 variant associated with eosinophil numbers, myocardial infarction, and blood pressure [29, 33, 42–44]. Extending these associations to stroke will provide further insights into the nature of these genetic variants and the pathophysiology of stroke. The goal is for information derived from GWA studies to eventually inform decisions on prophylactic and acute treatment in genetically at-risk patients and to identify new targets and pathways for drug development.

Many case-control GWA studies have adopted a multistage design to reduce the amount of genotyping and retain statistical power to detect genetic associations. In a multistage design, the initial genome scan is done in a subset of the study participants from which disease-associated SNPs are selected. The initial selection of candidate SNP is done at a less stringent P value threshold ~$P = 10^{-4}$. These SNPs are then used in a second stage to determine if the initial association between the SNP and disease can be replicated. In some instances, a third level is used to further refine the list of SNPs prior to significance testing. Because the number of SNPs tested is reduced after each stage, the threshold for significance is less affected by correction for multiple testing [45]. But without a doubt the most common approach remains the pooling of GWAs into meta-analyses of substantial sample sizes in order to cater for the stringent P value threshold ~$P = 5 \times 10^{-8}$ needed to counteract expected small effect sizes and multiple testing challenges.

Meta-Analysis in Genetic Association Studies

Meta-analysis uses a set of methods to combine genotype data from several studies. It is useful for both CGAS and GWAS designs. There are many examples of CGAS results that are confirmed on meta-analysis but that are not initially detectable on GWAS. For example, an association between chromosome 9p21 variants and ischemic stroke was first identified by CGAS [46, 47] and subsequently confirmed by meta-analysis [48], but has not reached genome-wide significance in GWAS.

Meta-analysis improves power to detect associations by significantly increasing the sample size and by examining more variants throughout the genome than originally genotyped within each individual study, as first shown by the meta-analysis of type 2 diabetes GWA study data [49]. Genetic meta-analysis with GWAs generally entails two steps: an imputation step followed by the meta-analysis *per se*. Although GWAs studies have revolutionized the field of human genetics by reliably identifying dozens of common genetic determinants of complex traits and diseases [50–52], it has become clear from these early successes that genetic effect sizes are quite modest, often with odds ratio of 1.2 or less for dichotomous traits [53, 54]. Large sample sizes and carefully designed studies are therefore needed to permit detection of small effect sizes. Thus the genome wide association meta-analysis (GWAMAs) combines summary level data from individual GWAs [35, 55]. For an excellent protocol and description of quality control in the conduct of a discovery level GWAMAs see Winkler et al. This can also be easily applied to follow up stages and imputed datasets [56].

Imputation in GWAs Meta-Analysis

Imputing the three million SNPs for which a haplotype structure has been described in HapMap from the directly genotyped SNPs in each study provides a means to improve the genetic coverage and combine the genetic datasets. HapMap data is used to calculate the probability of each genotype (or most likely genotype) for each non-genotyped SNP. It follows that the quality of imputed genotypes is critically dependent on HapMap data [57, 58], whose main limitation is small sample size especially when imputing low allelic frequency variants. However, the ongoing data releases from the 1000 Genomes Project [59] has added substantially to the quantity of data used for imputation analysis. Algorithms generally provide some measure of

imputation quality for each SNP, and it is recommended to remove low-quality imputation SNPs from analysis according to the instructions of the software developer [60]. Imputation is a computationally intensive process that can, in some cases, take weeks to complete depending on the study size and the number of SNPs to impute. Parallelization is possible by splitting the data by chromosome but care must be taken not to induce biases by, for example, imputing SNPs in cases and controls separately [61]. Widely used imputation tools include PLINK, IMPUTE, MACH, minimac [62], BEAGLE, and BIMBAM. For an excellent comparison of imputation methods see Table 3.1 from Marchini et al. [63].

Table 3.1 Comparison of imputation methods in Genotype imputation for genome-wide association studies

Properties	**Imputation method**				
	IMPUTE v1	**IMPUTE v2.2**	**MACH v1.0.16**	**fastPHASE v1.4.0 BIMBAM v0.99**	**BEAGLE v3.2**
Reference panels					
Can use a haplotype reference panel?	Yes	Yes	Yes	Yes	Yes
Can use a genotyped reference panel?	No	Yes	Yes	Yes	Yes
Can two haplotype or genotype reference panels be used in the same run?	No	Yes	No	No	No
Reference panels available in correct format	HapMap2	HapMap2	HapMap2	HapMap2	No
	HapMap3	HapMap3	HapMap3		
	1KGP pilot data	1KGP pilot data	1KGP pilot data		
Study samples					
Can take	No	Yes	No	No	Yes

genotypes specified with uncertainty?					
Can accommodate trios and related samples?	No	No	No	No	Trios and duos
Can impute into a study sample of autosomal haplotypes?	Yes	Yes	No	No	Yes
Can impute on the X chromosome?	Yes	Yes	No	No	Yes
Program options and features					
Does phasing as well as imputation?	No	Yes	Yes	Yes	Yes
Can impute sporadic missing genotypes?	No	Yes	Yes	Yes	Yes
Has internal performance assessment?	Yes	Yes	Yes	No	No
Can impute only in a specified interval?	Yes	Yes	No	No	No
Can handle strand alignment between data sets?	Yes	Yes	Yes	No	No
SNP and sample inclusion and exclusion options?	Yes	Yes	No	Yes	Yes
Joint model for imputation and association testing?	No	No	No	No	No

Operating system requirements	Linux, Solaris, Windows, Mac	Linux, Solaris, Windows, Mac	Linux, Windows, Mac	BIMBAM (source code + Windows) fastPHASE (Linux, Solaris, Windows, Mac)	Java executable
Computational performance					
Assessment 1[a]	43 m (1000 Mb)	75 m (180 Mb)	105 m (80 Mb)	855 m (16 Mb)	56 m (3100 Mb)
Assessment 2[b]	–	48 m (115 m)	–	157 m (211 m)	104 m (234 m)
Error rates [c]					
Rows correspond to the Scenario A, Scenario B (restricted) and Scenario B (full) data sets	5.42%	5.16%	5.46%	5.92%	6.33%
	–	3.4% (0.86%)	–	5.33% (1.32%)	3.46% (0.93%)
	–	3.4% (0.86%)	–	–	4.01% (1.04%)
Output files					
Genotype posteriors produced?	Yes	Yes	Yes	Yes	Yes
Information measures?	Yes	Yes	Yes	No	Yes
Easiest use of output files to test association	Feed files directly into SNPTEST. Test based on genotype posteriors, dosages or thresholded genotypes	Feed files directly into SNPTEST. Test based on genotype posteriors, dosages or thresholded genotypes	Genotype dosage files can be fed into MACH2DAT or MACH2QTL	BIMBAM can produce file formats used by BIMBAM. fastPHASE out files need to be processed	Best-guess phased haplotypes can be tested in BEAGLE. Processing required to use genotype posteriors or dosage

Marchini and Howie, Nature Reviews Genetics 11, 499–511 (July 2010) doi:10.1038/nrg2796

Properties are shown for the version of the method as listed

[a]Imputation of 1377 samples on the Affy500k chip from 120 CEU HapMap2

haplotypes: 7.5 Mb region. Data from [7]

[b]Imputation of 500 (1000) samples genotyped at 872 SNPs from 1000 haplotypes at 8712 SNPs in a 5 Mb region. Timings based on data sets simulated using HAPGEN and the pilot CEU haplotypes from the 1000 Genomes project in a 5 Mb region on chromosome 10

[c]Error rates from [7]. Except results for IMPUTE v2 have been updated. Scenario B error rates given are for Illumina SNPs imputed from Affymetrix SNPs. Error rates for Affymetrix SNPs imputed from Illumina SNPs are given in brackets

Meta-Analysis Following Imputation: Genome Wide Association Meta-Analysis (GWAMAs)

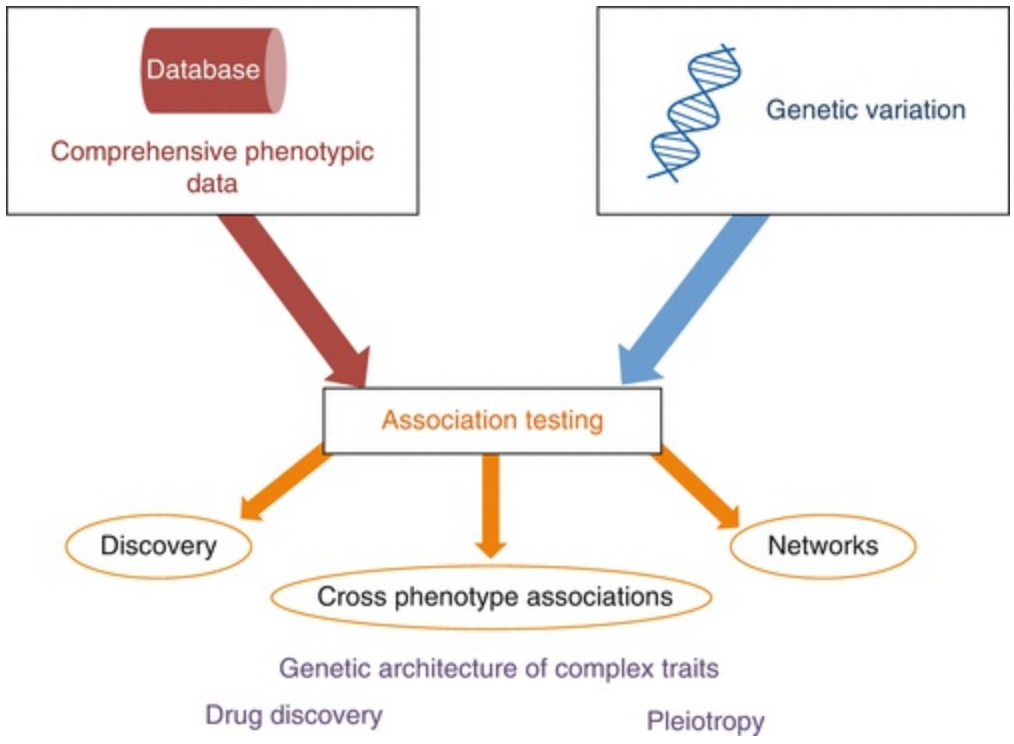
Once SNPs have been imputed and included in the meta-analysis, association results can be combined for each SNP [49]. GWAMAs is widely accepted as the preferred approach to combine summary level data from individual GWAs as it can generate the necessary sample size for the statistical power required. Only summary data are typically shared between investigators for each SNP and individual genotype data need not be provided. Datasets can be combined under either a fixed-effect or random-effect framework with weights given to each study under the principle that studies with more precision are assigned greater weight [55].

Fixed-effect models test the null hypothesis of no effect in any of the study populations analyzed and assume that a single common effect underlies each study in the meta-analysis. Examples include inverse-variance weighted meta-analysis for quantitative traits and study-size weighted meta-analysis for case-control data, although procedures further incorporating imputation uncertainty also exist. Random-effect models make the assumption that individual studies are estimating different effects. Random-effects meta-analysis therefore assumes a distribution of effects across different studies. In the absence of between-study heterogeneity, both fixed- and random-effects meta-analyses will give similar estimates and confidence intervals. However, in the presence of significant heterogeneity, random-effects estimates tend to have larger variance and accordingly smaller statistical significance. Between-study heterogeneity can be formally tested using either Cochran's Q or I^2 statistics, although the best approach to meta-analysis with significant heterogeneity remains controversial [64, 65]. Cochran's Q was developed to

test whether there is statistically significant heterogeneity or not. Its main disadvantages are that it is generally underpowered and its power increases with the number of studies included. The I^2 statistic [66] describes the percentage of variance across studies not due to chance. I^2 has the advantages of being easy to interpret and of not being dependent on the number of combined studies.

Phenome-Wide Association Studies

In this global research landscape rich with data and advanced computational ability a novel avenue for discovery of genetic architecture in disease is underway. To explore and unravel the relationship between genomic and phenotypic data a variation of design is emerging—the phenome wide association study (PheWAS). In this design, large datasets derived from epidemiological, clinical and genomic sources are interrogated through association analysis to test for linkage and unforeseen networks [67].



Overview of PheWAS. PheWAS can be used to evaluate the association between a comprehensive set of phenotypes and genetic variation. A relational database is useful for organizing and working with the phenotypic data. The phenotypic data can be collected through multiple types of studies, including epidemiological studies, de-identified electronic health records, clinical trials data, and animal breeding research. Genetic variation can be single nucleotide polymorphisms (SNPs), but any genetic variation

that can be evaluated for association with phenotypic variation can be used. The association testing results can be evaluated multiple ways, and while not shown, a relational database can assist with analyses of results. Novel discoveries can be identified along with cross-phenotype associations. Networks of connections between SNPs, genes, and phenotypes can be explored. These results can provide more information about the genetic architecture of complex traits, highlight biologically important pleiotropy, and can support drug discovery. Source: Pendergrass et. al. [67]

One of the exciting advantages of PheWAS design approach is the creative utilization of existing data with much broader simultaneous testing across multiple phenotypes and genotypes, resulting in more detailed data thus enhancing potential to better target underlying mechanisms. In addition, the identification of shared networks and etiologies across complex diseases may revolutionize future clinical practice as it teases out pleiotropic genes. For example, using PheWAS a recent paper highlights identification of novel markers after association testing between functional variants for pharmacogenes and International Classification and Disease, Ninth Revision billing codes extracted from de-identified electronic health records of 6892 patients [68]. Using this novel methodology highly informed polygenic scores were created from domains such as psychopathology, personality, cognitive abilities and educational achievement.

Sources of Bias in Genetic Association Studies

Genetic association studies are thought to be more robust against confounding than traditional (non-genetic) association studies. For example, recall bias, which can occur when cases overcall previous risk factor exposure knowing they have the outcome of interest does not influence genetic associations. There is also a lack of temporal bias which normally can occur when a risk factor of interest is measured after disease development and the direction of causality is unclear. Generally, this is less of a concern for genetic studies where alleles are inherited at birth and do not change. However, in the case of stroke, a stroke may not be recorded; therefore, the reported age of onset is inaccurate. There are new sources of bias to consider in genetic association studies and multiple articles have been written about these, both generally (e.g., [11]), and in the context of stroke [69, 70]. This has led to an extension of the guidelines for reporting observational studies geared specifically to genetic association studies (see STREGA [71]). The recommendations in those guidelines will act as a template for an epidemiological discussion of sources of bias here. Readers looking for more

details on the specific statistical issues in GWAs and CGAS are referred to an excellent discussion and reviews [72–74].

Misclassification of Cases and Controls

Selection of participants and careful case ascertainment is a key component in the validity of any case-control study as any misclassification of case and control patients leads to loss of sample size and diminish statistical power. This is even more pertinent for genetic association studies where losses can be more significant through the use of retrospective data with less than ideal case and control ascertainment. Ideally, control patients should be drawn from the same population as cases and should have the same risk of developing disease. So-called super controls, people with high risk but without evidence of disease have been used in a number of GWA studies [75]. Often case participants are selected from clinical sources and therefore may not include fatal, mild, or silent cases resulting in selection bias. Conversely, control subject may have an early undetected disease. In common diseases such as stroke, efforts must be made to ensure that controls are disease free. It is essential that well-established principles of epidemiologic design are employed such as description and comparison of key phenotypic characteristics for cases and controls. It is important that a GWA study provides a table comparing relevant characteristics of cases and controls allowing assessment of comparability and generalizability of the two groups. For a thorough discussion of phenotype standards in GWAs studies see recent detailed recommendations from the International Stroke Genetics Consortia [76].

Misspecification of Outcomes

The cases and controls in a CGAS or a GWAs are chosen based on their disease outcome (e.g., stroke *vs*. no stroke), and any difference in the frequency of a genetic variant is said to be related to that outcome. In practice, however, stroke case and control groups also differ in the frequency of many risk factors for stroke (e.g., diabetes mellitus, cigarette smoking, obesity, and hypertension), and it is also possible that the genetic variant is associated with one of these as a “covert” outcome. In this case, the risk factor is associated with both genetic variant and stroke outcome and acts like a confounder, leading one to say that a variant is associated with stroke when

in fact it is related to, for example, diabetes mellitus. This occurred with variants of the *FTO* gene, which were found to be associated with diabetes; however, diabetic cases had higher body mass index (BMI) than controls and when this was adjusted for, association between *FTO* and diabetes disappeared, indicating that the true association was between *FTO* and BMI [4]. The solution to this potential confounding problem is to adjust for imbalances in known risk factors or to match cases and controls on potentially confounding variables.

Misspecification can also occur when there is unsuspected heterogeneity of outcomes. Stroke itself is heterogeneous with many possible differences in the pathophysiologic mechanisms underlying the various subtypes. For example, it is biologically likely that ischemic and hemorrhagic strokes have fundamentally different etiologies and hence, any attempt to find an association between a genetic variant and all strokes could fail. Likewise, ischemic stroke may be considered homogenous by one group but heterogeneous by another, who may subdivide this further into large artery, small vessel, and cardioembolic stroke. GWAS and CGAS have been published for both ischemic stroke as a whole, and for subgroups and until recently, there was very little standardization in classifying ischemic stroke in GWAs or CGAS [77, 78].

Multiple Comparisons

The problem of multiple comparisons in CGAS is somewhat hidden as it is often unclear how many genetic variants had been tested prior to finding and reporting the one that showed an association; when this happens, the likelihood of type I error, that is, incorrectly declaring an association as statistically significant, is increased. Given the difficulty in publishing negative results, it is often the lone positive result that gets published, often using an unadjusted significance threshold of $p < 0.05$. Given the play of chance and the need to have a “strong” result in order to break the publication barrier, this first study also tends to overstate the effect size of the genetic variant, a phenomenon particularly in CGAS known as the “winner’s curse” [79]. Subsequent studies often show lower effect sizes or refute the original association completely.

For GWAs, multiple hypothesis testing is a major issue and must always be taken into consideration. Unless further replication can be obtained from independent study populations, a conservative Bonferroni correction should

be applied using either the actual number of SNPs that have been tested for association or assuming a total of 1,000,000 SNPs. One million is the estimated number of statistical tests that would be performed if all common genetic variations segregating in European Caucasian individuals were tested (corresponding to a P value threshold of $0.05/1{,}000{,}000 = 5 \times 10^{-8}$) [56, 70]. Several bioinformatics tools (i.e., PLINK [80]) are available to perform these analyses.

Population Stratification

In both CGAS and GWAS, population stratification (i.e. structure), refers to a situation where there is an imbalance of subgroups, usually ancestral subgroups, between cases and controls. If an ancestral subgroup is associated with a particular genotype and also has a higher incidence of disease, this can cause confounding; the genotype may appear to be related to disease due to systematic ancestral differences between cases and controls, rather than true association of genotypes with disease. The magnitude of confounding due to population stratification has been debated [81–83]. On the one hand, these effects are very likely to be small on a theoretical basis [84], and empirically [85], but on the other hand, it is argued that the genetic effects we are trying to detect are also small (odds ratios typically <1.4) and may be obscured and recently other methods have been tried [48, 83]. The major concern for GWA studies is it can lead to false-positive associations when genetically heterogeneous populations are analyzed together without further adjustment. Spurious associations will be observed when a trait varies from one population to another, and these populations have differing allelic frequencies at the genotyped SNPs. Because differences in allelic frequencies most likely reflect genetic drift between populations that have evolved apart and are not of functional relevance for the most part, the observed associations do not have a biological meaning.

Thus many methods for adjustment have been developed, including structured association [86, 87], genomic control (GC) [88, 89], and principal components analysis [90, 91]. EIGENSTRAT and EIGENSOFT are widely used packages to correct for population heterogeneity based on principal component analysis [82]. PLINK [92] offers a related analysis that relies on multidimensional scaling. The coordinates of the first components of the principal component or multidimensional scaling analysis can then be used as

covariates in linear or logistic regression analysis. PLINK can also match cases to controls based on identity-by-state of genotype data, resulting in strata of genetically similar individuals that can be analyzed with the Cochran-Mantel-Haenszel test. More recently, the linear mixed model (LMM) has been applied and is considered to be more powerful as it simultaneously accounts for both population structure and kinship [30]. Current practice is to use one of these adjustment methods, restrict cases and controls to a single ethnic group or adjust for self-reported ethnicity.

Genotyping Related Errors

Sources of error and associated bias relevant to genotyping in association studies also need to be considered and include:

- Case-control sampling differences—controls may be selected from population-based studies, with cases coming from hospital series. In practice, this means that samples are collected in different laboratories, under different conditions, potentially many years apart. Such differences in sample handling may lead to variable DNA quality and variable DNA amplification for cases and controls [93].
- Batch effects: if cases and control samples are processed separately in different batches, any errors affecting batch genotyping accuracy can lead to spurious genotype frequency differences between cases and controls [94, 95].
- Cross-platform effects: sometimes cases are genotyped in one laboratory on one type of array, and controls are processed in a completely different laboratory on a different array. Such cross-platform comparisons can lead to extreme batch effects [96].

Genotyping Measurement Error

Although measuring genetic polymorphisms may have the cachet of being exact and absolute, in fact, there is error, just as there is for traditional epidemiological factors such as blood pressure or diet. Multiple articles have outlined the sources and magnitude of error in genotyping assays, which can range up to 30% [2, 97]. However, high concordance between genotyping platforms was reported when technical replicates were tested [97], highlighting the importance of improving GWAs reliability through this

approach particularly when minor allele frequency was low [97].

Quality Control Measures

Stringent quality controls measures have been adopted for genetic association studies and include; screening for deviation from Hardy Weinberg Equilibrium, assessment for allele call rates, filtering SNPs based on differences in call rates between cases and controls, examining the pairwise genetic relatedness between samples in order to detect cryptic relatedness or frank duplication of samples, and comparing the genetic sex of individuals to the self-reported sex to identify sample mix-ups. Increased heterozygosity can also indicate poor DNA quality and can be used to exclude samples. PheWAS also uses these standard GWAS quality control procedures followed by imputation with principal components analysis used to control for any population stratification [98].

Hardy-Weinberg Equilibrium

In both CGAs and GWAs studies, genotyping error and population stratification can produce deviations from Hardy-Weinberg equilibrium (HWE) proportions in the control group, hence making HWE tests a useful quality control check. Briefly, Hardy-Weinberg equilibrium describes the relationship between genotype distribution and allelic frequency in an idealized population. Deviation from Hardy-Weinberg equilibrium [99] can theoretically occur because of selection, mixture of genetically heterogeneous populations, cryptic relatedness, or genotyping errors [2] (either selective dropout of a given allele or misclassification of alleles). There is no universally accepted Hardy-Weinberg P value threshold, but a typical cutoff for whole-genome experiments would be between 10^{-5} and 10^{-6}; this offers a good balance between a low P value due to chance versus the other reasons mentioned given the number of tests performed in a GWA study. SNPs are also screened for deviation from Hardy-Weinberg equilibrium.

Studies have found deviation from HWE for a larger than expected proportion of published studies, raising the possibility of occult error in many published association studies [99, 100]. As mentioned, the significance level for judging deviation from HWE, however, has been a moving target, with values anywhere between (alpha) $\alpha = 0.05$ and (alpha) $\alpha = 5 \times 10^{-6}$; this is further compounded by the fact that the estimated significance is dependent on sample size (i.e., two studies of different size could have the same HW

proportions, but one appears to be consistent with HWE while the other does not). Some have suggested that the measure of HWE should be based on measures of the degree of disequilibrium rather than the *p* value, since the former is independent of sample size [2]. These measures of degree of disequilibrium can then be used to adjust the association analysis [101].

Filtering Based on Call Rates, Missingness and Pairwise Relatedness

First, individual samples are checked for completeness of genotyping by calculating the percentage of SNPs for which no allele could be called, a process that must be repeated for every sample. Individuals with a high rate of missing genotypes likely reflect poor DNA quality and should be excluded. Although there is no universally accepted threshold, over 10% missing genotypes are usually considered unacceptable by most investigators. Second, individual SNPs are examined for missing genotypes (i.e., proportion of individuals without a proper allele call for each SNP). Again, no universally accepted threshold exists, but over 10% missing would be considered high. It is important to note that the order of these two procedures can be inverted and that one procedure can affect the other. In general, the standard for call rates should exceed 98% for both cases and controls and individual SNPs with current technology. All of the preceding procedures can be done in PLINK but could also be executed with other databases or statistical packages.

Analysis for Association

Testing each SNP for association with a trait of interest lies at the heart of any candidate, genome-wide or phenome wide association study. SNPs with low minor allele frequency (<1%) are usually removed since statistical power to detect any association will be low. The specific statistical test chosen will depend upon the a priori hypothesis being tested as well as the type of data available. Linear regression is preferred for continuous traits, and logistic regression or the Cochran-Armitage test for categorical traits. Additive genetic models, whereby each minor allele is assumed to have an equal effect on the trait of interest or risk of disease, are usually used, although recessive and dominant models are also considered.

Methodology for pooling results in traditional epidemiological studies is well established but this has focused mainly on pooling contrasts between

two groups. The main difference with genetic association studies is that there are, at minimum, three genotype groups (AA, Aa, and aa) and potentially more if testing a DNA repeat variant. One approach is to collapse the three groups to two by assuming a dominant or recessive genetic model. However, this may be an inaccurate assumption; there is often little evidence to indicate the genetic model operating in a disease association. Theoretical work has suggested that in the absence of any evidence about genetic models, an additive genetic model retains the most power with the least error, even when the true underlying genetic model is dominant or recessive [102], although newer approaches are now being implemented [103]. Indeed, in an additive model, risk increases with each copy of the risk allele. Alternatively, one can look at the two pairwise comparisons between the three genotype groups and by dividing them obtain a parameter (lambda) that can indicate the genetic model; this is an empirical approach that lets the empirical data indicate which genetic model might be operating [103, 104].

It is also increasingly recognized that associations identified via GWAS for stroke are largely indirect, as tested SNPs are unlikely to be causative but simply correlated with functional variants most likely due to linkage disequilibrium (LD). Hence, considerable subsequent fine mapping and sequencing is typically required to isolate the actual causative variants, which may not be a SNP but alternatively an insertion, deletion, or copy number variant (CNV). CGAS that have been identified within loci consequently identified by GWAS are now proving very useful in the arsenal for such fine mapping [105].

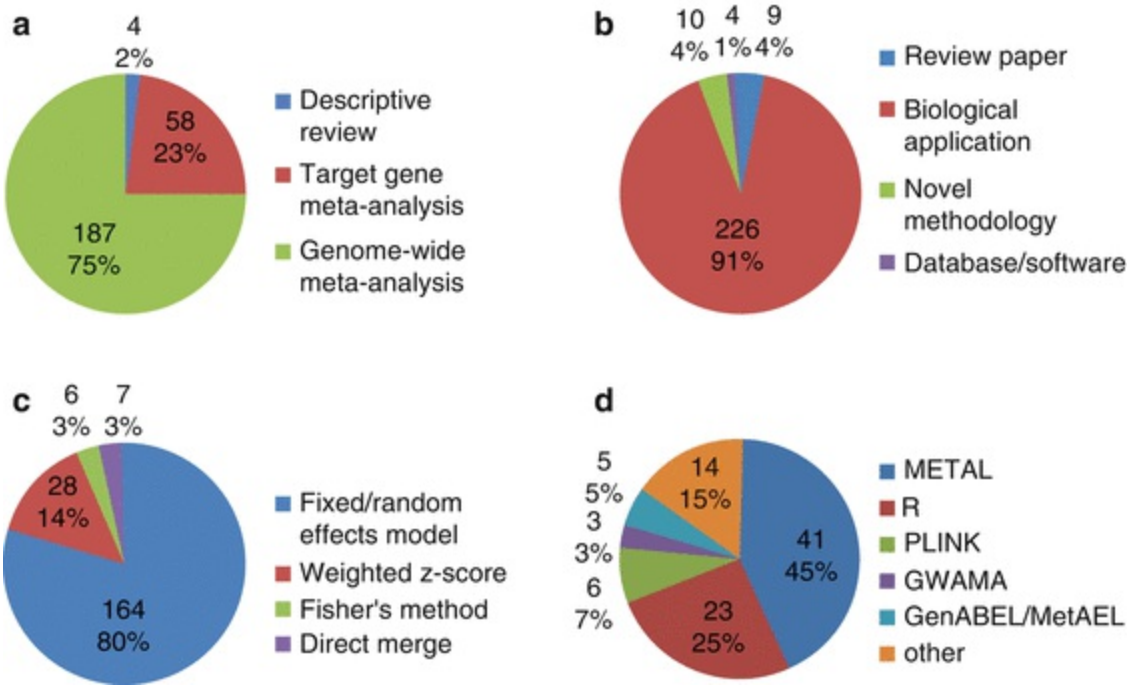
Analysis of GWA study data is a complex process that entails the successive completion of multiple steps [72, 103]. The mode of inheritance is rarely known *a priori* and testing of all possible models will lead to an increase in the type 1 error rate. The considerations and issues in analysis of GWAS have been well summarized elsewhere [103, 106, 107].

Many tools exist to facilitate analysis, but in most cases, advanced informatics and statistical skills are required. Several commercial packages, such as SNPMax and GoldenHelix, have been developed to help with the management and analysis of genetic data. These packages offer comprehensive solutions in a user-friendly environment requiring minimal informatics skills. Nevertheless, freely available tools such as PLINK (available both for Microsoft Windows and Linux operating system) can perform a wide range of analyses and are updated frequently to keep pace

with the development of novel GWA analysis methods. While each individual study group can determine the analysis plan that best suits its needs, choice of a specific method should be based on expert understanding of underlying statistical concepts and a good understanding with the adopted analysis framework.

Meta-Analysis

In a comprehensive 2012 review of GWAS meta-analysis literature where the focus was on methodology used and software options, Bagos et al. note that the most commonly used model chosen for pooling GWAs is fixed/random effects. Heterogeneity between studies originated from a variety of trait measurements, study designs, ethnic groups, environmental exposures, choice of genotyping chips and the potential for differing linkage disequilibrium patterns in different ethnic groups. To address heterogeneity they found that a major improvement over Fisher's method is a weighted Z-score method, in which P-values are transformed to Z-scores in a one-to-one transformation [103].



Summary of GWAS meta-analysis review: (**a**) type of meta-analysis; (**b**) type of paper; (**c**) type of meta-analysis methods used (**d**) software used. Source: Tseng et. al. [72]

The fixed effects model does not make allowance for any between-study variation, whereas the random effects model does; in essence, it allows for the fact that the genetic effect may not be identical in all studies. Where pooled genetic estimates are heterogeneous, it is still good practice to explore and understand the source of heterogeneity rather than just continuing with "brute force" pooling. This heterogeneity may indicate genotyping error, misclassification of outcomes, or, indeed, different interacting environmental variables between studies [108]. It appears unlikely that there are true differences in a genetic effect between ethnic groups; a survey of identical association studies between different ethnic groups overwhelmingly showed that although allelic frequencies vary widely across ethnic groups, if an allele is present, it has the same magnitude of effect across ethnic groups [109]. The current practice of pooling using random effects when heterogeneity is present does not necessarily increase power and in fact can result in power "deserts," where adding data sets produces no increase in power; using fixed effects in this situation is not a solution either, as it leads to many false-positive signals [64, 110].

Graphical displays of the CGAS data using Peto plots are a good way to show the effect sizes from different studies and the variation between them; an example of this plot is shown for the Thr/Met rs6065 polymorphism of glycoprotein 1bα (alpha) and ischemic stroke from Maguire et al. (Fig. 3.2).

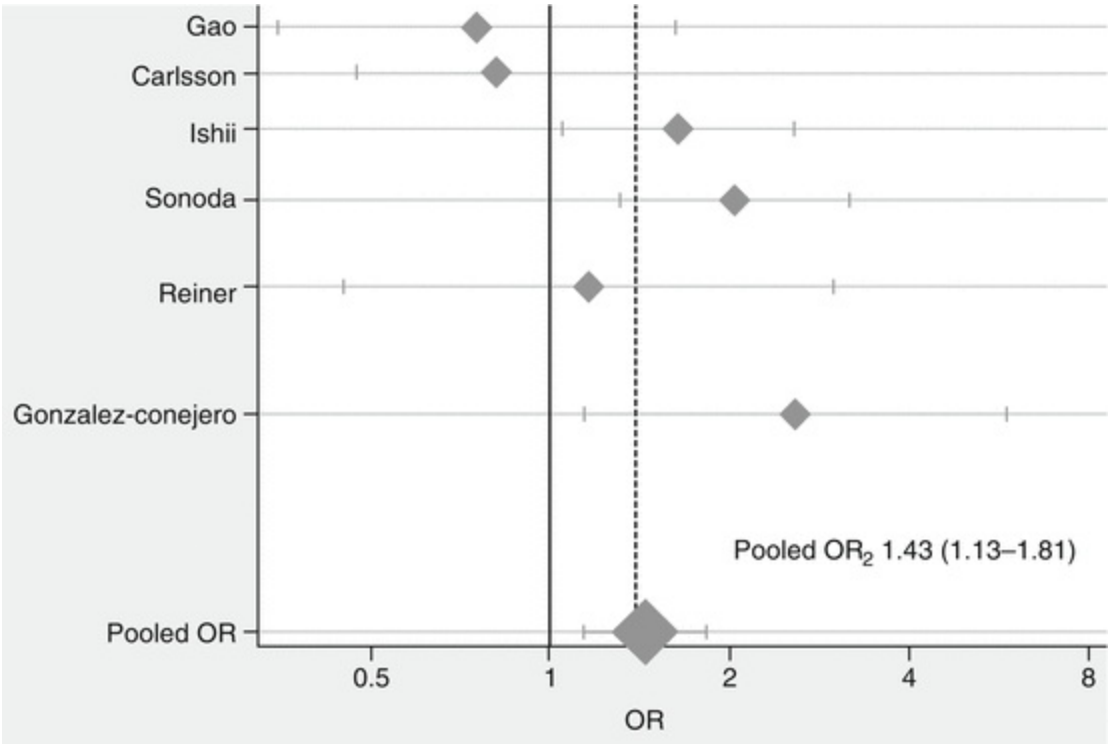


Fig. 3.2 This shows the odds ratio for the Met/Thr variant versus the Thr/Thr variant in glycoprotein GP1bα and ischemic stroke. In this case, significant heterogeneity was present (I^2 = 58%, p value = 0.04) (Reprinted with permission from Maguire et al. [111])

Mendelian Randomization: Another Option for Investigating Genetic Association

Mendelian randomization is a method to test and estimate the causal effect of an exposure of interest from observational data [112, 113]. The underlying idea is to use the random allocation of alleles at a particular gene at meiosis during conception to act as a randomized controlled trial. Since randomization avoids confounding, the random allocation of a gene allows an unconfounded assessment of the relationship between an environmental exposure (that is dependent on the gene) and a disease outcome (Fig. 3.3). The idea was first suggested by Katan [114]; the term "Mendelian randomization" was originally coined by Gray and Wheatley [115] but was popularized by Davey Smith and Ebrahim [112]. For example, the effect of methylenetetrahydrofolate reductase (MTHFR) genotypes on homocysteine levels (exposure) and stroke risk (outcome) was examined using Mendelian randomization [116]. This analysis required that the genetic variants in

MTHFR had a well-understood effect on the exposure (homocysteine levels) and affected the outcome solely through modification of the exposure (homocysteine levels). Since genotypes are passed randomly from parents to offspring during meiosis, Mendelian randomization is assumed to be devoid of confounding by social, environmental, behavioral, or reverse causation factors that can plague observational studies. Mendelian randomization can thus be considered a "natural" randomized trial experiment, with genotypes acting as an instrumental variable for the exposure of interest and can be used as a tool to assess causality. In the aforementioned example, stroke risk between MTHFR genotype groups was similar to that expected given the effect of MTHFR on homocysteine concentrations, indicating that homocysteine concentrations likely have a direct role in mediating stroke risk. Similarly, this Mendelian randomization was used in a clinical trial to investigate the causal role of high density lipoprotein cholesterol and triglycerides in coronary artery disease and reported a robust causal role for triglycerides in CAD [117].

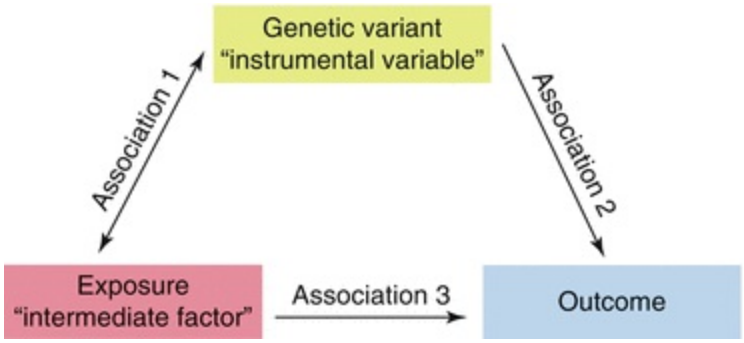


Fig. 3.3 Mendelian randomization (Adapted from Thanassoulis et al. [66])

Care must be taken in the presence of linkage disequilibrium, genetic heterogeneity, pleiotropy, or population stratification as these factors can lead to false conclusions when using Mendelian randomization. Genetic heterogeneity describes the phenomenon in which a phenotype is caused by any one of a number of polymorphisms. Conversely, pleiotropy is the phenomenon in which a single gene influences multiple phenotypic traits. Indeed, the Mendelian randomization approach can fail when there is pleiotropy (i.e., a gene has multiple biological effects that can influence an outcome) or when there is "canalization," a term referring to the homeostatic mechanisms of the human body that seek to restore a phenotype toward

normal values. Indeed, randomized controlled trials of folate and vitamin B, which lowered homocysteine levels did not influence cardiovascular event rates [118] indicating that the Mendelian randomization approach is not foolproof.

One weakness of MR studies to date is that a SNP has been a poor instrumental variable, given the small allelic OR associated with most exposure variables. A recent advance is multivariable mendelian randomization, where multiple genetic variants, potentially linked to multiple risk factors can strengthen the magnitude of the association and improve the performance of the SNP as an instrumental variable [119].

The Human Genome Project

The large sample size requirement of GWAs has led to most GWAS meta-analyses being assembled from consortia of investigators contributing data from similar traits. The International Stroke Genetics Consortia is one such group. Many publically available databases are now also available and one of the most commonly used is NIH Database of Genotype and Phenotype (dbGaP). But the most significant contribution made to our understanding of human disease through genetics began with the NCBI initiative—the Human Genome Project.

Genotyping the Human Genome

The Human Genome Project [120] was completed in 2003 and made reading the entire human genome possible. This combined with the SNP Consortium [121], the International HapMap Project [122], and the 1000 Genomes Project [123] became essential components in the development and future success of GWA studies. To capture human genetic variation, the International HapMap Project was launched in 2002 with the aim of characterizing SNP frequencies and linkage disequilibrium patterns across the human genome. In 2016 this project was retired by the NCBI as it has fulfilled its aims and the 1000 Genomes Project and International Genome Sample Resource took precedence. By its completion, HapMap had successfully collected genotype information from 270 representative individuals and 11 global populations including European, African, and Asian populations. The Human Genome Project has already instigated the

discovery of more than 1800 disease genes and genes suspected of causing an inherited disease can be identified much more rapidly as opposed to previously taking years of painstaking investigation. Over 2000 diagnostic and predictive genetic tests for human conditions are available and translation into clinics is more imminent every day. With this enormous number of catalogued SNPs, it was essential that researchers determined if all ten million common variants needed to be tested for association to disease (i.e., if one million SNPs were directly measured, would 90% of associations be missed?). Previous work indicated that for each disease-associated mutation that arises on a copy of the genome, there will be a set of common alleles in cis at nearby loci—this is termed a haplotype. Since the recombination rate is low in humans (~1 crossover/100 megabases/generation), disease alleles are typically found in association with nearby marker alleles for many generations, this phenomenon is called linkage disequilibrium (LD).

LD patterns are important in determining the location and number of SNPs that must be directly genotyped to achieve genome-wide coverage. We now know that the human genome is composed of LD blocks divided by regions with higher degrees of recombination called recombination hotspots. LD blocks rarely undergo recombination, and therefore, knowledge of the genotype of a few SNPs within an LD block allows assignment of the haplotype for that portion of the genome. The haplotype map produced by the HapMap project allowed researchers to make informed choices about the SNP density and distribution for optimal genome coverage with a subset of SNPs that tag each haplotype. In practice, multiple SNPs are used to tag each region of high LD. Current empirical estimates suggest approximately 500,000 SNPs for non-African populations and 1,000,000 SNPs for African populations to ensure genome-wide coverage for alleles with a frequency of at least 5% [122].

While genome-wide coverage can be achieved with 500,000 to 1 million carefully selected SNPs, greater SNP density is required to finely map the loci associated with disease, especially for low minor allele frequency variants (i.e., 1–5%). New technologies for genotyping continue to be developed, resulting in dramatic increases in sample throughput, precision and SNP density per sample. Development of such genotyping platforms required overcoming two major challenges: (1) miniaturization of assays and ensuring decreased reagent cost, a major barrier to large-scale genotyping projects, and (2) parallelization of assays, a nontrivial problem since

amplification of >12 amplicons with PCR is fraught with technical difficulties. Evaluation of these technologies is based on the number of SNPs assayed, call rates, error rates, and concordance with published data (i.e., HapMap samples). Many different classes of platform exist but can be broadly classified into those suitable for GWA studies (greater than 100,000) or replication/validation experiments (1–several thousand SNPs). Commonly used technologies for high-throughput genotyping include Illumina's Infinium Beadchips and Affymetrix—GeneChip but as new versions are frequently released please refer to specific company websites for the latest versions and product updates.

Next Generation Sequencing (NGS)

The faster, wider and more precise capability of NGS has magnified data readouts to a genome wide scale with unprecedented potential. NGS is now replacing array-based methods in some GWA studies due to its increased resolution and coverage. NGS has been applied in two ways:

- Whole exome sequencing: which yields sequence information primarily from protein coding regions of the genome and adjacent regulatory regions. The underlying assumption here is that variations that influence the amino acid sequence are the most likely to be relevant to disease. Unfortunately the reality is that over 85% of all signals in GWAS to date for complex disease have been in non-coding and intergenic regions, and there are many examples of diseases related to non-coding regions [124].
- Whole genome sequencing: which yields sequence information on the entire genome. Although there are a number of ways of doing this, the principle is to "cut" the genome into manageable segments, read the sequences of these fragments and then reassemble them into the entire genomic sequence. Due to errors in reading the DNA, fragments must be sequenced multiple times, called "read depth", typically up to 40–50 times per fragment, and a consensus sequence created. These consensus sequences are then assembled into a longer genome read, based on the existing human genome map. Needless to say, this generates a significant amount of alignment and bioinformatic work. Much of this simply recapitulates sequences that are largely identical from one

individual to another, given that the site of common variation were already tagged using microarray SNPs.

There has been much discussion about how to identify causal variants in WGS from the broader background of individual-specific, family specific or ethnic-specific variation [125] Work to date has been mixed and although some rare variants from WGS have been linked to complex disease, the overall impression is that rare variants are failing to identify the large amount of missing heritability in complex disease [126].

Some of the first GWAs using NGS are now being published [127] with opportunities to rapidly translate findings. In one small vessel disease focused study the authors combined a systematic genetic screening with detailed phenotyping to advance the biological understanding for this subtype [1]. Some caution has been voiced with the use of NGS in association testing and while it appears that the NGS is here to stay, there is still much to be done in regards to absorbing and interpreting the enormous data outputs and development of best practice statistical and design methods [128].

Conclusion

Our still limited understanding of relevant biological pathways from which to select CGAS candidate genes for stroke research and the “agnostic” approach of GWAS maintains GWAs as methodologically superior. The reductions in the cost of microarrays for GWAS and the advent of faster, cheaper NGS techniques have also added to this popularity. New creative hybrid designs such as the PheWAS bring more detailed phenotype and genotype data together and better target underlying mechanisms. Over the last decade, much has been learned about the genetics of common complex diseases and stroke specifically. As more GWA studies of complex diseases are completed, some contribution of multiple rare variants to overall genetic risk of disease has become apparent [55, 129–131]. Identification of rare alleles and attributing causality will require careful study design, NG high-density genotyping, a large sample population, and sophisticated data analysis techniques. In addition, the contribution of multiple common variants of individually weak effect will continue to be investigated; this will also require large sample population and sophisticated data analysis techniques. The need for very large sample populations to detect rare variants has promoted collaboration between large consortia resulting in mega-meta-analyses including well over

100,000 participants [131]. Indeed, the International Stroke Genetics Consortia has assembled over 60,000 stroke cases and mega-meta analyses conducted in early 2016 (unpublished) have resulted in significant novel hits and replication of previously reported associations. The future for stroke genetics is indeed enticing.

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4. The Genetics of Cerebral Aneurysms and Other Vascular Malformations

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Introduction

There are several types of structural vascular abnormalities of the blood vessels supplying the brain. The most common among the general population—in approximate order of descending frequency—include developmental venous anomalies, intracranial aneurysms, arteriovenous malformations, capillary telangiectasias, and cavernous malformations. Developmental venous anomalies and capillary telangiectasias are typically benign and often found incidentally either radiographically or pathologically. The remaining three have a greater tendency to cause neurologic sequelae such as seizures, headaches, or intracranial hemorrhage. In this chapter, we review the growing

evidence supporting a genetic influence on the occurrence of aneurysms and vascular malformations, which has been aided with recent advances in molecular biological techniques, gene expression profiling, and large multicenter collaborative studies.

Intracranial Aneurysms

Saccular aneurysms are the most common type of intracranial aneurysms (IA). They are berry-like protuberances that arise most commonly at arterial bifurcations in the circle of Willis. Other less common aneurysmal types include blister aneurysms, which are outpouchings that typically occur at non-branch points and fusiform aneurysms, which are longitudinally dilated areas of vessel wall associated with atherosclerotic disease or dissections. For the purposes of this chapter we will be focusing on the genetics of saccular aneurysms.

Epidemiology and Prevalence

The prevalence of intracranial aneurysms in the general population is estimated to be anywhere from 1% to 10%, depending on the population being studied and the method of screening used [1–5]. Aneurysmal subarachnoid hemorrhage (SAH), the cause of 80% of non-traumatic subarachnoid hemorrhages, occurs in a minority of patients with intracranial aneurysms.

Risk factors for intracranial aneurysms include older age, cigarette smoking, arterial hypertension, binge alcohol drinking [6–10], female sex (3:1 ratio) [3, 5, 11], Finnish or Japanese ethnicity [12], positive family history (2 + members with brain aneurysms), and family history of polycystic kidney disease [5].

Genetics of Sporadic Aneurysms

There have been several genetic linkage and association studies performed with the aim to find genetic loci associated with intracranial aneurysm formation and rupture. Several genome-wide association studies have replicated significant associations in endothelin receptor A (EDNRA) and cyclin-dependent kinase inhibitor 2BAS (CDKN2BAS) on chromosome 4 and 9 respectively with IA in the Japanese population [13–15]. Other studies

have also shown associations in chromosome 7 near HDAC9 [14] and in SOX17 at chromosome 8 [15, 16].

Over the last decade there have been several studies showing significant genetic polymorphisms with mixed results. There have been two recent meta-analyses that found that the eNOS gene T786C polymorphism showed a significant association with intracranial aneurysms [17, 18]. However, there have been some conflicting results regarding the IL-6 gene G572C and G174C polymorphisms [17, 19].

Unfortunately, despite several genetic linkage and candidate gene studies over the last few years showing a potential association with IA formation, there are still no diagnostic tests to assess genetic risk for IA formation and rupture in patients with a family history of IA or SAH. However, current practice guidelines recommend screening for unruptured IA with CTA or MRA in those with more than one first-degree relative with IA or SAH, and state that it may not be inappropriate to consider screening those with a first-degree relative with a SAH, although this remains controversial [20].

Familial (Non-hereditary) Intracranial Aneurysms

The overall risk of having a relative with IA has been investigated in several studies. In a case-control, population-based study in Rochester, Minnesota, patients with aneurysmal SAH over a 19-year period were studied, and 20% of patients had a first- or second-degree relative with aneurysmal SAH [21]. Meanwhile, another study from an isolated area of Quebec compared 533 individuals with IA to 1599 controls [22]. Forty-eight patients (9%) had a first-degree relative with IA (parent-child or sibling relationships) compared to 1.9% of controls. In general, first-degree family members of those with intracranial aneurysms have an increased risk of having an IA compared to the general population, with studies showing a prevalence of 4–9% [23, 24] (Fig. 4.1).

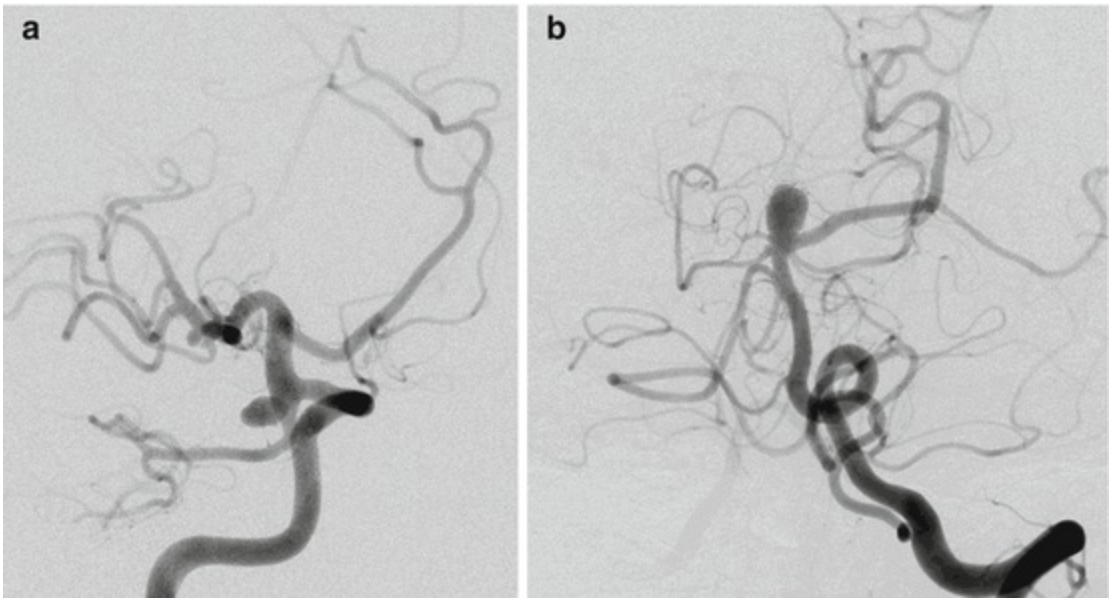

Fig. 4.1 Cerebral catheter angiogram of a patient with familial intracranial aneurysms. A 58-year-old man who is a current smoker with a family history of aneurysmal subarachnoid hemorrhage in two sisters underwent a screening MRA followed by a cerebral catheter angiography demonstrating (**a**) 7 mm right posterior communicating artery aneurysm, a 3 mm right MCA bifurcation aneurysm and (**b**) an 8-mm basilar bifurcation aneurysm

The Familial Intracranial Aneurysm study (FIA) has formed much of our understanding of the heritability and genetics of IA formation. Patients with familial IAs in the FIA were defined as individuals with at least three family members with an IA or an affected sibling pair in the absence of ADPKD, Ehlers-Danlos syndrome, Marfan syndrome or moyamoya. The first large whole-genome linkage study in familial IAs (including 192 families) detected possible evidence of linkage to four regions: chromosomes 4, 7, 8, and 12 [25]. The overall data suggest that there is no single gene exerting a large effect in familial IA formation, but rather the risk seems to be mediated through the action of multiple loci [25].

Familial aneurysms may be at greater risk of rupture than sporadic aneurysms and tend to rupture at a smaller size and younger age than sporadic aneurysms [23]. A recent study suggested that those with an anterior circulation aneurysm ≤6 mm with a positive family history had a 17-fold increased risk of aneurysmal rupture compared to patients in the International Study of Unruptured Intracranial Aneurysm (ISUIA) [26, 27]. However, the number of patients being followed was small, and only two hemorrhages occurred, leading to large confidence intervals in the estimated risk of hemorrhage. Additionally, in a population-based, case-control study, the odds

ratio (OR) for SAH was 2.5 in non-smokers with a positive family history (first-degree relative affected with SAH) compared to non-smokers with a negative family history [28]. Cigarette smoking appears to compound this risk, as the OR for smokers with a positive family history was 6.4 compared to 3.1 in smokers with a negative family history.

Intracranial Aneurysms in Hereditary Disorders

There are several hereditary diseases associated with intracranial aneurysms such as connective tissue diseases, neurocutaneous disorders (i.e. Neurofibromatosis I [NF1] and tuberous sclerosis complex [TSC]), and autosomal dominant polycystic kidney disease (ADPKD).

Autosomal Dominant Polycystic Kidney Disease

Epidemiology

ADPKD is an autosomal dominant condition due to genetic mutations in PKD1 and 2 on chromosomes 16 and 4, respectively. These patients have multiple renal cysts that may lead to renal failure, generally during adulthood. These patients are affected by cysts in other locations as well including the liver and pancreas. Population-based studies of ADPKD patients demonstrate a prevalence of IA of 10%, with the prevalence being double in those with a family history of aneurysm [5] (Fig. 4.2).

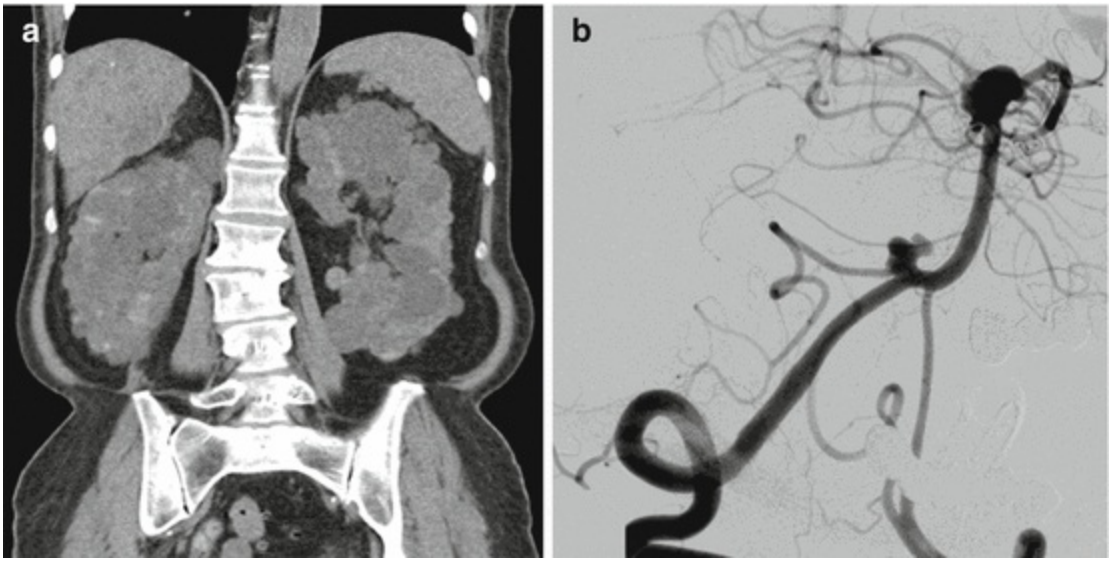

Fig. 4.2 A 65 year old female with a history of ADPKD underwent screening MRA followed by cerebral catheter angiography. CT abdomen/pelvis shows (**a**) large kidneys with multiple renal cysts.

Cerebral angiogram shows (**b**) a large multilobed 6-mm right posterior inferior cerebellar aneurysm and a 9-mm basilar apex aneurysm

Genetics

The mechanism by which PKD mutations result in formation of IAs is unclear. The PKD genes code for polycystins. Polycystins play an important role in the maintenance of the vascular system and are thought to play a role in cellular response to shear stress. Some studies have suggested that endothelial cells that lack normal polycystic function are defective in their release of nitric oxide. This failure to react normally to wall shear stress is thought to predispose these patients to aneurysm formation [29]. Some authors advocate a two hit hypothesis by which aneurysm formation occurs as a result of local somatic loss of the wild-type PKD gene in the intracranial vasculature. Meanwhile, others argue for the haploinsufficiency model, which states that patients need to have two normal PKD alleles in order to maintain normal homeostatic functions. Both of these theories are extremely difficult to prove, however.

Screening

The question of screening patients with ADPKD for IAs is controversial. Current guidelines recommend screening patients with ADPKD, particularly if there is a family history of IA [20]. Some experts recommend initially screening patients with ADPKD only if they are undergoing major elective surgery with potential hemodynamic instability [30, 31]. Others suggest that screening should be performed in all ADPKD patients with follow-up MRAs at 2- to 10-year intervals depending on various patient risk factors [32]. Some centers screen all ADPKD patients prior to kidney transplantation. It is important to point out, however, that the average age of rupture of intracranial aneurysms in the ADPKD population is 40 years, while the age of transplantation for most patients is 55 years. This suggests that there may be a limited benefit in pre-transplant screening [29].

Neurocutaneous Syndromes

There are some data suggesting that neurocutaneous disorders such as NF1 and TSC can be associated with IAs. TSC is a neurocutaneous autosomal dominant disorder due to genetic mutations in TSC1 and 2 genes. While IAs have been reported in association with this disorder, no large case-control screening study has been performed to date demonstrating a higher

prevalence of aneurysm in the tuberous sclerosis population when compared to controls [33–35]. In one retrospective study of over 400 tuberous sclerosis patients, the prevalence of IAs was 0.74% compared to 0.35% in controls, and the difference was not statistically significant. It is important to point out however that this prevalence was based on reviews of MRI brain evaluation and not MRA. MRA is known to have a significantly higher sensitivity in detection of IAs than MRI. Furthermore, the mean age of the patients included in this study was 25 years, and it is known that aneurysm prevalence increases with age [36].

A number of theories exist to explain the genetic basis for formation of aneurysms in tuberous sclerosis patients. Some authors have postulated that because the TSC 2 gene is near the PKD1 gene on chromosome 16p13.3, a potential deletion of intervening genetic material could result in a contiguous gene syndrome [34, 35]. Meanwhile, other studies have suggested that aneurysm formation is the result of disruption of the mTOR pathway, which plays a role in the differentiation and function of smooth muscle cells.

NF1, formally known as Von Recklinghausen disease, is an autosomal dominant disease due to mutation of the NF1 gene on chromosome 17. NF-1 codes for neurofibromin, which is expressed on endothelial and smooth muscle cell layers of blood vessels and is postulated to result in vascular pathology if there is reduced expressivity. These patients often present with café-au-lait macules and axillary and/or inguinal freckling, they may also have neurofibromas or optic gliomas and Lisch nodules. Patients with NF1 can present with a variety of vascular phenotypes, including intracranial aneurysms, AV fistulae (i.e. vertebro-vertebral AVFs), moyamoya disease and arteriovenous malformations [37, 38]. No prospective screening studies using angiographic imaging have been performed to date in the NF1 population. However, retrospective studies suggest that the prevalence of IAs in NF1 patients is about 10% [38]. There are no current aneurysm screening guidelines for NF1 patients.

Connective Tissue Diseases

There are several connective tissue diseases that are associated with IAs. All of these disorders result in weakened connective tissue in the media of the arterial wall, thus predisposing them to a wide range of intracranial and systemic arteriopathies, including dissections, aneurysms and stenoses. Connective tissue diseases associated with IAs include Marfan syndrome,

Ehlers-Danlos Syndrome and Loeys-Dietz Syndrome.

Marfan syndrome is autosomal dominant connective tissue disease disorder due to a mutation in Fibrillin 1 (FBN1) gene. Fibrillin 1 is a glycoprotein involved in microfibril formation. It is characterized by: hypermobile joints; *ectopia lentis*; arachnodactyly; aortic root dilatation or dissection; and mitral valve prolapse. There is also a propensity to form IAs that can be saccular but also fusiform and dissecting as well [38, 39]. Prevalence estimates for IAs in the Marfan population are about 10% [38]. However, no large prospective screening study has been performed.

Ehlers Danlos (EDS) type IV, also known as vascular EDS, is due to a genetic mutation of COL3A1 on chromosome 2 that codes for type III procollagen. These patients typically have hypermobile joints, easy bruising and diminished facial subcutaneous fat. IAs and cerebral and cervical arterial dissections are potential arterial findings. Arterial dolichoectasias have also been described [38]. Prevalence estimates for IAs in the Ehlers-Danlos population are about 10% [38]. However, no large prospective screening study has been performed.

Loeys-Dietz syndrome (LDS) is an autosomal dominant connective tissue disorder due to four known genetic loss of function mutations in transforming growth factor B receptor 1 and II (TGFBR1/2), and ligand 2 (TGFB2), and decapentaplegic homolog 3 (SMAD 2) which are all involved in the transforming growth factor B signaling cascade. It is unclear how these contribute to aortic and cerebral aneurysm formation. However, the incidence of intracranial aneurysms has been reported to be 10–32% [38].

Other Diseases

There are numerous other conditions that have been reported to be associated with intracranial aneurysms including: pseudoxanthoma elasticum (PXE); glucocorticoid-remediable aldosteronism (GRA), also known as familial hyperaldosteronism type I; bicuspid aortic valve; microcephalic osteodysplastic primordial dwarfism; and arterial tortuosity syndrome [40, 41]. Some current practice guidelines recommend screening for aneurysms in patients with any hereditary disorders associated with increased risk of IA occurrence and a family history of IA or subarachnoid hemorrhage (i.e., ADPCKD, Ehlers Danlos type IV, microcephalic osteodysplastic primordial dwarfism, aortic coarctation, or bicuspid aortic valve) [20]. A summary of the abovementioned disorders is provided in Table 4.1.

Table 4.1 Heritable disorders associated with intracranial aneurysm

Hereditary disease	OMIM	Inheritance pattern	Gene	Phenotype/clinical symptoms
Autosomal dominant polycystic kidney disease	601313 613095	AD	PKD1/2	Multiple renal cysts, hepatic and pancreas cysts, colonic diverticula, aortic dilatation/dissection, cardiac valvular disease, inguinal hernias.
Marfan syndrome	154700	AD	Fibrillin 1 (FBN1)	Tall stature with long extremities, hindfoot deformity, scoliosis, arachnodactyly, pectus carinatum, pneumothorax, ectopia lentis, aortic root aneurysm/dissection, mitral valve prolapse, carotid artery dissection, cerebral and cervical arterial dissections.
Ehlers-Danlos type IV	130050	AD	COL3A1	Diminished facial subcutaneous fat, easy bruising, hypermobile joints, skin laxity and atrophic skin scarring, rupture of uterus and colon, cerebral and cervical arterial dissections.
Loeys-Dietz syndrome Type I/II	609192 610168	AD	TGFBR1/2, SMAD3, TGFB2, SKI	Marfanoid habitus, craniosynostosis, scoliosis, pectus carinatum or excavatum, hypertelorism, cleft palate, bifid uvula, generalized arterial tortuosity, cerebral and cervical arterial aneurysms.
Microcephalic osteodysplastic primordial dwarfism, type II	210720	AR	MOPD2	Mental retardation (in some), microcephaly, short stature, intrauterine growth retardation, brachy-clinodactyly, facial dimorphisms, scoliosis, hip dysplasia, subglottic stenosis, moyamoya angiopathy
Pseudoxanthoma elasticum (PXE)	264800	AR	ABCC6	Yellowish skin papules or plaques in flexor areas, oblique mental creases, retinopathy, coronary and peripheral artery disease, gastrointestinal bleeding.
PXE-like syndrome	610842	AR	GGCX	Generalized cutis laxa with leathery skin folds, mild retinopathy, coagulation disorder
Tuberous sclerosis	191100 613254	AD	TSC1/2	Facial angiofibroma, subependymal nodule, renal angiomyolipoma, cardiac rhabdomyoma, subependymal giant cell astrocytoma, shagreen patch, retinal hamartoma.
Neurofibromatosis type I	162200	AD	NF-1	Café-au-lait macules, neurofibroma, Axillary or inguinal freckles, optic glioma, Lisch nodules, aqueductal stenosis, pheochromocytoma, renal artery stenosis
Glucocorticoid-remediable aldosteronism	103900	AD	Fusion of CYP11B1 and	Childhood-early adulthood hypertension, adrenal hyperplasia

(GRA) aka. Familial hyperaldosteronism type I			CYP11B2	
Bicuspid aortic valve Disease	109730	AD	NOTCH1	Bicuspid aortic valve, aortic dilatation/dissection, dissection of carotid and cerebral arteries.
Arterial tortuosity syndrome	208050	AR	SLC2A10	Facial dimorphisms, arachnodactyly, joint and skin laxity, pectus excavatum and carinatum, ventricular hypertrophy, arterial elongation, tortuosity and aneurysms, gastric hernias

AD autosomal dominant, *AR* autosomal recessive

Arteriovenous Malformations

Arteriovenous malformations (AVM) are high-flow vascular abnormalities involving a shunt with direct connection of arteries to veins, bypassing intervening capillary bed. These include nidal-AVMs that involve a tangle or nidus of multiple “feeding” arteries to multiple draining veins and arteriovenous fistulas (AVFs) characterized by a fistula of a single artery-vein or venous sinus. Micro-AVMs (also known as capillary vascular malformations) have a nidus of <1 cm and a single feeding artery and draining vein [42].

Epidemiology and Natural History

AVMs are the most frequently detected symptomatic vascular malformations, accounting for 2% of all strokes [43, 44]. The prevalence of brain AVMs is estimated at 0.2–0.5% of the general population [44–46]. The detection rate in a population-based study in Olmsted County, Minnesota, was found to be 1.11/100,000 persons [45], similar to the detection rate in the New York Islands AVM Study of 1.34/100,000 person-years [47]. Detection rates in other population-based studies have ranged from 0.56 to 0.89/100,000 person-years [48, 49]. These lesions tend to affect young patients (20–40 years old) with equal sex predominance [50]. However, because they are rare and most affected individuals are likely asymptomatic, the precise prevalence is unknown [51].

Most AVM-associated hemorrhages are intraparenchymal [52]. However, AVMs account for up to 10% of non-traumatic subarachnoid hemorrhages

[53, 54]. The rupture rate of symptomatic brain AVMs is 1–4% per year [55–58]. For lesions that have already bled, recurrent bleeding risk doubles in the first year, then decreases to a baseline rate of 1–2% per year [46, 59–61].

Etiology and Pathogenesis

The etiology of AVM formation is unknown and continues to be debated. In general, these lesions are thought to be congenital or related to an embryonic defect attributable to a persistent primitive arteriovenous connection [62, 63]. However, this theory is being challenged because, aside from the presence of vein of Galen malformations, *in utero* AVMs have not been reported, and because there are multiple case reports of *de novo* AVM formation [64–66]. More likely, AVMs form for multiple reasons consistent with the "multiple-hit hypothesis" that genetic predisposition and acquired environment factors (e.g., cerebral ischemia, venous thrombosis, hemorrhagic event, trauma, and infection) work together to cause an angiogenic reaction [67].

Sporadic AVM Genetics

Most cerebral AVMs are sporadic, non-familial and not attributable to a single genetic locus. However, there has been a substantial amount of research aimed at identifying various single nucleotide polymorphisms (SNPs) associated with sporadic brain AVM formation. Patients with sporadic brain AVMs are more likely to have SNPs in genes involved in inflammation, embryonic and post-natal angiogenesis and tissue remodeling and repair [68, 69]. While it is unclear how these SNPs affect gene function and what their exact role is in AVM formation, they strongly suggest aberrant angiogenesis and inflammation contributing to AVM pathogenesis. A summary of the SNPs associated with brain AVMs is provided in Table 4.2.

Table 4.2 Sporadic mutations and SNPs associated with AVMs

SNP	Gene	Gene product and function
rs1143627	IL-1B	Interleukin-1, involved in inflammation. Produced mainly by monocytes
rs1800795	IL-6	Inflammatory cytokine produced by endothelium.
rs16944	IL-1B	Interleukin-1, involved in inflammation. Produced mainly by monocytes
rs3025010	VEGFA	Vascular endothelial growth factor. Induces angiogenesis in vivo
rs7015566	GPR124	G Protein-Coupled Receptor 124. Important in CNS-specific angiogenesis. Over-expression results in increased endothelial sprouting and migration

rs522616	MMP-3	Matrix metalloproteinase-3. Produced by connective tissue cells. Involved in wound repair and tissue remodeling.
rs11672433	ANGPTL4	Angiopoietin-Like 4. Vascular growth factor plays role in embryonic and post-natal angiogenesis.

SNP single nucleotide polymorphism

Genetic Syndromes Associated with Brain AVMs

While most AVMs are sporadic, there are multiple genetic syndromes associated with an increased prevalence of brain AVMs. Many of these syndromes are associated with various distinguishing extra-CNS clinical manifestations (Table 4.3).

Table 4.3 AVM-associated syndromes

Syndrome	OMIM#	Inheritance pattern	Gene product	Mutated gene
HHT1	187300	AD	Endoglin	ENG
HHT2	600376		Activin receptor-like kinase 1	ACVRL/ALK1
Juvenile Polyposis-HHT Overlap	175050		SMAD4 protein	SMAD4
Capillary Malformation-Arteriovenous Malformation	608354	AD/Sporadic in 30%	RAS p21 protein activator	RASA1
Parkes Weber Syndrome	139150	AD/Sporadic	RAS p21 protein activator	RASA1

HHT hereditary hemorrhagic telangiectasia, *AD* autosomal dominant

Hereditary Hemorrhagic Telangiectasia

HHT is the most common of the AVM-associated syndromes affecting anywhere from 1:5000 to 1:10,000 people worldwide. HHT is diagnosed clinically using the Curacao criteria [70]. The four Curacao criteria are: (1) spontaneous/recurrent epistaxis, (2) mucocutaneous telangiectasias, (3) visceral AVM's (including brain AVMs, pulmonary AVMs and gastrointestinal AVMs), and (4), diagnosis of HHT in a first-degree relative. When patients meet three or more of the four criteria they have "definite HHT," while those with two of the four criteria have "possible" or "suspected" HHT [71]. HHT commonly manifests with epistaxis, gastrointestinal bleeding, and pulmonary AVMs.

There are at least five different genes responsible for HHT; however, the most common and the most well-characterized are HHT1 and HHT2. About 10–20% of patients with HHT have a brain AVM, with a higher prevalence in HHT1 patients [72]. HHT1 is caused by mutations in the ENG gene, which codes for endoglin. Endoglin plays a role in the transforming growth factor-beta complex and is essential to the development and regulation of human vascular endothelium. HHT2 is caused by mutations in the ACVRL1 gene, which codes for an activating receptor-like kinase. This protein is involved in the TGF-beta system and regulates vasculogenesis and inflammation. Although HHT has a strong genetic basis for AVM formation, there is phenotypic heterogeneity within the same family, suggesting epigenetic factors that would support a "multiple hit" hypothesis in AVM formation in HHT.

HHT-associated AVMs are classified as (1) large single-hole pial arteriovenous fistulae (AVF), (2) nidal-type arteriovenous malformations and (3) micro-AVMs or capillary vascular malformation [73]. The large AVMs/AVFs are more frequently symptomatic at an early age and thus typically manifest in young children while small AVMs are typically discovered in older age groups [73]. AVFs represent about 10% of cerebral vascular malformations while nidus-type AVMs represent about 70% of cerebral vascular malformations [73]. Approximately 30–40% of patients with cerebral vascular malformations have micro-AVMs/capillary vascular malformations. When compared to nidal AVMs in the sporadic AVM population, nidal AVMs in HHT patients are generally smaller, more commonly located in non-eloquent areas, and tend to have superficial drainage [74–76]. AVM features that should make one consider a diagnosis of HHT include (1) AVM multiplicity, (2) the presence of a large pial AVF and (3) the presence of a brain AVM in a patient with a history of epistaxis, GI bleeding or mucocutaneous vascular lesions (Fig. 4.3).

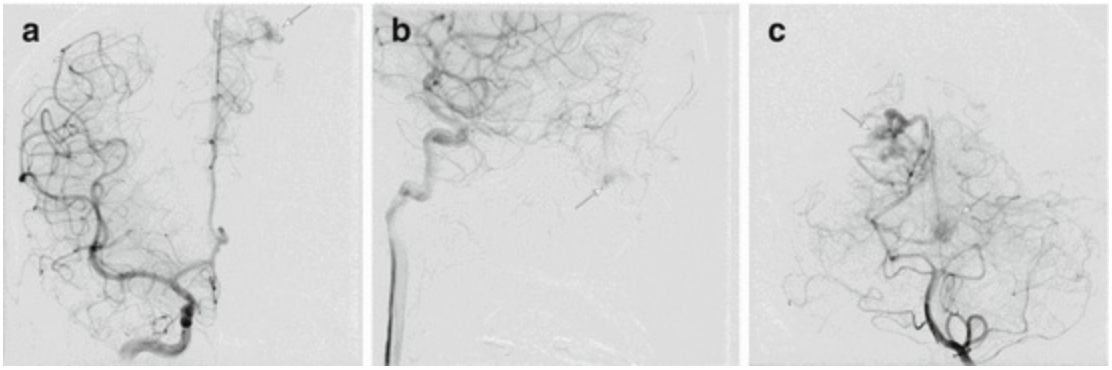

Fig. 4.3 Cerebral catheter angiogram of a 30-year-old female with HHT, and family history of ENG mutation of HHT presented with recurrent epistaxis. Right internal carotid angiogram shows (**a**) cross filling of left anterior cerebral artery filling a parasagittal left posterior frontal AVM (*arrow*). Left internal carotid angiogram demonstrates (**b**) a superior nasal ethmoidal telangiectasia, which is a common source of epistaxis (*arrow*). Left vertebral angiogram demonstrates (**c**) two AVMs, one is located in the right occipital lobe posterior to the splenium and draining into dilated veins and then into the vein of Galen (*superior arrow*) and the other is located in the midbrain (*inferior arrow*) and draining into the right basal vein of Rosenthal and then the vein of Galen

Studies of natural history of cerebral AVMs in HHT patients are few in number and limited by the fact that the angiographic characteristics of the lesions are not generally well characterized in these studies. Willemse et al. found a bleeding risk of 0.4–0.7% for HHT-AVMs [74]. The Brain Vascular Malformation Consortium HHT Investigator Group found an overall rate of bleeding of 1% per year with a rate of rupture of 0.4% per year (95%CI = 0.1–1.7%) for unruptured HHT-AVMs and 10% per year for ruptured HHT-AVMs (95%CI = 3.3–31.2%). Capillary vascular malformations have a benign prognosis, with no reports of hemorrhage or growth of these lesions [73, 77, 78]. Micro-AVMs are more abundant in HHT patients than in patients with sporadic AVMs [42, 78].

The most recent HHT International Foundation 2011 guidelines recommend screening children and adults with possible or definite HHT for cerebral AVMs. For children, the recommendation is to perform an unenhanced MRI in the first 6 months of life or at the time of diagnosis. Adults should be screened with an MRI with and without contrast. If the initial MRI is negative there is no indication for further or repeat testing [79].

Capillary Malformation-Arteriovenous Malformation Syndrome

CM-AVM syndrome is one of many RAS-opathies secondary to mutations in genes involved in the Ras/MAPK pathway. Mutations in the RASA1 gene encoding the p120-RasGAP protein are responsible for this syndrome. This protein is involved in cellular signaling for differentiation, proliferation, and migration. Many malignancies have somatic mutations of the Ras oncogene, which accounts for the higher rate of malignancy seen in patients with CM-AVM syndrome [80].

Like HHT, CM-AVM syndrome is an autosomally dominant inherited disorder. The prevalence of RASA1-related syndromes is estimated at 1:100,000 [81]. Patients are affected by multifocal capillary malformations, and AVMs and AVFs affecting multiple tissues including the brain [82]. Ten percent of patients with CM-AVM syndrome suffer from various tumors including optic gliomas, superficial basal cell carcinoma, neurofibromas, and vestibular schwannomas [80, 83]. About 10% of patients with RASA1 mutations have brain AVMs, 15% have AVMs in other vascular beds and nearly 100% have capillary malformations [83]. In addition, about 10% of patients suffer from spinal AVMs [83].

The key feature distinguishing CM-AVM syndrome patients from those with sporadic AVMs is the presence of skin vascular malformations. However, since these vascular malformations are similar to the cutaneous lesions seen in HHT patients, dermatological evaluation of these patients is generally recommended. Some authors advocate for genetic testing of patients who present with both intracranial/facial AVMs and cutaneous capillary malformations. While no evidence-based recommendation regarding screening for brain AVMs has been published, many experts believe that brain AVM screening is important in patients with known CM-AVM syndrome or a family history of the disease [84–86].

Parkes Weber Syndrome

Parkes Weber is RASA1 mutations, similar to CM-AVM syndrome [87]. PWS can be sporadic or inherited in an autosomal dominant fashion. PWS patients present with cutaneous capillary malformations associated with underlying multiple AVFs and limb hypertrophy [88]. AVM in PWS are most commonly located in the lower extremities [89].

While, the association is commonly reported in the literature, the exact proportion of PWS patients with brain AVMs is unknown. AVMs seen in PWS are similar to those seen in patients with CM-AVM syndrome because

the mutated gene is often the same (RASA1) [81, 90, 91]. In addition these patients can present with spinal vascular malformations [92, 93].

There are no evidence-based recommendations for management of these patients. In general, PWS patients should be managed at highly specialized centers because they require high-level multidisciplinary care in managing their multiple malformations. Many advocate screening these patients with a brain MRI for an intracranial vascular malformation at least once during the patient's lifetime. RASA1 genetic testing is generally recommended.

PTEN Mutations

PTEN mutations have also been associated with high flow vascular malformations including AVMs. This gene is located on chromosome 10q23.3 and codes for a tumor suppressor protein that is responsible for cell growth, proliferation and angiogenesis via the phosphoinositide-3 kinase (PI3K) pathway. This genetic mutation has been found in multiple syndromes with reported AVM lesions such as Cowden, Bannayan–Riley–Ruvalcaba syndrome and 'Proteus-like' Syndrome [94, 95] and exhibits variable expressivity and reduced penetrance. No definite AVM screening recommendations have been published for families and patients with these mutations.

Other AVM Syndromes

There are several less common syndromes associated with AVMs, such as CLOVES, Wyburn-Mason syndrome, and Klippel-Trenaunay syndrome. These are typically sporadic. CLOVES is an acronym for congenital lipomatous tissue overgrowth, vascular malformation, epidermal nevi, and skeletal/spinal abnormalities. It is associated with spinal high-flow vascular malformations. A somatic genetic mutation in PIK3CA has been identified and is responsible for regulating cell growth and proliferation [96, 97].

Klippel-Trenaunay is similar to Parkes-Weber syndrome in that it is associated with limb hypertrophy. Other characteristics are port wine stain, varicose veins and slow-flow vascular malformations. However, there have been rare reports of spinal AVMs with this syndrome [98, 99]. Upregulation of VG5Q angiogenic factor (previously known as AGGF1) has been discovered in association with this syndrome [100, 101] but no clear genetic mutation has been identified yet.

Wyburn-Mason syndrome is also known as Bonnet-Dechaume-Blanc syndrome or retinoencephalofacial angiomatosis. It is characterized by facial vascular nevi, retinal, optic pathway AVMs, and intracranial AVMs. No known genetic abnormalities have been discovered.

Cavernous Malformations

Histopathological Characteristics

CMs are well circumscribed, multi-lobulated, angiographically occult vascular malformations (Fig. 4.4). Histologically, they are comprised of sinusoidal leaky capillary-like channels (caverns) separated by collagenous stroma lacking elastin, smooth muscle, and tight junctions. CMs characteristically lack intervening brain parenchyma (Fig. 4.5). However, the surrounding parenchyma often shows evidence of prior microhemorrhages with hemosiderin discoloration and hemosiderin-filled macrophages. A surrounding gliomatous reaction may form a capsule around the lesion.

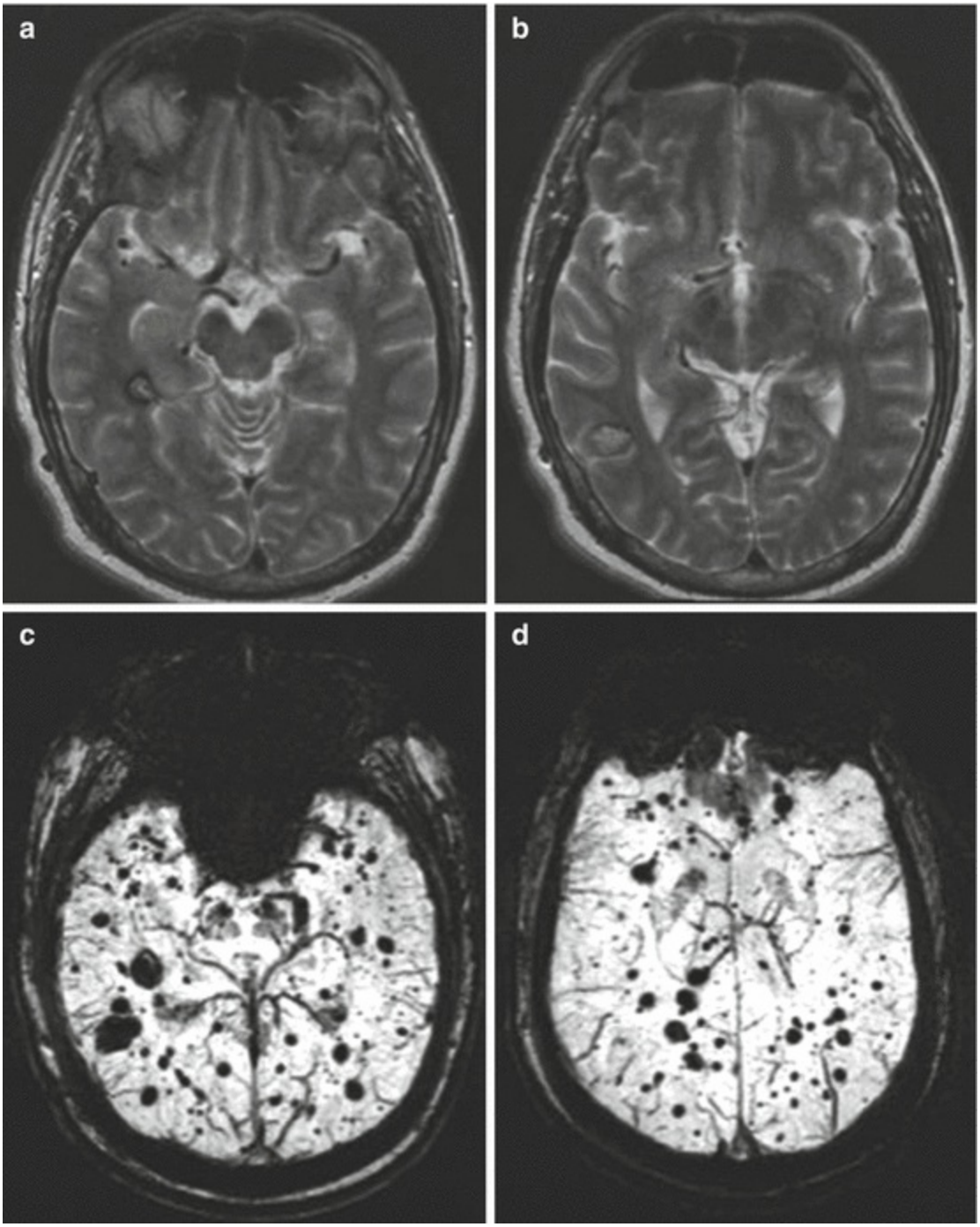

Fig. 4.4 MRI findings in familial cerebral cavernous malformation syndrome. A 57-year-old man with a family history of cavernous malformation (an affected son) presented with a seizure. Axial T2-weighted brain MRI images show (**a**, **b**) two cavernous malformations with characteristic lobulated appearance of mixed signal intensities surrounded by a rim of hypointense signal. (**c**, **d**) Innumerable foci of abnormal hemosiderin, consistent with prior microhemorrhages, are shown on axial susceptibility-weighted MRI sequences

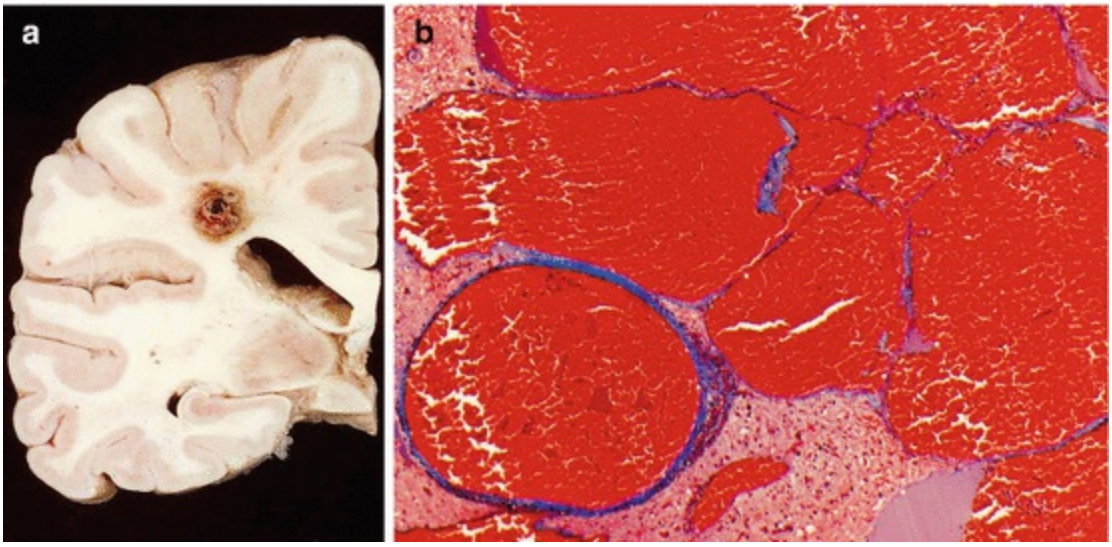

Fig. 4.5 Gross coronal brain section (**a**) showing a well circumscribed multi-lobulated cavernous malformation surrounded by hemosiderin stained tissue. Trichome stain at high power magnification (**b**) shows irregular vascular channels lined by one layer of endothelium without intervening neuronal tissue

Epidemiology and Natural History

Cavernous malformations (CMs) account for about 1–5% of all cerebral vascular malformations [102, 103]. Their prevalence in the general population is around 0.5–1% based on radiographic and autopsy studies [104–108]. While most CMs are benign, asymptomatic lesions, they can present with headache, seizure and hemorrhage. In general, studies on the natural history of CMs demonstrate a risk of hemorrhage of 2–4% per year [52, 109, 110] which increases to 4–60% per year after the first hemorrhage [111–115]. Factors that are predictive of future hemorrhage include brainstem location, prior history of cavernous malformation hemorrhage, and a focal neurologic deficit. Age, sex and multiplicity have not been found to be risk factors for future hemorrhage [116].

Hereditary Versus Sporadic Cavernous Malformations

Most CMs are sporadic, but hereditary forms exist [103]. In general, hereditary CMs are transmitted in an autosomal dominant fashion. About 10–20% of Caucasians have a familial form of CM compared to 50% of Hispanics [117]. Patients with the familial forms of CM generally have a similar age at presentation when compared to those with sporadic CMs [117,

118].

There are some key differences in the characteristics of hereditary and sporadic CMs. As mentioned previously, there is an increased prevalence of familial CMs among Hispanic patients. In addition, there is a tendency for familial CMs to be multiple rather than solitary [103, 108, 117, 119]. While developmental venous anomalies are commonly associated with CM in the sporadic form, they are seen less often among those with familial CMs [120].

Pathogenesis of Cavernous Malformations

While CMs were originally thought to be congenital, cases of *de novo* formation have been documented, arguing against this theory [108, 121–123]. *De novo* formation has been documented in association with radiation therapy [124, 125], along the path of stereotactic biopsy [126], and in association with development venous anomalies [127].

Whether the genetic mechanisms by which these CMs occur are via the two-hit hypothesis or haploinsufficiency is debated. The two-hit hypothesis was originally suggested as a potential mechanism in the genetics of CCMs because most sporadic cases have single lesions, whereas most familial cases have multiple lesions. This is supported by the fact that a CCM1 gene knockout alone does not result in CM formation—rather a second hit is required for the expression of a cavernous malformation [128].

Haploinsufficiency is a condition that arises when a normal phenotype requires the product of both alleles and a reduction of 50% gene function results in an abnormal phenotype. Haploinsufficiency can result from a deletion or a mutation of one of two copies of a gene. CM lesions, particularly CCM3 mutations, may be the consequence of haploinsufficiency [129–131].

Familial Cavernous Malformation Syndromes

There are three known genotypes of familial CMs, identified as CCM1-3. These are mostly a result of loss of function mutations (Table 4.4). CCM1 mutations account for over half of the familial forms of CMs, while CCM 2 and 3 are less common [132]. There is variable penetrance amongst the different genetic loci with full penetrance in CCM2, 62% penetrance in CCM3, and 60–88% penetrance in CCM1 [133, 134].

Table 4.4 Summary of genetic cavernous malformation syndromes

	OMIM	Locus	Gene	Phenotype
CCM1	116860	7q11–22	KRIT-1/KREV1 interaction trapped 1	Common in Hispanic population.
CCM2	603284	7p13	MGC4607/malcavernin	
CCM3	603285	3q25.2-q27	PDCD10/Programmed cell death 10	Presents early in childhood. Associated with increased risk of ICH.

The CCM1 locus encodes for KRIT1 protein, a microtubule associated protein. It has been mapped to chromosome 7q11.2-q21 [128, 135, 136] and accounts for over half of familial CCMs [132, 137, 138]. The KRIT1 protein has been found in vascular endothelium, astrocytes, and pyramidal cells within the brain [117]. The amino terminal of the KRIT1 protein binds to ICAP1α (alpha) and may be involved in the integrin signaling pathway, cell adhesion and migration. The KRIT1 protein also co-localizes with beta tubulin in endothelial cells and is thought to interact with the cytoskeleton. It has been hypothesized that this link may result in impaired endothelial cell junctions during a critical phase of angiogenesis thus resulting in the dilated, leaky capillaries that are characteristic of these lesions [136].

Mutations of the CCM2 gene are located on chromosome 7p and are responsible for 10–20% of mutations. In addition, large deletions in the CCM2 gene have also been shown to be association with familial CMs [132, 134, 137–139]. The CCM2 gene encodes for a protein (malcaverin or MCG4607) that interacts with KRIT1 and may influence the p38 MAPK pathway, an intracellular kinase that transduces signals necessary for vascular remodeling and maturation [117].

The CCM3 locus has been mapped to chromosome 3q25.2-q27 and is responsible for around 10–22% of mutations in familial forms of CM, which is a lower representation than previously reported linkage studies [132, 134, 137, 138, 140, 141]. The CCM3 gene encodes for a protein (PDCD10), which has a role in apoptosis [128, 142], angiogenesis, endothelial cell integrity and endothelial cell survival [143, 144]. In a multicenter study of 163 families affected with familial CM, patients with CCM3 mutations had a higher risk of cerebral hemorrhage, particularly in childhood. In this study, cerebral hemorrhage was the initial manifestation in 53% of symptomatic CCM3 mutation carriers compared with 26% of CCM1 and 39% of CCM2 patients, but this was not statistically significant ($p = 0.07$). Among patients who presented with cerebral hemorrhage, CCM3 carriers were affected at earlier

ages. These data suggest that CCM3 mutation carriers are more prone to symptomatic hemorrhages in childhood than CCM1 or CCM2 carriers [132]. Since then two other recently published studies also confirmed early-onset cerebral hemorrhages in CCM3 as compared to other familial CCMs [145, 146]. Interestingly, CCMs is also associated with the presence of multiple meningiomas [146, 147].

Up to 15% of familial CCM patients do not have a known associated genetic mutation [131, 132, 137]. However, there have been several studies proposing the existence of a fourth unidentified locus near PDCD10, 3q26.3-27.2 [140, 148]. Another study proposed a genetic translocation of ZPLD1, between Chromosome 3q and X, near PDCD10 [148]. Although the exact pathophysiology of these mutations is unknown, the proteins associated with these genes are thought to act in the same pathways as the gene products of KRIT1, malcaverin, and PDCD10, the genes associated with CCM 1, 2 and 3 respectively [145, 149–151].

Indications for Genetic Testing and Imaging Screening

Genetic screening in this patient population is controversial. Genetic counseling should take place prior to any genetic screening. Genetic screening is generally discouraged in those with a single cavernous malformation, as the yield of finding a genetic mutation is low. One could consider genetic screening for counseling purposes in sporadic cases with multiple CCMs, but it otherwise would not change clinical management. It is important to note that a negative test does not exclude a genetic cause as there are likely more CCM genes that have not been discovered yet. The benefit of MRI in an asymptomatic individual with affected family members is unclear.

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5. Intracerebral Hemorrhage and Cerebral Amyloid Angiopathy

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Introduction

Intracerebral Hemorrhage: A Cerebral Small Vessel Disease

Intracerebral hemorrhage (ICH) is the acute manifestation of a chronic progressive disease of the cerebral vessels [1]. Rarely, the underlying vessel disease can be a vascular malformation. However, for patients over the age of 55 years, the overwhelming majority of ICH cases occur in the presence of cerebral small vessel disease [2]. ICH is routinely classified according to the region of the brain in which it occurs: the thalamus, basal ganglia, brainstem, cerebellum (“deep” or “nonlobar” ICH), or at the junction of the cortical gray

matter and subcortical white matter (“lobar” ICH). Pathological studies demonstrate that ICH location frequently correlates with different underlying small vessel diseases. For example, while chronic hypertension has long been recognized as the leading cause of deep ICH, cerebral amyloid angiopathy (CAA) has been recognized as a leading cause of lobar (ICH) [3–7].

The public health impact of ICH is substantial. It is the most fatal form of stroke, with a 90-day mortality rate between 30% and 50%. Furthermore, of those who survive, only 10–25% achieve functional independence [1, 8, 9]. Survivors are also at increased risk of recurrent ICH. At present, the only therapy demonstrated to reduce the risk of ICH is control of hypertension [10–12]. However, population-attributable risk estimates suggest that improved control of hypertension is unlikely to prevent more than one-third of all of ICH in the elderly [6]. Additional primary and secondary prevention measures are therefore urgently needed.

Genetic Variation and Risk of ICH

Multiple familial ICH syndromes, manifesting only in selected families with highly consistent phenotypes and a clear autosomal dominant inheritance pattern, have been described [7, 13]. Nonetheless, the vast majority of ICH in the general population occurs as a sporadic event, unaccompanied by an easily identifiable strong family history. In the case of genetically complex sporadic disorders, heritability estimation, which allows quantitative measurement of genetic contribution to disease risk, has traditionally been performed with twin studies. Unfortunately, no twin studies of ICH have been published to date. However, evidence from epidemiological studies consistently suggests that genetic variation plays a substantial role in the pathogenesis of sporadic ICH.

Familial aggregation—that is, increased disease risk among family members of ICH patients compared to the general population—points to a strong genetic contribution to ICH. An early study of 48 ICH patients in North Carolina found that a family history of ICH was present in 15% [14]. In their population-based case-control analysis of ICH genetic risk factors, the Greater Cincinnati/Northern Kentucky investigators found that having a first-degree relative with ICH was strongly associated with risk of ICH (odds ratio [OR] = 6.3) after adjustment for confounding variables, with the effect of family history equally strong in deep and lobar ICH subtypes [6]. Standardized incidence ratios allow comparison of disease incidence rates

between groups with and without a particular risk factor, in this case family history of disease. A population-based hospital discharge register in Sweden involving 7961 cases of first-hospitalized hemorrhagic stroke (subarachnoid hemorrhage and ICH) found that sibling history of hemorrhagic stroke more than doubled ICH incidence (standardized incidence ratio [SIR] = 2.14) [15]. Of particular interest, the effect of family history appeared to increase with age: there was no effect of sibling hemorrhage on hemorrhage risk among those <50 years of age, while risk was highest (SIR = 2.45) in the group aged 60–69. Finally, no effect of ICH history for spouses on stroke incidence was found: this supports the conclusion that genetic risk factors or (less likely) shared early environmental exposure contribute to risk of ICH, but not shared later environmental exposure. This is in contrast to ischemic stroke in the same study, where spousal ischemic stroke did increase SIR for ischemic stroke.

The advent of Genome Wide Association Studies (GWAS) has enabled investigators to use genome-wide genotype data for common variants (minor allele frequency [MAF] >5%) obtained from unrelated subjects with ICH and disease-free controls to estimate heritability using alternative strategies [16, 17]. Investigators within the International Stroke Genetics Consortium (ISGC) utilized these methodologies to provide heritability estimates for sporadic ICH risk. ICH risk heritability was estimated at 44% (standard error, 11%) in this study [18]. Of note, genetic variation at the APOE locus alone (see below) accounted for ~35% of genetic effects on sporadic ICH risk. While the precision of such estimates is limited by the small sample sizes available thus far, these results provide compelling evidence for a substantial contribution of genetic variation to risk of sporadic ICH.

This chapter will summarize identified genetic risk loci for both familial and sporadic ICH, focusing in particular on CAA-related ICH (CAA-ICH), both in its familial and sporadic forms. While many candidate gene studies of ICH have been published, the vast majority of reported positive findings have not been successfully replicated. In particular, no replication of previously reported candidate gene association studies has been reported by groups performing GWAS of ICH, suggesting that published positive results could be due to the known limitation of most candidate gene studies (e.g., small sample size and inability to account for confounding due to population structure). Recently, large collaborative GWAS studies have identified and replicated novel risk loci for sporadic ICH [19].

Cerebral Amyloid Angiopathy and Intracerebral Hemorrhage

CAA is characterized by β(beta)-amyloid peptide (Aβ[beta]) deposition and destruction of the vessel walls of capillaries, arterioles and small- and medium-sized arteries of the cerebral cortex, leptomeninges, and cerebellum [4, 7]. Vascular beta-amyloid is composed chiefly of a 39- to 43-amino acid proteolytic fragment of the β(beta)-amyloid precursor protein (APP). Pathological evidence of CAA, regardless of severity, was found in 10–40% of elderly brains at autopsy, with increasing prevalence with age. Prevalence increases to almost 80% for autopsy samples from patients with concomitant Alzheimer's disease (AD) [20]. Advanced CAA pathology (i.e., moderate or severe) increases with age as well: it was found in 2.3% of 65- to 74-year-olds, 8.0% of 75- to 84-year-olds, and 12.1% of subjects over 85 (samples from the Harvard Brain Tissue Resource Center, prevalence estimates corrected for overrepresentation of AD referrals) [21]. Prevalence of severe CAA has been reported to be as high as 20% in a series of autopsied individuals aged 85–86 [22]. CAA can be unaccompanied by clinical manifestations, but the effect of amyloid on vessel integrity and function contributes to a range of clinical consequences, including CAA-related ICH. CAA-related ICH accounts for between 15% and 40% of all nontraumatic ICH in the elderly and is associated with mortality rates of 30–50%, with <20% recovering functional independence [4, 7].

Genetically, CAA is classified as either sporadic or familial. In the first case, strong family history is usually absent, and a clear inheritance pattern cannot be discerned upon pedigree examination. Sporadic CAA accounts for the vast majority of lobar ICH events in the general population. In contrast, familial CAA is invariably characterized by strong family history (usually following an autosomal dominant inheritance pattern of vertical transmission) [7, 23–29].

Sporadic CAA-Related ICH

While definitive diagnosis requires pathological examination, CAA can be reliably diagnosed during life noninvasively using neuroimaging and clinical data, according to the Boston Criteria (Table 5.1) [30]. Criteria for probable CAA in the absence of histologic examination of brain tissue are based on the

tendency of CAA-related hemorrhages (identified on computed tomography scan) to be multiple and strictly confined to lobar brain regions. A much more powerful imaging technique in this setting, however, is gradient recall echo (GRE) magnetic resonance imaging (MRI). By identifying even very small chronic iron deposits (remnants of microbleed events) as well as acute ICH, gradient-echo MRI provides what can be considered a lifetime record of an individual's history of hemorrhages. Integration of clinical and imaging data allows for reliable diagnosis of CAA, with specificity and sensitivity above 90% [30].

Table 5.1 Boston Criteria for the diagnosis of cerebral amyloid angiopathy

1. Definite CAA
Full postmortem examination demonstrating:
Lobar, cortical, or corticosubcortical hemorrhage
Severe CAA with vasculopathy[a]
Absence of other diagnostic lesion
2. Probable CAA with supporting pathology
Clinical data and pathologic tissue (evacuated hematoma or cortical biopsy) demonstrating:
Lobar, cortical, or corticosubcortical hemorrhage
Some degree of CAA in specimen
Absence of other diagnostic lesion
3. Probable CAA
Clinical data and MRI or CT demonstrating:
Multiple hemorrhages restricted to lobar, cortical, or corticosubcortical regions (cerebellar hemorrhage allowed)
Age ≥55 years
Absence of other cause of hemorrhage[b]
4. Possible CAA
Clinical data and MRI or CT demonstrating:
Single lobar, cortical, or corticosubcortical hemorrhage
Age ≥55 years
Absence of other cause of hemorrhage[b]

Note: INR.3.0 or other nonspecific laboratory abnormalities permitted for diagnosis of possible CAA

[a]As defined in Von Sattel et al. [34]

[b]Other causes of intracerebral hemorrhage include the following: excessive warfarin dosing (INR > 3.0), antecedent head trauma or ischemic stroke, central nervous system (CNS) tumor, vascular malformation, CNS vasculitis, blood dyscrasia, and coagulopathy

Common Genetic Variation and Sporadic CAA-ICH

Apolipoprotein E (APOE)

The biological overlap between CAA and Alzheimer's disease has been leveraged to identify CAA genetic risk factors, in the absence of well-powered candidate gene and GWAS studies. Multiple groups have reported an association between the ε(epsilon)2 and ε(epsilon)4 alleles of the APOE gene and CAA-ICH [31–33]. Indeed, in the population-based Greater Cincinnati/Northern Kentucky Study, the risk factor accounting for the largest proportion of cases of lobar ICH was possession of the APOE ε(epsilon)2 or ε(epsilon)4 allele, resulting in a population-attributable risk of 29%. Until recently, the small size and lack of control for confounding due to population stratification of all candidate gene studies of *APOE* and CAA-ICH yielded inconsistent results [34, 35]. Finally, in 2010, the ISGC definitively established the role of APOE in lobar ICH risk in a large, multicenter meta-analysis that included more than 2000 ICH cases and more than 4000 controls [36]. Both the ε(epsilon)2 and ε(epsilon)4 alleles were shown to associate with lobar ICH risk at genome-wide significance levels ($p < 5.0 \times 10^{-8}$), and the association was also extended to individuals of African-American ancestry.

Genetic association analysis of APOE has uncovered associations with specific features of ICH. ICH volume and ICH expansion on follow-up imaging were found to be associated with APOE ε(epsilon)2. Each copy of this allele increases CAA-ICH hematoma size by approximately 18% and potently influences risk of CAA-ICH hematoma expansion (OR = 2.72), associations that appeared to mediate a role for ε(epsilon)2 allele in post-ICH mortality and disability [37, 38]. Results from these follow-up analyses are consistent with previous findings from pathological studies of APOE and CAA-ICH, which suggested a distinct role to the ε(epsilon)2 allele in increasing risk of CAA-related bleeding, possibly because of increased vasculopathy-mediated small vessel wall damage [39, 40].

Other Sporadic CAA-ICH Risk Loci

Rare mutations within the amyloid precursor protein (APP) gene are responsible for most forms of familial CAA. This gene was therefore investigated to identify possible variants associated with sporadic CAA. A study evaluating a total of 58 CAA-ICH subjects, however, failed to identify any APP mutation or duplication [41]. Based on the available sample size in this study, it is estimated that, if relevant for sporadic CAA pathogenesis, APP genetic variation would account for less than 8% of all CAA-ICH cases. Similar findings were reported from a study enrolling 78 CAA-ICH subjects of Spanish (European) descent [42]. It is therefore likely that, while much larger studies will be required to gather conclusive evidence, the role of APP variants in sporadic CAA is likely to be secondary at best.

A number of additional loci have been investigated for association with sporadic CAA-ICH. While none have been confirmed at genome-wide significant levels of significance, those that have been previously associated with Alzheimer Disease (AD) at the genome-wide level, can be treated with the assumption that they have a higher probability of being truly associated with CAA. A variant within the CR1 gene (rs6656401), previously associated with AD risk and pathology burden [43–45], was found to be associated with both CAA-ICH risk and vascular amyloid burden [46]. Multiple variants within the translocase of outer mitochondrial membrane 40 (*TOMM40*) gene, lying in close proximity to the *APOE* locus, were associated with amyloid plaque and vascular burden, but not with CAA-ICH risk [47]. A consistent trend towards an association between CAA pathology and rs1800470 in the transforming growth factor-β1 (*TGF-β1*) gene was reported by two studies (total of 449 participants) [48, 49]. None of these associations have been confirmed by lobar/CAA-ICH GWAS studies to date.

Familial CAA

Familial forms of CAA (Table 5.2) generally present with more severe clinical manifestations than sporadic CAA and are almost always characterized by earlier age of onset, more severe clinical course, and earlier age of death [7]. Unlike sporadic CAA, these familial forms are very rare in the general population; indeed, they present only in selected families and usually are transmitted as autosomal dominant disorders. From a clinical perspective, both sporadic and familial CAA are often responsible for

substantial cognitive impairment, but lobar ICH is not a consistent feature of all familial forms (see Table 5.2).

Table 5.2 Familial CAA forms

Amyloid peptide	Precursor protein	Chrom	Disease	Notes	CAA-ICH
Aβ	APP	21	CAA related to familial AD	Associated with Presenilin-1, Presenilin-2 and APP mutations	+
Aβ	APP	21	CAA in Down syndrome	Lobar ICH has been reported in some cases	+/−
Aβ	APP	21	CAA in APP duplication	CAA pathology prominent Increased risk of lobar hemorrhage. Also causes early-onset autosomal dominant familial AD	+
Aβ	APP	21	Hereditary Cerebral Hemorrhage with Amyloidosis: Dutch type	Described in 2 large families from the Netherlands Age at onset: 50 years Lobar hemorrhages, focal neurological deficits, dementia, and leukoencephalopathy	+
Aβ	APP	21	Hereditary Cerebral Hemorrhage with Amyloidosis: Italian type	Described in 3 Italian families Age at onset: 50 years Lobar hemorrhages and dementia	+
Aβ	APP	21	Hereditary Cerebral Hemorrhage with Amyloidosis: Flemish type	Described in a Dutch family (discovered in Belgium, therefore called “Flemish”) and a British family Age at onset: 45 years Progressive AD-like dementia, in some patients associated with a lobar hemorrhage	+/−
Aβ	APP	21	Hereditary Cerebral Hemorrhage with Amyloidosis: Iowa type	Described in a Iowa family and a Spanish family Age at onset: 50–66 years Memory impairment, expressive language deficit, personality changes, myoclonic jerks, short-step gait No clinically manifest ICH (family from Iowa) or lobar hemorrhages (family from Spain)	+/−
Aβ	APP	21	Hereditary Cerebral Hemorrhage with Amyloidosis: Piedmont type	Described in one family from Piedmont (Italy) Age at onset: 50–70 years Recurrent lobar hemorrhages,	+

				cognitive decline	
Aβ	APP	21	Hereditary Cerebral Hemorrhage with Amyloidosis: Arctic (Icelandic) type	Described in one family from northern Sweden Age at onset: ~60 years Progressive cognitive decline (no strokes)	−
ACys	Cystatin C	20	Hereditary Cerebral Hemorrhage with Amyloidosis: Icelandic type	Described in 9 sub-families in Iceland Causes systemic amyloidosis Age at onset: 20–30 years. Recurrent lobar hemorrhages	+
ATTR	Trans-thyretin	18	Meningovascular amyloidosis	Causes systemic amyloidosis Polyneuropathy is the main clinical symptom Rarer findings: ataxia, spasticity and dementia	in some families (rare)
AGel	Gelsolin	9	Familial Amyloidosis: Finnish Type	Causes systemic amyloidosis Progressive corneal lattice dystrophy, cranial and peripheral neuropathy, cutaneous amyloidosis	−
PrPSc	Prion Protein	20	Gerstmann-Sträussler-Scheinker syndrome	Described in one family Progressive cognitive decline	−
ABri	ABri precursor protein	13	Familial British Dementia	Described in 4 families Age at onset: 45–50 years Progressive dementia, cerebellar ataxia, and spastic tetraparesis	−
ADan	ABri precursor protein	13	Familial Danish Dementia	Described in 1 family from Denmark Age at onset: 30 years Cataracts, deafness, progressive ataxia, dementia (previously known as “heredopatia ophtalmo-oto-encephalica”)	−

AD = Alzheimer’s Disease, *CAA* = Cerebral Amyloid Angiopathy, *Chrom* = Chromosome

Familial CAA can be further classified as Aβ(beta) and non-Aβ(beta) forms, depending on the nature and source of the accumulating peptide (or fragment). Peptide deposition in the cerebral vasculature has been described in all familial CAA syndromes, but lobar ICH rarely dominates the clinical picture of non-Aβ(beta) CAA (with the remarkable exception of the non-Aβ[beta] Icelandic type). As for familial Aβ(beta) forms, some alterations in APP processing have been associated with individual mutations (particularly

the Flemish mutation). However, Aβ(beta) CAA largely seems to be caused by modification of the biochemical properties of the peptide itself, including conformation, aggregation, and fibril generation. APP duplication also leads to Aβ(beta)-CAA, most likely as a result of gene duplication. This mutation has been proven to cause both early-onset Alzheimer's disease and Aβ(beta)-CAA, and additional phenotypic features (including seizures and Lewy body dementia) have been described in several large families. Of note, *APOE* alleles ε(epsilon)2 and ε(epsilon)4 have very limited relationship with disease risk and evolution over time in familial CAA, possibly reflecting the overwhelming effect of the autosomal dominant rare mutation in causing amyloid accumulation. Of note, different individuals with the same mutation may present with substantially different clinical phenotypes (pleiotropy). For example, one kindred with the Iowa mutation (substitution of asparagine for aspartate at position 23) had a history of recurrent ICH; in another kindred, individuals presented with dementia and leukoaraiosis, but not ICH. These findings suggest that additional genetic factors likely modify the strong effect of this mutation (and other familial CAA mutations), although it does not appear that *APOE* is such a factor [29].

COL4A1/COL4A2 Related Intracerebral Hemorrhage

The *COL4A1* and *COL4A2* genes are located in tandem on chromosome 13q34, and encode the collagen chains α1(IV) and α2(IV), which constitute a major component of the vascular basement membrane. Rare mutations within the *COL4A1* gene on chromosome 13q34 (encoding the alpha1 chain of type IV collagen) gene have been associated with autosomal dominant syndromes manifesting variably with perinatal intracerebral hemorrhage (ICH) with consequent porencephaly, adult-onset ICH (all anatomical locations), small foci of chronic blood products in normal (or near normal) brain tissue known as microbleeds, lacunar strokes, and leukoaraiosis (white matter damage). Most disease-causing mutations in this setting are missense variants involving a highly conserved hydrophobic glycine residue, which results in inhibition of heterotrimer deposition into the vascular basement membrane and altered structural properties. When imaged with electron microscopy, the basement membrane is uneven, with inconsistent density and focal disruptions [50]. Ultimately, these changes lead to increased fragility of vessel walls and ICH. Of note, *COL4A1* appears to play a major role in determining cerebral vessel tolerance to minor head trauma, as surgical

delivery of mouse pups bearing a mutated *COL4A1* allele can prevent the severe perinatal cerebral hemorrhages that occur in spontaneous live births [51]. In humans, impaired responses to even mild trauma may include variable clinical manifestations such as subclinical microbleeds, subarachnoid hemorrhage, and devastating ICH.

A number of studies have explored potential associations between genetic variation at *COL4A1* and sporadic ICH. Rare, non-synonymous variants in *COL4A1* were identified in sporadic ICH cases, but not controls. Furthermore, these mutations impaired *COL4A1* secretion, in striking similarity to mutations causing familial syndromes [52]. Previous studies had suggested that genetic variation within *COL4A2* (which is structurally and functionally associated with *COL4A1*) was also associated with sporadic ICH risk [53]. More recently, a multi-consortium effort uncovered evidence of association between common genetic variants at the *COL4A1*/*COL4A2* locus and a number of phenotype associated with cerebral small vessel disease. An intronic variant at *COL4A2* was found to be associated with deep ICH, lacunar ischemic stroke and white matter disease severity among stroke patients [54]. Taken together, these findings strongly support a role for collagen IV deposition and function in sporadic ICH and other manifestations of cerebral small vessel disease.

Deep Hemispheric (Hypertensive) ICH

Deep (nonlobar) ICH is generally thought to be due to cerebral small vessel damage due to long-standing hypertension. To date, no candidate gene study has specifically reported genetic risk factors for deep ICH. Results from the population-based Greater Cincinnati/Northern Kentucky Stroke Study suggest that a large proportion of the population-attributable risk for deep ICH (about 20%) remains unexplained by known risk factors (including hypertension, which accounts for roughly 50% of the risk) [6]. This suggested that genetic risk loci for deep ICH exist. This hypothesis was recently confirmed by findings from the ICH GWAS study conducted by the ISGC [19]. This study included [1] a discovery phase comprised of a case cohort of 1545 individuals (664 lobar and 881 nonlobar cases) and a control cohort of 1481 individuals; [2] a replication phase comprising a case cohort of 1681 individuals (484 lobar and 1194 nonlobar cases) and a control cohort of 2261 individuals. The investigators identified and replicated an association

between deep ICH risk and rs2984613 at the 1q22 locus ($p = 2.2 \times 10(-10)$. The 1q22 locus contains a number of genes, with *PMF1* and *SLC25A44* being of highest interest for further biological dissection. *PMF1* codes for polyamine-modulated factor 1, a nuclear protein regulated by polyamines required for normal chromosome alignment and kinetochore formation during mitosis. *SLC25A44* encodes solute carrier family 25-member 44, a member of the SLC25 family of mitochondrial carrier proteins. Of note, multiple variants at 1q22 associated with deep ICH risk were subsequently associated with cerebral white matter lesion burden, providing independent evidence of a biological role in cerebral small vessel disease [55, 56].

Previously published evidence suggested that *APOE* ε(epsilon)4 might also increase risk of deep ICH, albeit with a significantly smaller effect size when compared to the effect on lobar (CAA-related) ICH [36]. This finding suggests that multiple, beta-amyloid-independent mechanisms could underlie the impact of *APOE* on ICH, both lobar and deep. A subsequent analysis of risk of ICH recurrence found that APOE ε(epsilon)4 carriers were at elevated risk of deep ICH recurrence (Hazard Ratio 1.31; 95% confidence interval 1.02–2.69) [57]. Low-Density Lipoprotein (LDL) cholesterol was found to modulate part of the effect of *APOE* on deep ICH risk, further supporting the hypothesis that *APOE* influences ICH risk by both beta-amyloid-dependent and independent mechanisms.

Conclusion

The genetic investigation of ICH remains in its infancy. Multiple familial ICH/CAA syndromes have been identified and their genetic underpinnings elucidated. Replicated loci for sporadic ICH have emerged from large collaborative GWAS efforts. Investigators now face the challenge of translating these findings into a more extensive dissection of the pathobiology of ICH/CAA, identifying novel pathways and disease mechanisms and, ultimately, new treatments.

For familial disorders, one research strategy that has had an impact in other single-gene disorders involves the genetic dissection of differences in phenotypes among carriers of the same familial mutation, as has been done in Huntington’s disease [58, 59]. Lessons from Huntington’s also point to the identification of genetic modifiers as a promising approach for uncovering novel biological pathways.

For sporadic ICH, *APOE* appears to account for only a small proportion of cases of lobar ICH, and even fewer cases of nonlobar ICH [6, 36]. Collaborative GWAS studies continue to grow in sample size and will identify novel genetic risk factors for sporadic ICH [19]. The hope is that discovery of novel risk loci for sporadic ICH will transform our understanding of disease biology in ways similar to what has happened in other complex disorders. Genetic discoveries were what yielded the role of the alternate complement pathway in age-related macular degeneration and the role of pancreatic β(beta)-cell dysfunction in type 2 diabetes [60, 61]. Furthermore, the discovery of sufficient numbers of risk loci to be able to develop reliable and powerful predictive models for bleeding risk could have a positive effect on clinical practice, influencing, for example, the decision to initiate anticoagulation in patients at risk for thromboembolism. While currently available data do not allow such a model to be adequately implemented in clinical practice [62], discovery of additional risk loci and of their relationship to other disease phenotypes (e.g., MRI and computed tomography [CT] neuroimaging traits) may bring us closer to this goal. Finally, identification of novel loci implicated in ICH/CAA will provide novel targets for therapeutic interventions, as has been demonstrated in the field of lipids genomics. Identification of rare, protective variants within the Proprotein convertase subtilisin/kexin type 9 (*PCSK9*) gene associated with favorable phenotypes for lipid traits led to development of an entirely new class of medications (PCSK9 inhibitors), that may revolutionize clinical approaches to dyslipidemia [63].

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6. Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL)

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Introduction

CADASIL (Cerebral Autosomal Dominant Ateriopathy with Subcortical Infarcts and Leukoencephalopathy) [1] is the most frequent hereditary disease among the different types of cerebral small vessel disease [2, 3]. Up to 1993, several families with hereditary cerebral small artery disease mimicking manifestations observed in CADASIL were reported using numerous eponyms; “Chronic familial vascular encephalopathy” [4], “Familiäre zerebrale arteriosklerose” [5], “Familiäre zerebrale Gefäßerkrankung” [6], “Démence sous-corticale familiale avec leucoencéphalopathie artériopathique” [7], “Familial disorder with Subcortical ischemic strokes, dementia and leukoencephalopathy” [8], “Slowly progressive familial dementia with recurrent strokes and white-matter hypodensities on CT-scan” [9].

CADASIL was identified in the 1990s after the development of efficient genetic and imaging tools. The disease is caused by mutations of the *NOTCH3* gene located on chromosome 19 [10]. This mutation leads to an accumulation of the ectodomain of the NOTCH3 receptor within the vascular wall. CADASIL is responsible for migraine with aura, transient ischemic attacks and stroke, mood disturbances cognitive decline, and leads progressively to dementia associated with gait and balance disturbances associated with pseudobulbar palsy. The disease was first reported in European families but is now recognized in American, African and Asiatic pedigrees and worldwide. The disease remains largely underdiagnosed, particularly in countries where MRI and genetic testing are not available. Several hundreds of families have been reported in France, Germany, Netherland and in United Kingdom. The minimal prevalence of the disorder varies between 2 and 5 per 100,000 among studies obtained in West-Scotland [11] or North-East England [12]. This prevalence is about 1–2% in populations aged less than 70 years and with diffuse white-matter changes on Magnetic Resonance Imaging (MRI) presumably related to small vessel diseases [13]. The disease is much rarer in non-selected populations of stroke or demented patients (Figs. 6.1, 6.2, and 6.3) [15].

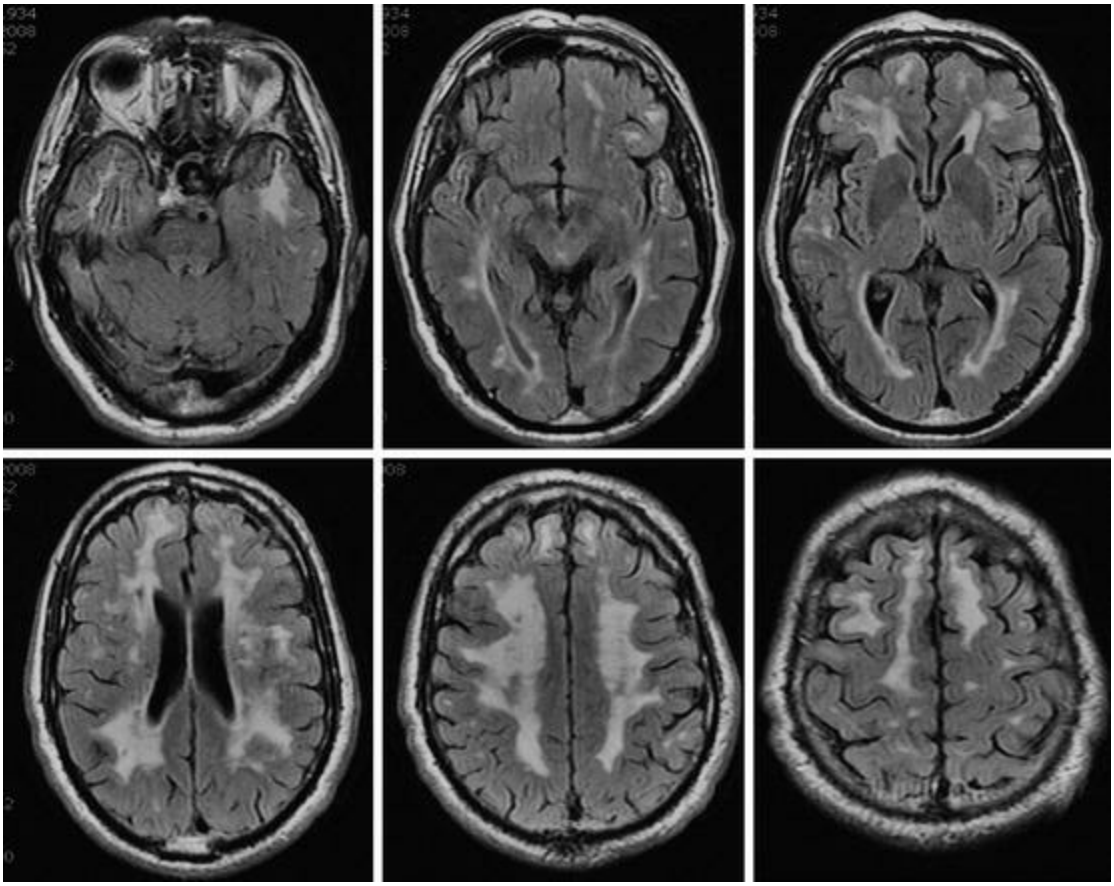

Fig. 6.1 Typical MRI features observed in a CADASIL patient aged 74 years old with no previous history of stroke. Note the very low number of lacunes in this patient, the extent of white-matter hyperintensities and the presence of dilated perivascular spaces under the insular and temporal cortex ribbon in the temporal lobes

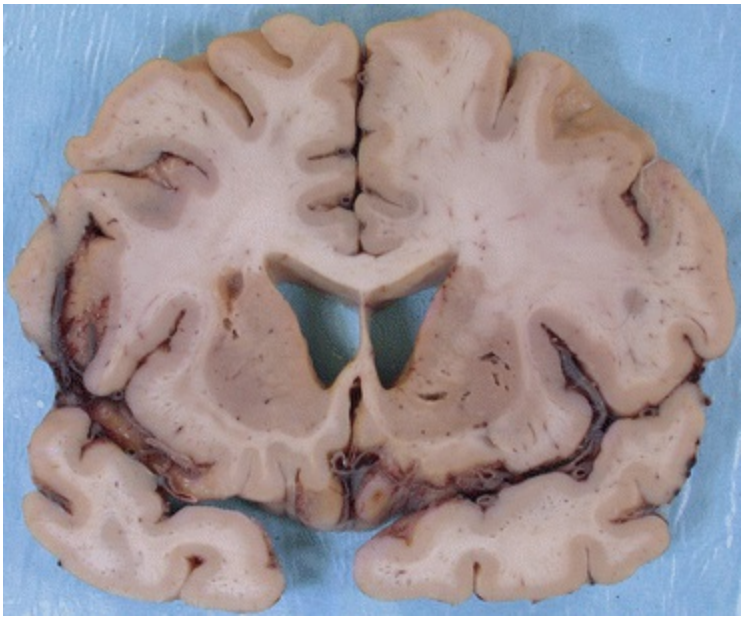

Fig. 6.2 Macroscopic post-mortem data obtained with the courtesy of Pr. Françoise Gray (Department of Pathology, Hôpital Lariboisiere, University Denis Diderot) showing in contrast to Fig. 6.1, numerus lacunes in basal ganglia and dilated perivascular spaces

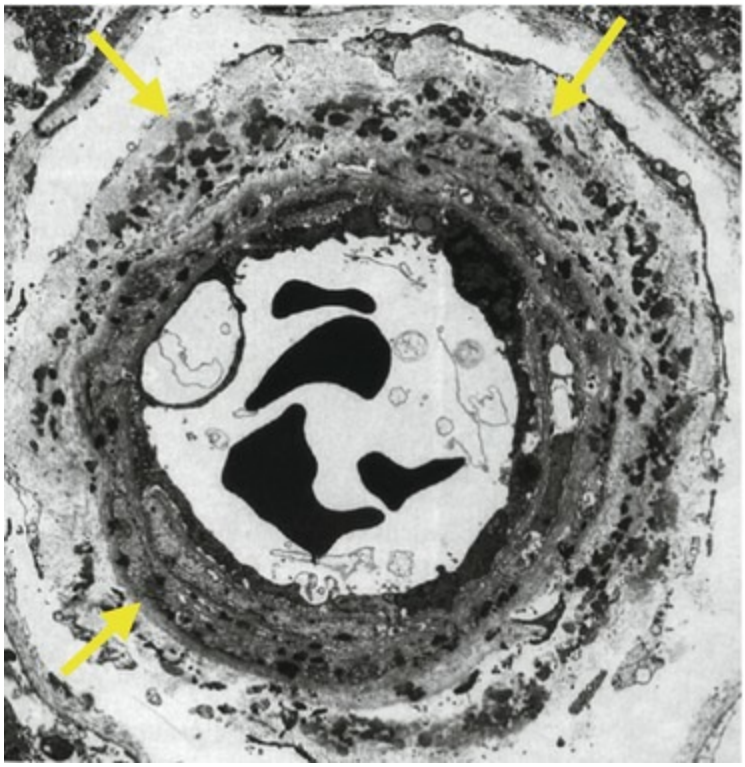

Fig. 6.3 Granular osmiophilic material detected in the vascular wall of a cerebral artery in a CADASIL patient (*yellow arrows*) corresponding to the accumulation of the NOTCH3 ECD from a post-mortem study [14]

Clinical Features

The most frequent clinical manifestations at onset are attacks of migraine with aura. They are inaugural in about 40% of symptomatic patients. Despite their frequency [16–18], migraine with aura are reported in only about half of symptomatic subjects [19]. Migraine with aura occurs therefore five to ten times more frequently in CADASIL than in the general population. By contrast migraine without aura should not be considered as a specific manifestation of the disorder. When present, migraine with aura is usually the earliest symptom, with an average onset of 30 years (range: 6–48 years) and occurs earlier in women than in men [20]. Most frequently, attacks are typical with visual or sensory aura symptoms, lasting 20–30 min, followed by a throbbing headache lasting a few hours but about two third of patients also

have atypical attacks of migraine with aura with basilar, hemiplegic or prolonged aura and a few patients have very severe attacks with confusion fever, meningitis or coma [19–23]. Trigger factors are unsurprising (e.g. stress, anxiety, alcohol) [19, 20]. Migraine with aura may be the prominent symptom in some families. However, the frequency of migraine attacks can also largely differ among affected subjects from one attack in life to several attacks per month [19].

Stroke is the most frequent clinical manifestation of the disease. About two third of symptomatic subjects have had transient ischemic attacks or a completed stroke [17, 24]. These events usually occur at a mean age of 45–50 years but the first stroke manifestations have been reported from 20 to 80 years [16, 18, 24]. Most of them are classical lacunar syndromes: pure motor stroke, ataxic hemiparesis, pure sensory stroke, sensory motor stroke. Other focal neurologic deficits of abrupt onset are less frequently reported: dysarthria either isolated or associated with motor or sensory deficit, monoparesis, paresthesiae on one limb, isolated ataxia, non fluent aphasia, hémianopia [16]. The onset of focal neurological deficits can be sometimes progressive on several hours as already reported in small deep infarcts. Less often, stroke events can occur suddenly in association with headache. When they are transient, they can mimic attacks of migraine with aura. Ischemic events manifestations usually occur in the absence of vascular risk factors. However, vascular risk factors are often detected in CADASIL patients as in the general population.The number of lacunes on baseline MRI, a past history of stroke events and smoking are strong predictors of stroke events in CADASIL [25]. In contrast, the relationships between the genotype and the clinical phenotype are not clearly delineated but appear limited [26].

Severe episodes of mood disturbances are observed in 20–30% of CADASIL patients. The frequency of these manifestations is variable among families [16, 27]. Severe depression episodes of melancholic type that can alternate with typical manic episodes have been repeatedly reported in anecdotal cases [18, 23, 27, 28]. The location of ischemic lesions in basal ganglia and/or in frontal white-matter might play a key role in their occurrence [29, 30].

Dementia is a major clinical manifestation of CADASIL. It is reported in one third of symptomatic patients and appears nearly constant at the end stage of the disease [17, 27]. The location of cerebral lesions explains the “subcortical” profile of the cognitive deficit. The cognitive deficit mainly

consists in attention deficit, memory impairment and slowness [7, 27, 31]. Apathy characterized by a lack of motivation associated with a reduction in voluntary behavior, has recently been recognized as another major clinical manifestation of the disease that is observed in more than one third of patients, most frequently in men than in women [32]. Apathy has a strong impact on the quality of life of patients and their families and is more often observed at the end course of the disorder in association with significant disability. Aphasia, apraxia or agnosia are very rare and are detected only at the very end stage of the disease [7, 9]. The cognitive deficit is often subtle, at the onset of the disease but can be easily detected using an adapted battery of neuropsychological testing. Alteration of speed processing and of executive functions are the earliest cognitive changes [33]. They do not necessarily have a significant impact on daily life. They are presumably related to lesions observed on MRI in strategic white-matter tracts [34] such as the anterior thalamo-cortical tracts and/or the forceps minor. The cognitive decline develops most frequently progressively and can be observed in the total absence of clinical ischemic events, mimicking a degenerative dementia [23, 27]. The frequency and severity of cognitive decline largely can largely vary among different members of a given family. The severity of cognitive impairment is strongly related to gait and balance disturbances. The severity of motor disability strongly predicts cognitive decline and vice-versa in CADASIL patients observed over a 3-year period [25]. The variable location and severity of cerebral tissue damage seems to play a key role in their development. The clinical severity was found strongly correlated to the load of infarctions within the white-matter [35] and degree of cerebral atrophy [36]. The accumulation of lacunes is also associated with reduced cognitive performances in follow-up studies (unpublished data) and is associated with remote cortical degeneration [37–39]. Therefore, the degree of tissue loss, accumulation of focal ischemic lesions and location of these lesions are crucial in the development of cognitive decline in CADASIL patients. When dementia is present, at a mean age of 60 years, it is observed in the absence of any other clinical manifestations in only 10% of cases. Dementia is always associated with pyramidal signs, pseudobulbar palsy, gait difficulties and/or urinary incontinence [40]. The cognitive and functional decline is usually observed over decades. The patient becomes bedridden and often deceased after pulmonary complications of swallowing difficulties. In a large retrospective study, dementia was present in 90% of cases before [18, 41].. In

contrast to the normal median survival time observed in women, men with CADASIL may have a mean decrease of 5 years of their life expectancy [41].

Other clinical manifestations have been reported in CADASIL. Focal or generalised seizures are observed in 6–10% of cases [18, 27]. Deafness (not profund) has been detected in several cases [42]. The lack of cranial nerve palsy, spinal cord disease and of symptoms of muscular origin is noteworthy although these clinical manifestations can be observed fortuitously.

Imaging Features

MRI is a key diagnostic tool in CADASIL. MRI is abnormal in all cases with permanent symptoms or stroke manifestations [2, 27, 40]. MRI signal abnormalities can be detected at the presymptomatic stage of the disorder during a period that can largely vary among affected individuals. MRI signal abnormalities have been reported as early as 20 years of age. They usually develop between 20 and 40 years. After the age of 35 years, all subjects having the affected gene were found to have an abnormal MRI in the first CADASIL reports [3, 27]. The frequency of totally asymptomatic individuals with abnormal MRI decreases progressively with aging and becomes extremely low after 60 years particularly after a detailed neuropsychological evaluation is performed.

MRI shows on T1-weighted images punctiform or round hypointensities in basal ganglia and white-matter. T2-weighted images show hyperintensities in the same regions often associated with widespread areas of increased signal in the white-matter that can be distinguished from lacunes and dilated perivascular spaces on FLAIR images [43] The severity of MRI signal abnormalities is variable. White-matter lesions dramatically increase with age. In subjects of age under 40 years, T2 hyperintensities are usually punctuate or nodular with a symmetrical distribution, and predominate in periventricular areas and within the centrum semi-ovale. Later, white-matter lesions are diffuse and can involve the whole of white-matter including the U fibres under the cortex. The frequency of signal abnormalities in the external capsule (2/3 of the cases) and in the anterior part of the temporal lobes is noteworthy and appears extremely useful for the differential diagnosis [44]. Brainstem lesions are mainly observed in the pons [45].

On T2*-weighted or susceptibility-weighted images, microbleeds are easily detected in one third of patients. Age, blood pressure and haemoglobin

A1C were found associated with microbleeds in a large sample [46]. The location of microbleeds differs from that of lacunes and white-matter hyperintensities, they can be observed within the cerebellum [46]. Other MRI findings include dilated perivascular spaces with a typical “etat criblé” in very few cases [47, 48]. Their number appears strongly correlated to the extent of white-matter hyperintensities [48]. Dilated perivascular spaces can be detected using thin slices on FLAIR images just under the cortical ribbon in temporal lobes and external capsules. This may represent a specific feature of CADASIL [44].

Cortical or cerebellar lesions are exceptional in CADASIL. They have been observed in anecdotal cases older than 60 years. Cerebral angiography obtained in 14 patients belonging to seven affected families was normal except in one case with a detectable narrowing of small arteries [27]. Stenosis of large arteries has been however occasionally observed with magnetic resonance angiography [49]. Weller et al. reported clinical worsening in two CADASIL patients after intra-arterial angiography which was found normal with a possible vasospasm in one. One subject had a severe headache, vomiting, confusion, somnolence and a grand mal seizure that resolved within several hours [50]. Dichgans et al. confirmed the high frequency of neurological complications after angiography in CADASIL patients [51]. Echocardiography is usually normal, although a high frequency of patent foramen ovale (47%) has been reported in an Italian series [52]. CSF examination is usually normal. Oligoclonal bands with pleïocytosis have been previously reported [53]. Electromyogram examination is normal. A monoclonal immunoglobulin was detected in few anecdotal cases [42].

Pathological Data

Post-mortem studies show a diffuse myelin pallor with rarefaction of the hemispheric white-matter usually sparing the U fibres [2, 54]. Lesions predominate in the periventricular areas and centrum semi-ovale. They are associated with lacunar infarcts located in the white-matter and basal ganglia (lentiform nucleus, thalamus, caudate) [14, 54]. The most severe hemispheric lesions are the deepest [2, 7]. In the brainstem, the lesions are more marked in the pons and associate pontine white-matter rarefaction and lacunes [55]. Lesions detected within the temporal lobes are associated with multiple dilated perivascular spaces and myelin degeneration [56].

Microscopic pathological studies showed that the wall of cerebral and leptomeningeal arterioles is sometimes thickened and can present with a significant reduction of the lumen [2]. Such abnormalities can also be detected by leptomeningeal biopsy [57]. PAS positive staining that can be detected in the vessel wall suggested the possible presence of glycoproteins [2, 14, 58, 59]. However, immunohistochemistry does not support the presence of immunoglubulins in the vessel wall. By contrast, the endothelium of the vessels is usually spared. In some vessels, smooth muscle cells are replaced by collagen fibres [60]. On electron-microscopy, these cells can appear swollen and are often degenerated, some of them with multiple nuclei. There was a granular, electron-dense, osmiophilic material within the media of small arteries [54]. This material consists of granules of about 10–15 nm of diameter [60]. It is localized close to the cell membrane of the smooth muscle cells where it appears very dense. The smooth muscle cells are separated by large amounts of this material. There is accumulating evidence from preclinical studies showing that this material mainly consists of NOTCH3 extracellular domain accumulating close to smooth muscle cells in arterioles and to pericytes in capillaries [61] with aggregates of various extracellular matrix proteins such as TIMP3 and Vitronectin [62]. Ruchoux et al. made the initial crucial observation that the vascular abnormalities observed in brain vessels were also detectable in other organs [14, 63, 64]. The granular and osmiophilic material (GOM) surrounding the smooth muscle cells as seen with electron microscopy is also present in the media of arteries located in the spleen, liver, kidneys, muscle and skin and also in the wall of carotid and aortic arteries [14, 64]. These vascular lesions can also be detected by nerve biopsy [65]. The detection of this material in skin vessels allows to confirm the intra vitam diagnosis of CADASIL in difficult cases using punch skin biopsies [66], but the sensitivity and specificity of this method have not yet been established [14, 64, 67]. Skin biopsy immunostaining with a Notch3 monoclonal antibody revealing the accumulation of the NOTCH3 protein in the vessel wall is another potential diagnostic tool with high sensitivity (85–95%) and specificity (95–100%) [68, 69].

Genetics

CADASIL is caused by stereotyped mutations of the NOTCH3 gene. This

protein made of 2321 amino-acids is a transmembrane receptor that includes an extracellular domain containing 34 EGF repeats (including 6 cystein residues) in addition to its intracellular and transmembrane domains [3]. All pathogenic mutations are located within epidermal-growth-factor-like (EGF-like) repeats in the extracellular domain of the protein [59]. In 70% of cases, they are within exons 3 and 4 which encode the first five EGF domains. All mutations responsible for CADASIL lead to an uneven number of cystein residues [58, 59, 70–74] presumably responsible for neoporphic [75] and/or functional changes [76] of the receptor. Biochemical fractionation of brain and artery samples recently showed that mutant NOTCH3 extracellular domain (ECD) accumulates in insoluble aggregates both in mutant mice and patients with CADASIL. Two functionally important extracellular matrix proteins, tissue inhibitor of metalloproteinases 3 (TIMP3) and vitronectin with different functional effects on the microvasculature are sequestered with NOTCH3(ECD) within the GOM. The abnormal recruitment of such functionally important extracellular matrix proteins may ultimately cause toxicity by impairing extracellular matrix homeostasis in small vessels [62].

Genetic testing is now currently used for the positive diagnosis of CADASIL. Over 95 % of mutations in the NOTCH3 gene are missense mutations. Others are small in frame deletions or splice site mutations [72, 77–80]. De novo mutations have been reported but their exact frequency is unknown [81, 82]. Today, genetic testing is obviously the gold standard for diagnosis. Screening of the 23 exons encoding the 34 EGFR has a 100 % specificity when it detects a mutation leading to an odd number of cysteine residues within an EGFR. Its sensitivity is also close to 100% [59, 74, 83].

Treatment

No preventive treatment has been evaluated in CADASIL. In order to improve executive dysfunction and cognitive performances in patients, a controlled clinical trial comparing donepezil to placebo was performed in CADASIL considered as a unique and “pure model” of vascular dementia. A significant improvement was detected on variable measures of speed processing and executive dysfunction but without a clear benefit in activities of daily life, the primary endpoint was not reached and the trial was considered negative [84]. Because CADASIL is a vascular disorder responsible for cerebral ischemic events, different authors prescribe aspirin

for secondary prevention, but its benefit in the disease has not been shown. The occurrence of intracerebral hemorrhage in several anecdotal cases [85] and in one patient, at the time of death [2], suggests that anticoagulant therapy may be dangerous in CADASIL. Whether this risk is related to the number of silent microbleeds is still unknown. Hypertension may have some deleterious effects but this is not demonstrated although intracerebral hemorrhages have been reported in different hypertensive cases [85]. Vasoconstrictive drugs such as ergot derivatives and triptans are usually not recommended in presence of a vascular disorder. Treatment of migraine should be restricted, if possible, to analgesic agents and nonsteroidal anti-inflammatory drugs. As reported for other ischemic diseases, rehabilitation procedures are crucial, particularly after a new ischemic event and when disability is progressing. If stroke occurs at an early stage of the disease, recovery is often complete. In all cases, social and psychological support for the patient and family are crucial and genetic counseling and testing should be performed at specialized centers.

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7. Monogenic Disorder: Fabry Disease

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Introduction

Fabry disease (FD) (OMIM 301500) [1], also known as Anderson-Fabry disease, is an X-linked lysosomal storage disorder caused by deficiency of the enzyme α-galactosidase A (EC 3.2.1.22) [2]. As a consequence of this inborn error of metabolism, there is widespread accumulation of neutral glycolipids, particularly globotriaosylceramide (Gb3) (Fig. 7.1), including in neurons and vascular endothelial and smooth muscle cells. The deacylated form of Gb3 (lyso-Gb3) also accumulates and may contribute to the pathogenesis of the disease [3]. The birth frequency of fabry disease has been quoted as lying between 1:40,000 and 1:117,000, though studies based on newborn screening have suggested a much higher frequency of 1:3100 [4].

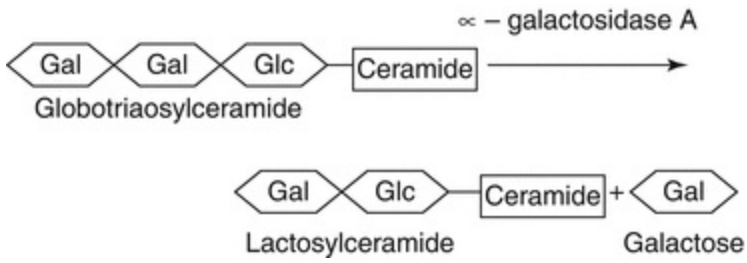

Fig. 7.1 α-galactosidase A: enzyme, substrate, and product. *Gal* galactose residue, *Glc* glucose residue

Nonneurological clinical features of fabry disease include a characteristic skin rash (*angiokeratoma corporis diffusum*), corneal and lens changes (*cornea verticillata* and cataract, respectively), hypohidrosis, and gastrointestinal complications. These manifestations often develop in childhood or adolescence [5]. Later, patients (particularly males) are at risk of life-threatening complications—renal failure and cardiac disease (arrhythmias, valvulopathy, ischemic heart disease, cardiomyopathy) [6].

Neurological Manifestations

Fabry disease affects both the central (CNS) and peripheral (PNS) nervous systems (Table 7.1). Though Gb3 is deposited in CNS neurons, fabry disease is not associated with clinical features prodromal for neurodegeneration, unlike Gaucher disease, another lysosomal storage disorder [8]. Instead, the most prominent CNS manifestations are a consequence of vasculopathy (see below). In the PNS, a painful, small-fiber neuropathy commonly occurs in early childhood, but the pain may persist into adult life. Patients experience acroparesthesia and “Fabry crises” or “pain attacks.” These crises are episodes of severe pain, which can last several days or even weeks, and may be triggered by intercurrent illness, stress, temperature change, or exercise. The pain may be accompanied by fever and elevation of the erythrocyte sedimentation rate. Fabry neuropathy is not generally associated with neurological signs beyond some distal impairment of pain and temperature sensation, though Charcot joints occasionally occur. Large-fiber nerve conduction studies are usually normal, but abnormalities may be detected on thermal threshold testing [9, 10]. The pattern of these small-fiber abnormalities, with greater impairment of cold than warm sensitivity, implies early damage to small myelinated nerve fibers. This deduction is supported by morphometric analyses of nerve biopsies from fabry disease patients, where selective loss of the small myelinated fiber population was also seen [11]. Although there is Gb3 deposition in peripheral nerves, the primary injury may be at the level of the dorsal root ganglion cells, where lipid has been identified in the perikaryon [12]. In addition, lyso-Gb3 may be directly nocigenic by enhancing voltage-gated calcium currents in sensory neurons [13].

Table 7.1 Neurological features of Fabry disease

Central nervous system	Other
Ischemic stroke	Peripheral neuropathy
Transient ischemic attack	Autonomic neuropathy
Cognitive impairment	Vision[a]
Psychiatric/behavioral	Hearing and balance
Hemorrhage	
Neurovascular conflict[b]	
Aseptic meningitis[c]	

[a]Ophthalmological manifestations of fabry disease include cornea verticillata, cataract, and tortuous vessels (in the conjunctiva and the retina)
[b]Ectatic blood vessels have been reported to compress cranial nerves in fabry disease and to obstruct the normal flow of cerebrospinal fluid, causing hydrocephalus
[c]Several fabry disease patients with lymphocytic cerebrospinal fluid have been described [7]

Ischemic Stroke

In the CNS, the dominant complication of fabry disease is an increased risk of stroke, with an early age of onset [14]. Initial small-scale studies pointed to a prevalence of stroke in fabry disease as high as 16–25%, but these findings are likely to have been overestimates, arising as artifacts of small sample size with selection bias [9, 15–17]. Results from the Fabry Registry, a database of more than 2000 fabry disease patients, gave stroke frequencies of 6.9% for males and 4.3% for females [18]. While these values markedly exceed stroke prevalence in the general population, they may be contrasted with the frequency encountered in CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy), the other classic monogenic disorder associated with an increased risk of ischemic cerebrovascular disease, where stroke is almost a *sine qua non* (see chap. 6). The Fabry Registry data also underline the fact that fabry disease can be severe in women. Despite the X-linked pattern of inheritance, women are not merely carriers of fabry disease but can occasionally express the disease as severely as men [16, 19]. The median age at first stroke in the Fabry Registry was 39.0 years for men and 45.7 years for women. At 13.2%, the proportion

of patients in this registry having hemorrhagic strokes does not differ significantly from that found in the general population. Other important findings from the Fabry Registry include the fact that many patients presented with stroke before experiencing major renal or cardiac events, indeed before a diagnosis of fabry disease had been reached [18]. Paradoxically, however, results from the Fabry Outcome Survey (FOS), another large database with a comparable number of patients to the Fabry Registry, indicate that fabry disease stroke patients are particularly at risk of cardiovascular and renal complications compared to the general fabry disease population [20, 21]. The FOS data also suggest that ischemic stroke in fabry disease more commonly affects the vertebrobasilar circulation than in the general population (in keeping with a suggestion from a meta-analysis of early case reports) [14]—typically with small vessel events resulting in moderate disability [21].

MRI Findings

In addition to infarcts, fabry disease is associated with several magnetic resonance imaging (MRI) features [22]. These include nonspecific white matter lesions (Fig. 7.2), which increase with age [23, 24], affect both sexes [23, 25], and may be present even in childhood [26]. They are generally presumed to be ischemic in basis and apparently correlate with stroke risk in fabry disease [27]. Chronic microbleeds, detected on gradient-echo imaging, are less commonly encountered than these presumed ischemic lesions [28] but may be present in up to 30% of patients [29]. Another abnormality is a striking appearance of high signal in the pulvinar on T1-weighted images (Fig. 7.3) [30, 31], most likely signifying microangiopathic calcification (occasionally also visible on computed tomography scans). This sign is present in up to 20% of fabry disease patients, particularly males. It is not pathognomonic of fabry disease but can be helpful diagnostically in the appropriate clinical context. A final finding on MRI and MR angiography is marked distortion and dilatation of the larger vessels, especially in the posterior circulation, known as dolichoectasia (Fig. 7.4). This may be a consequence of Gb3 deposition in the vessel wall. In extreme examples, the diameter of the basilar artery may exceed 1 cm—the “megadolichobasilar” anomaly—with a high risk of basilar artery thrombosis and intracranial hemorrhage [32]. Quantitative analyses of MRI changes in fabry disease

initially suggested that basilar artery diameter could be a useful MR discriminant between fabry disease and the general young stroke population [33, 34], but this was not confirmed in larger scale studies [35].

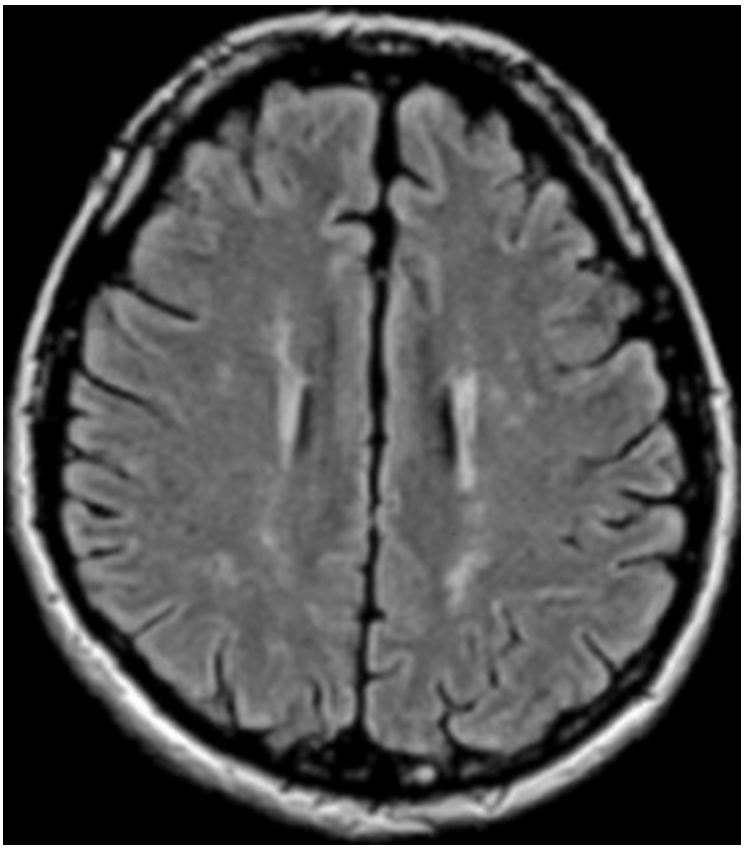

Fig. 7.2 White matter lesions in the cerebral hemispheres in Fabry disease: axial FLAIR MRI section (Reproduced with permission from Ginsberg et al. [23])

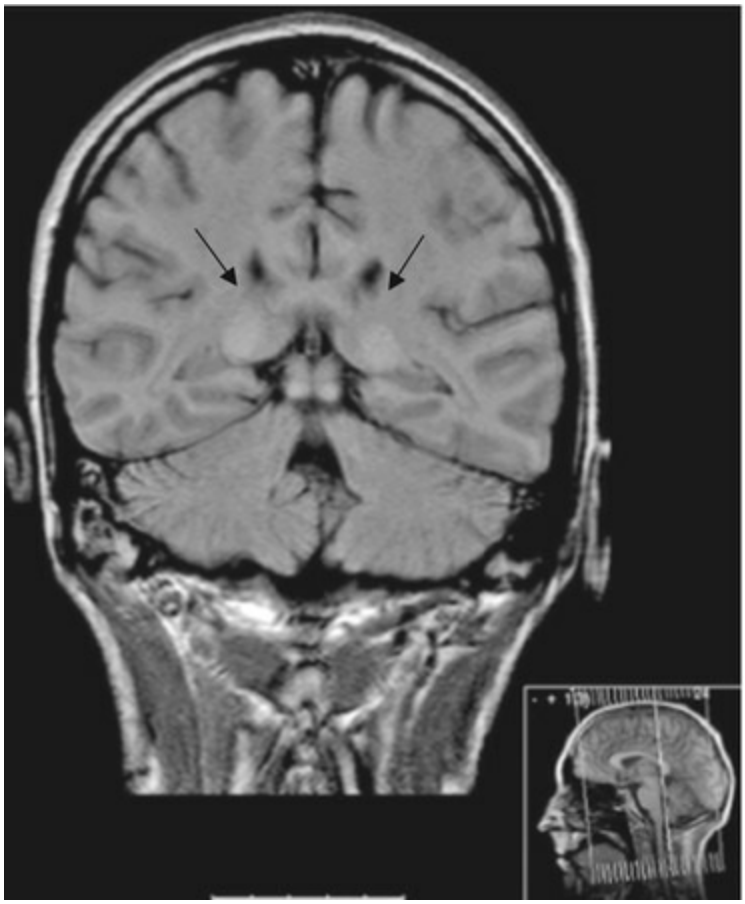

Fig. 7.3 High signal in the pulvinar (*arrowed*) in Fabry disease: T1-weighted coronal MRI section

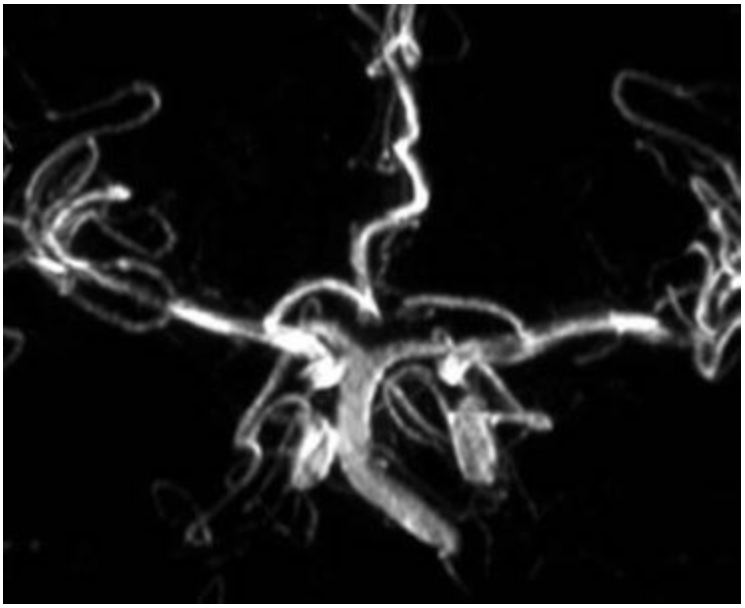

Fig. 7.4 Vertebrobasilar dolichoectasia in Fabry disease: MR angiography (Reproduced with

permission from Ginsberg et al. [23])

Genetics of Fabry Disease

The gene responsible for encoding α-galactosidase A, known as *GLA*, is located on chromosome Xq22.1. Its coding region comprises 1290 base pairs, in seven exons, which translate to a polypeptide of 429 amino acids. Over 600 pathogenic mutations of *GLA* have been described in fabry disease, including point mutations (missense, nonsense, and splice site) and rearrangements [36]. Unlike some other lysosomal storage disorders, such as Gaucher disease, there is a very high proportion of unique or "private" mutations in fabry disease. This complicates the study of genotype-phenotype correlation, as does the phenotypic heterogeneity encountered in fabry disease patients sharing the same mutation, even within the same family. In broad terms, there may be a trend towards milder disease in those patients with missense mutations that leave them with significant residual enzyme activity. A preliminary study of stroke in fabry disease is consistent with this trend, strokes being more likely to occur in patients with more severe mutations [37]. That this pattern is an oversimplification is shown by the existence of heterozygous female patients with near normal blood enzyme activity who can express the disease with at least moderate severity, including having strokes. However, there are particular genetic considerations that apply to female heterozygotes, notably the likelihood of variable lyonization in different organ systems.

Some patients, usually with residual enzyme activity, are labeled as having cardiac [38] or renal "variants" of fabry disease because their phenotype is dominated by involvement of one of these systems. Again, this may be a simplification as fabry disease is progressive, and a patient with the so-called cardiac variant may ultimately develop renal complications. Variants are therefore best interpreted as manifestations of the wide phenotypic heterogeneity of fabry disease. The finding that many patients in the Fabry Registry presented with stroke in relative isolation [18] may be construed in the same way, but gives rise to three corollaries. First, a distinct subgroup of fabry disease patients does not follow the "classical" model of the disease, characterized by early skin changes, neuropathic and abdominal pain, and then the development of life-threatening complications only later in the course of the illness. Second, the existence of this subgroup may go some

way towards explaining the discrepancy between earlier estimates of fabry disease prevalence and that based on newborn screening [4]. Finally, the presence of a pathogenic fabry disease mutation in the general population may be regarded as a risk factor for vasculopathy and stroke, potentially acting in concert with conventional risk factors. Having said this, no single fabry disease mutation (or type of mutation) is exclusively associated with high stroke risk.

Stroke Etiology and Pathogenesis

The ultimate cause of the clinical findings in a patient with fabry disease is the presence of a pathogenic mutation in the *GLA* gene. But the molecular and cellular processes leading from such a mutation to the clinical phenotype remain incompletely understood. At the level of gene and enzyme, Gb3 storage, as a consequence of the enzyme deficiency, is unlikely to be the sole pathogenetic mechanism, as indicated in preceding sections, and other processes, including toxic effects of lyso-Gb3, are likely to be involved. At the level of cell, tissue, and organ, fabry disease has highly atypical features when compared with “mainstream” lysosomal storage diseases. These usually present with a combination of visceromegaly, skeletal deformity, and profound neurodevelopmental disorder—features that are perhaps easier to relate to storage.

The pathogenesis of stroke in fabry disease is likely to represent a particularly complex interplay of potential mechanisms. Despite ample potential cause [6], cardiogenic embolism seems relatively rare, as judged by FOS data [21] and studies of microembolism in fabry disease patients [39]. If the majority of fabry disease strokes arise instead from autochthonous thrombosis, it seems reasonable to invoke Virchow’s triad as a starting point in the analysis. There is evidence that all three components of the triad are affected in fabry disease—changes in the vessel wall, changes in the pattern of blood flow, and changes in blood constituents. That Gb3 deposition leads to mechanical changes of the vessel wall seems likely from the macrovascular abnormalities seen in fabry disease (Fig. 7.4). But lyso-Gb3 may also contribute, by stimulating smooth muscle cell proliferation, and hence increasing vascular intima-media thickness [3]. How can such large-vessel structural changes relate to the small vessel clinical events most typically encountered in fabry disease? In fact, the disease may affect vessels

of all calibers, thereby potentially leading directly to occlusion of the smallest. But it has also been suggested that dolichoectasia could exert an indirect effect on small vessels by distorting and narrowing the ostia of the perforating arteries emerging from the main basilar trunk [14]. Beyond such changes to the vessel wall, stroke pathogenesis in fabry disease is likely to involve abnormalities of cerebral blood flow [40, 41]. In particular, regional cerebral hyperperfusion has been described in positron emission tomography (PET) studies, predominantly in the posterior circulation, and attributed to release of reactive oxygen species [40]. Finally, there are abnormalities of blood constituents in fabry disease, which are likely to result in a prothrombotic state, including increased soluble intercellular adhesion molecule-1, vascular cell adhesion molecule-1, P-selectin and plasminogen activator inhibitor, and reduced thrombomodulin, along with increased monocyte CD11b expression [42].

Diagnosis and Management

The observation that many patients in the Fabry Registry presented with young-onset stroke before a diagnosis of fabry disease had been reached [18] is an argument for screening the general population of young stroke patients for fabry disease, given the availability of treatment. A prospective study of this kind yielded a prevalence of fabry disease of around 1% among stroke patients presenting in the 18–55 years age range [27]. Subsequent studies (summarized by Kolodny et al. [43] and culminating in the SIFAP study of >5000 young stroke patients [44]) have indicated that the prevalence is more probably below 1%. This is a relatively low yield and there are counterarguments to the universal adoption of fabry disease screening programs in young stroke patients. In particular, there is concern that patients with *GLA* genetic variants of unknown or neutral significance may be identified, with the risk of wrongly labeling individuals as having fabry disease, when they are only harboring non-pathogenic gene variants [45, 46].

Outside screening programs and ascertainment of new patients from families known to have the condition, the diagnosis of fabry disease may be difficult because of the variable multisystem phenotype. Many patients have been misdiagnosed or have had a delayed diagnosis [5], despite the imperative to reach a diagnosis as early as possible, now that specific treatment is available (see later). In males, the diagnosis may be made by

measuring plasma and/or white cell enzyme activity. This is not possible for females, as the enzyme activity in heterozygotes may overlap the normal range; hence, genetic testing is necessary.

Management of the neurological complications of fabry disease includes supportive measures. Thus, standard approaches may be used for neuropathic pain. As is the case for the general population, fabry disease patients presenting with stroke are best managed on a stroke unit. There is no specific contraindication to thrombolysis in fabry disease patients with ischemic stroke, indeed this has been used, albeit without benefit [47]. In the absence of evidence to the contrary, conventional vascular risk factors should be managed appropriately, including use of antiplatelet therapy, statins, antihypertensive agents, and anticoagulation in the context of atrial fibrillation.

Specific treatment of fabry disease has been revolutionized by the advent of enzyme replacement therapy (ERT). Two products are available—agalsidase alfa and agalsidase beta—both given fortnightly intravenously and both extremely expensive and subject to manufacturing shortages. To date, this treatment has been shown in pivotal trials [48, 49] and many subsequent studies to benefit non-neurological manifestations of fabry disease. Some neurological complications also respond, including the neuropathy [50] and sensorineural hearing loss [51]. The effect of enzyme replacement therapy on stroke frequency has been more difficult to establish, though the treatment can reverse the abnormalities of cerebral blood flow seen in fabry disease [40]. Strokes certainly continue to occur in patients on enzyme replacement therapy [52, 53]. There have been conflicting reports of the effects of enzyme replacement therapy on white matter lesions [54, 55].

Several potential reasons for the apparent lack of benefit of enzyme replacement therapy in fabry disease stroke have been advanced. These include issues relating to enzyme dose and frequency of administration. Treatment may not have been given for long enough or may have been started at a time when irreversible changes in the cerebral circulation had already occurred. There is also evidence that the ability of enzyme replacement therapy to clear lipid deposits may not extend far beyond the vascular endothelium [56].

General consensus guidelines for starting and stopping enzyme replacement therapy in patients with fabry disease are now available [57]. Within this framework, for the particular case of patients in whom young-

onset stroke is the first indication of a diagnosis of fabry disease, it is reasonable to start enzyme replacement therapy, as treatment for non-CNS complications of the disease. There is also the hope of preventing further cerebrovascular events, despite uncertainty about treatment efficacy in the context of stroke. For patients with the more “classical” form of the disease, where the diagnosis is known before a stroke has occurred, but treatment has not yet been initiated for non-neurological reasons, the challenge is to select patients for enzyme replacement therapy who may be at particular risk of cerebrovascular events. Possible strategies could include identifying vulnerable patients by the presence of genetic modifiers of stroke risk [58], or by subtle early indicators of white matter disease detected, for example, by diffusion tensor imaging [59], or by means of a prognostic index, based on readily determined input variables [60].

Beyond enzyme replacement therapy, newer specific treatments for fabry disease are being developed, including substrate reduction and gene therapy. There is particular current interest in oral pharmacological chaperone therapy with migalastat [61, 62].

Conclusion

Fabry disease is an X-linked lysosomal storage disorder. In its “classical” form, a painful peripheral neuropathy develops in childhood or adolescence, along with non-neurological features. Later, life-threatening complications include renal failure, heart disease, and young-onset stroke. However, patients can present with stroke before a diagnosis of fabry disease has been reached. Despite its mode of inheritance, women with fabry disease may be severely affected, including presenting with young-onset stroke. Characteristic, albeit non-pathognomonic, MRI abnormalities in fabry disease include white matter lesions, pulvinar hyperintensity on T1-weighted images and vascular dolichoectasia. enzyme replacement therapy has revolutionized the management of fabry disease, but its role in stroke prevention remains unclear.

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8. Stroke-Like Episodes in Mitochondrial Myopathy, Encephalopathy, Lactic Acidosis, and Stroke-Like Episodes (MELAS)

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Introduction

More than 30 years have passed since the initial description of MELAS: mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes [1]. The factors that mediate the pathogenesis and mechanism of MELAS syndrome remain elusive, as have effective therapies. In this review, we discuss the current basis of understanding and theories relating to the

common phenotypic features of MELAS, in particular the pathognomonic "stroke-like episodes," as well as implications towards future therapeutic developments.

Overview of the Mitochondria and Mitochondrial Disease

While a thorough discussion is beyond the scope of this review and has been effectively presented elsewhere [2, 3], any study of MELAS syndrome would be remiss without at least a brief review of mitochondrial function and genetics. The mitochondrion performs a multitude of cellular tasks, its central role being oxidative phosphorylation (OXPHOS), the process that generates adenosine triphosphate (ATP). OXPHOS is a multienzyme pathway through which electrons enter at the level of complex I (NADH dehydrogenase) and complex II (succinate dehydrogenase) via nicotinamide adenine dinucleotide (NADH) or flavin adenine dinucleotide ($FADH_2$) and drive protons from the mitochondrial matrix into the intermembrane space through complexes I, III, and IV. This generates an ion gradient that is relieved by movement of protons back through the channel formed by complex V, with the concomitant generation of heat and ATP. The respiratory chain complexes comprise multiple subunits. Complexes I, III, IV, and V are composed of polypeptides encoded by both nuclear and mitochondrial genes, whereas complex II is encoded entirely by nuclear DNA. Nuclear-encoded genes are imported into the mitochondria and coassembled with mitochondrial DNA (mtDNA)-encoded genes into the respective enzyme complexes.

The mitochondrial genome in humans is a small prokaryotic remnant, a double-stranded circle of 16,569 base pairs encoding a mere 37 genes [4]. Of these genes, two specify ribosomal RNAs (12S and 16S rRNA) and 22 encode mitochondrial transfer RNAs (tRNA) [3]. The 13 remaining genes specify polypeptide components of the OXPHOS system, subunits of complexes I, III, IV, and V. No other proteins involved in mitochondrial function are encoded by mtDNA. Despite its small size and paucity of polypeptide end products, the mitochondrial genome is the locus of a surprisingly large number of pathogenic mutations. Over the last 23 years, more than 200 disease-causing point mutations in the mitochondrial genome have been recorded in the MITOMAP[1] database [5]. At least 30 of these

mutations have been associated with MELAS syndrome [6].

With a solitary exception [7], all mitochondria are inherited matrilineally. Thus, disease related to a mtDNA mutation comes exclusively from the proband's mother. Depending on the specific energy requirements of a given tissue, there may be hundreds to even thousands of mitochondria within each cell and each mitochondrion will contain several mtDNA copies [3]. Mitochondrial replication appears to be a stochastic event unrelated to the cell cycle. During cell division, there is a random distribution of mitochondria and its mtDNA between each of the daughter cells. This process, termed mitotic segregation, is the basis for the complicated and heterogeneous clinical phenotypes often seen with mtDNA mutations. The development of a mutation to one or more mitochondrial DNA genomes in a cell produces a state of heteroplasmy, simply defined as the presence of two or more different genomes within an individual cell. Should the mutation localize to cells in the female germ line, the mutation can be passed along to subsequent generations. The actual number of mtDNA copies passed from mother to child appears to be quite small (5–200 copies) [8], producing a genetic bottleneck and making it very possible that a pathogenic mutation may comprise a much higher proportion of the total mtDNA in the child than in the mother. Unsurprisingly, the ratio of mutant to wild-type mtDNA can also vary widely between children born to the same mother.

Heteroplasmy and mitotic segregation of mtDNA within developing tissues produce variation in the proportion of mutant mtDNA, not just between tissue types but from cell to cell. Moreover, due to ongoing mitochondrial and cellular division, the relative burden of mutation can be expected to evolve over the lifetime of the individual, even within the cells of terminally differentiated tissues. It is believed that there is a "threshold" level for each mutation beyond which the cell will manifest pathology. This results in extremely variable clinical phenotypes, ranging from asymptomatic to oligosymptomatic (milder or isolated symptoms) to fully symptomatic, even among affected members of a common pedigree. The significant tissue-to-tissue variability, the difference in threshold between tissues, and our inability to quantify the tissue-specific mutational burden for key organs such as the brain make it very difficult to give a prognosis for individuals harboring a pathogenic mtDNA mutation.

Genetics and Molecular Pathophysiology

There is a striking range of pathology stemming from individual point mutations. Relevant to discussions regarding MELAS is the adenine-to-guanine transition mutation at position 3243 of the mitochondrial genome (m.3243A>G). This mutation, which affects the mitochondrial $tRNA^{(Leu)}$ gene, is the most common mutation associated with MELAS [9, 10]. MELAS is a polygenetic disorder with at least 30 specific identified point mutations associated with the disease. In addition to at least seven identified point mutations in the mitochondrial $tRNA^{(Leu)}$ gene, mutations affecting numerous other mitochondrial tRNA genes (His, Lys, Gln, Glu) and protein-coding genes (MT-ND1, MT-CO3, MT-ND4, MT-ND5, MT-ND6, and MT-CYB) have been associated with MELAS [5]. Interestingly, many of these mutations have been implicated in other mitochondrial syndromes (Leber hereditary optic neuropathy, Leigh disease, myoclonic epilepsy with ragged red fibers [MERRF]) [5] with clinical phenotypes markedly different from MELAS. A unifying theory to explain the paradox of one gene (such as m.3243A>G) causing multiple, classical syndromes is lacking and remains a yet unexplained characteristic of many mitochondrial diseases.

Clinical and Diagnostic Features

Following the initial description and case series of patients with MELAS [1, 9, 10], there has been an increasing recognition of phenotype variability from oligosymptomatic presentations to significant systemic pathology. The central and classical clinical features, however, remain the triad of lactic acidosis, seizures, and stroke-like episodes. In their initial description, Pavlakis and colleagues noted the combination of seizures and progressive language and visual impairment with evidence of mitochondrial cytopathy (lactic acidosis and ragged red fibers on muscle biopsy). Summarizing the available literature and case reports years later, Hirano et al. [9] and Hirano and Pavlakis [10] confirmed this association and defined the clinical syndrome by three criteria: (1) stroke-like episode before age 40 years; (2) encephalopathy characterized by seizures, dementia, or both; and (3) lactic acidosis, ragged red fibers, or both [9]. Lactic acidosis, either measured in serum or cerebrospinal fluid (CSF), was a near universal finding, occurring in 94 of 101 (94%), as were seizures (97/102, 96%) and stroke-like events

(106/107, 99%) [9, 10].

Mitochondrial diseases are far more common than recognized. While numbers vary depending on methodology and subject group, an overall incidence of 12.48/100,000 in England has been reported [11]. The absolute carrier prevalence of the m.3243A>G mutation has been estimated to be as high as 0.06% [11, 12] or 60/100,000 individuals in the general population. Incidence among a cohort of Finnish children was 18.4/100,000 [13], and a report from Australia reported an even higher prevalence for the m.3243A>G mutation [14]. A retrospective database-based study of Italian individuals carrying the m.3243A>G mutation found that 33 out of 51 patients with stroke-like episodes were male, indicating a possible gender effect [15].

More subtle signs of mitochondrial disease can go unrecognized prior to the onset of stroke-like episodes and resulting neurological impairment. In addition to the classical pathognomonic features of MELAS, other neurological and psychiatric manifestations include: depression, psychosis, migraine headaches, and sensorineural hearing loss. Mood disorders and migraine headaches are particularly common among MELAS patients and in their oligosymptomatic family members. Sensorineural hearing loss is also extremely common in MELAS, to the point of near universality among affected individuals. The presence of hearing impairment, in combination with other classical features, should prompt consideration of the disorder [9, 10].

Many systemic abnormalities are also common among mitochondrial diseases and MELAS in particular. Every organ system can be potentially affected. Short stature, related or unrelated to growth hormone deficiency, is nearly universal in MELAS syndrome. Cardiomyopathy, both hypertrophic and dilated, is a frequent complication, as is Wolff-Parkinson-White syndrome [16]. The specific underlying pathogenesis may be related to dysregulation of energy utilization by developing heart tissues during embryogenesis. The PRKAG2 gene codes for an adenosine monophosphate-activated protein kinase described as a “cellular fuel gauge.” Abnormality of this gene has been linked to the development of Wolff-Parkinson-White syndrome in two families with an autosomal dominant form of the disorder [17]. It has been speculated that mitochondrial disease may lead to an energy-depleted state, impairing the maturation of the atrioventricular insulating ring necessary to generate a normal conductive circuit. Regardless of cause, a full cardiology evaluation and regular, routine electrocardiography are indicated

in all patients with mitochondrial disease. Diabetes mellitus, related to both pancreatic islet cell dysfunction (resulting in insulin dependence) and glucose resistance in peripheral tissues (resulting in type II diabetes mellitus), is nearly universal in mitochondrial disease. Almost all patients carrying the m.3243A>G mutation will develop diabetes mellitus over the course of their lives. Mitochondrial diseases underlie 1–4% of diabetes cases. MELAS should be considered as a potential cause of seemingly isolated diabetes mellitus should other clinical features suggest a mitochondrial cytopathy. Cyclic vomiting also frequently affects patients with MELAS syndrome. Renal, dermatological, hepatic, and pulmonary complications can also, though less commonly, affect patients with MELAS syndrome and other mitochondrial diseases [2].

Stroke-Like Episodes

The term “stroke-like episode” was developed to emphasize the metabolic origin of these events in contrast to conventional strokes that follow an identifiable vascular distribution (Fig. 8.1). Stroke-like episodes seen in MELAS syndrome have an irregular distribution, more consistent with small vessel etiology. Clinically, cases are marked by episodes of at least partially reversible aphasia, hemianopsia, and cortical blindness. One case report of an 8-year-old Japanese girl described a transient ischemic attack-like episode. Her symptoms of blindness, headache, and repeated seizures resolved completely within 24 h with treatment [18]. While stroke-like episodes are often partially reversible, there will typically be a gradual radiological and clinical accumulation of disease burden over time resulting in neurological deficits and eventual dementia. Cortical involvement can be of any size or severity but usually presents in an asymmetric pattern that affects predominantly the temporal, parietal, and occipital lobes. It is often restricted to the cortex with relative sparing of the deep white matter.

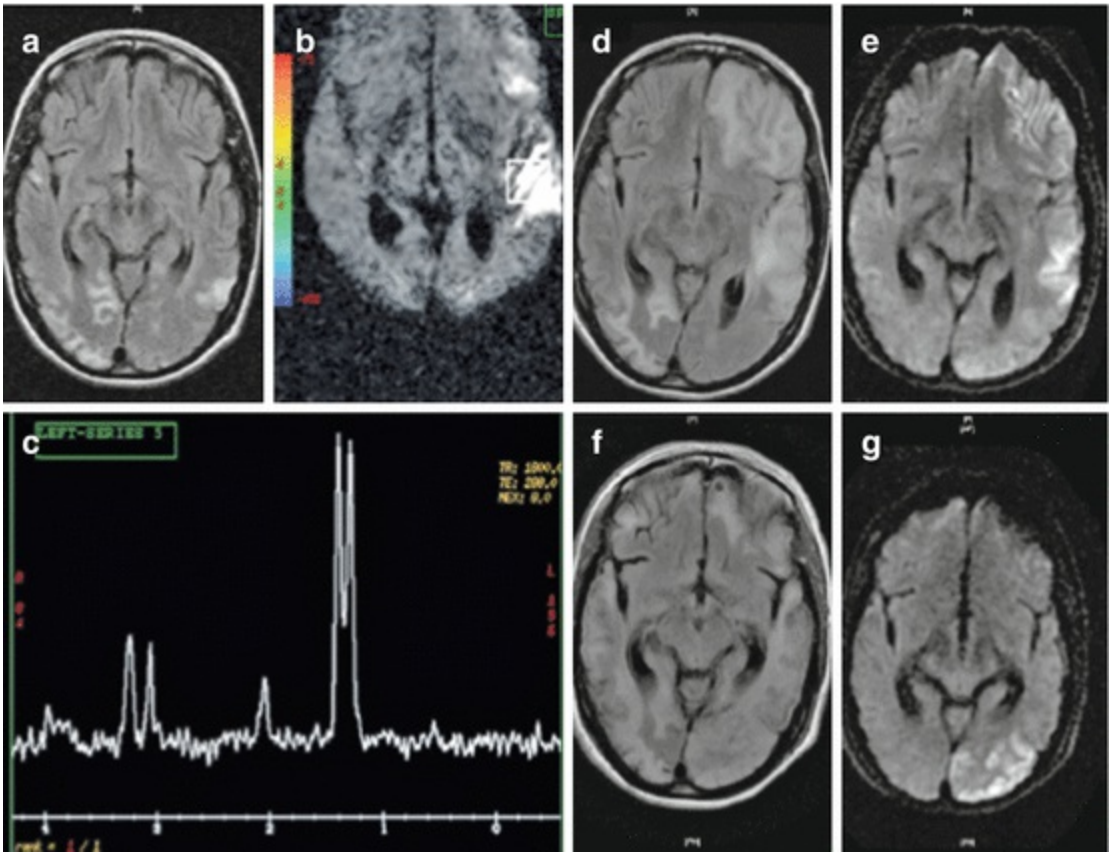

Fig. 8.1 Radiographic features of MELAS syndrome: sequential MRI findings observed in a patient with MELAS syndrome. (**a**) FLAIR imaging sequence from a scan performed early in this patient's disease course demonstrating bilateral areas of increased signal scattered within the cortex of the temporal, occipital, and parietal lobes. (**b**) Sequence demonstrating voxel placement for MR spectroscopy. (**c**) MR spectroscopy demonstrating a large lactate doublet and low NAA-to-creatine ratio. (**d**) FLAIR sequence from a scan 3 years later demonstrates new areas of signal intensity in the left frontal and left parietal regions, in addition to an area of signal abnormality in the right temporal and occipital regions. (**e**) DWI sequence from same scan as (**d**) demonstrates restricted diffusion within the left frontal, temporal, and parietal lobes. (**f**) FLAIR sequence from scan performed 2 years following that shown in (**d**) and (**e**) demonstrating, again, confluent areas of increased signal intensity within the left greater than right frontal, biparietal, bitemporal and bioccipital lobes, as well as mild cerebral atrophy and moderate ventricular dilatation. (**g**) DWI sequence from same scan as (**f**) demonstrating two large new areas of restricted diffusion within cortices of the left occipital and right parietal lobes consistent with progression of the underlying disease. Reproduced with permission from [2]

Radiographic Features of "Stroke-Like Episodes"

The stroke-like episodes seen in MELAS are ischemic events resulting in cerebral injury. The pattern of involvement can be demonstrated using diffusion-weighted (DWI) sequences on magnetic resonance imaging (MRI) (Fig. 8.1). There are asymmetric lesions of the occipital and parietal lobes

that mimic ischemia, except they usually do not respect vascular territories and are often restricted to the cortex with relative sparing of deep white matter [9, 10]. There is a characteristic fluctuation of lesions [19] with repeated imaging over time. MR angiography (MRA) is typically normal. In a review of 31 angiograms in MELAS patients, 11 were abnormal [10]. The most common abnormalities were increased caliber of arteries or veins, or a capillary blush, seen in six subjects. In two patients, there were abnormalities of major vessels [10]. The abnormalities were nonspecific and unlikely secondary to atherosclerosis. More recent studies suggest that there is vasodilatation of cerebral arteries early on in stroke–like episodes as evidenced by changes in computer tomography angiography and MRA [20]. Another study revealed hyperperfusion on arterial spin labeling perfusion MRI even before changes were seen on routine MRI [21]. Finally, quantitative measurements of cerebral extraction fraction using MRI shows reduced utilization of oxygen in stroke-like episode regions at all phases of the episode [22].

Magnetic resonance spectroscopy (Fig. 8.1c) is frequently used to identify elevations of lactate in CSF compartments and brain parenchyma among patients with suspected mitochondrial disease. The most common abnormalities seen with MR spectroscopy in MELAS include a reduced N-acetyl aspartate/creatine ratio and a lactate peak, especially in the stroke-like episode region or cerebral ventricles. The presence of a lactate peak is a sensitive metabolic marker of disease and has been correlated to disease progression and regression [23–26]. There is also a strong correlation between measures of high ventricular lactate and neuropsychological and neurologic impairment [27]. Ventricular CSF may be the simplest and most sensitive site for screening by spectroscopy. With CSF lactate >4.0 mmol/L, the lactate peak is easily detected [28, 29] and therefore serves as a confirmatory test for elevated lactate within the central nervous system.

Pathogenesis of Stroke-Like Episodes in MELAS

The pathogenesis of the signature stroke-like episode seen in MELAS is unclear. Although reported, large vessel disease is rare and may be secondary to abnormal endothelium causing a nidus for thrombus formation [10]. Most stroke-like episodes are felt to be secondary to abnormalities in areas not subserved by large vessels. While several potentially complementary mechanisms have been proposed, none appear to fully explain the clinical

phenotype and relationship with the molecular defect.

Neuronopathy Hypothesis

A mitochondrial neuronopathy may be the underlying cause for seizures, migraines, and stroke-like episodes seen in MELAS. Failure of energy-dependent ion transport due to an OXPHOS defect would result in increased extracellular potassium or glutamate within the synaptic cleft. Under such a hypothesis, increased capillary permeability and neuronal vulnerability would drive neuronal hyperexcitability and the development of the described clinical phenotype. The state of episodic neuronal hyperexcitability may lead to prolonged seizures, a predisposition to migraines, and progressive spread of stroke-like lesions [30]. The neuronal hyperexitability hypothesis is further buttressed by the two imaging studies performed by Ikawa and Yu [21, 31].

Angiopathy Hypothesis

Initially, it was hypothesized that stroke-like episodes were simply a metabolic insufficiency in a brain region that mimicked a stroke. Over the last several years, accumulating clinical insights have suggested that abnormalities in endothelial tissues may play a role in the specific pathophysiology of MELAS syndrome. Unlike other mitochondrial cytopathies, angiopathy has been demonstrated. There is an increased number of enlarged mitochondria with complicated cristae found in the pericytes of capillaries, endothelial cells, and smooth muscle cells of arterial pial vessels and small intracerebral arteries [32]. Endothelial vessels that stain strongly with succinate dehydrogenase, indicative of mitochondrial proliferation, have been reported in pathological specimens from patients with MELAS [33, 34] (Fig. 8.2c and d). This angiopathy appears to mainly affect small cerebral arteries, arterioles, and capillaries. Furthermore, microangiopathy is seen in many patients with mtDNA disease on autopsy of the cerebellum [35].

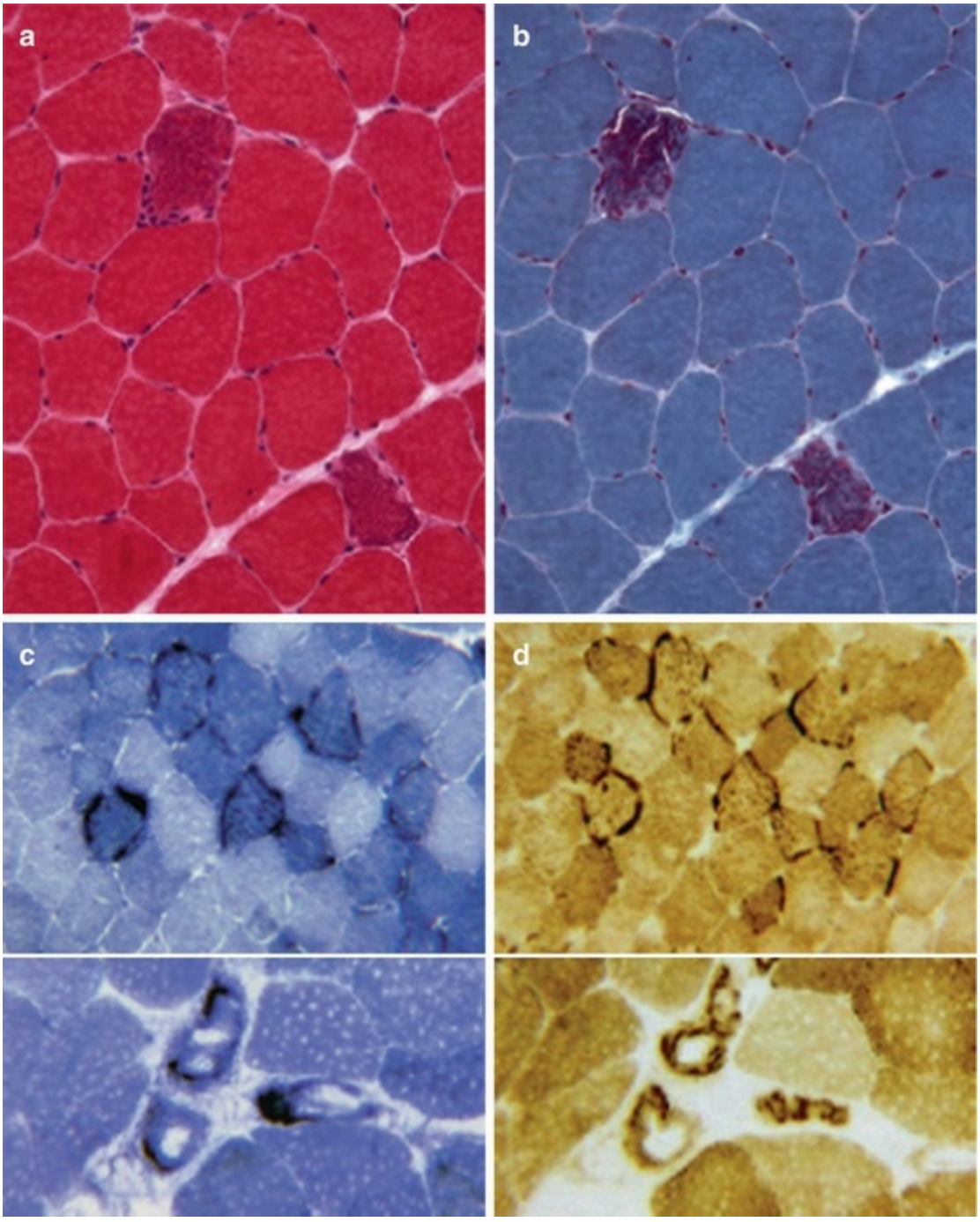

Fig. 8.2 Histopathological features of MELAS syndrome. (**a**) Hematoxylin and eosin staining demonstrates scattered vacuolated muscle fibers containing numerous, small basophilic inclusions. (**b**) Gomori trichrome staining demonstrates similar basophilic inclusions. (**c**) Staining with succinate dehydrogenase of muscle (*top*) and blood vessel walls (*bottom*) shows scattered muscle fibers and blood vessel endothelium with increased staining, suggestive of mitochondrial proliferation. (**d**) COX staining demonstrates muscle cells (*top*) exhibiting decreased, normal, and increased staining. Increased COX staining is also noted in blood vessel walls (*bottom*). Reproduced with permission from [2]

Whether these histopathological abnormalities relate to the stroke-like episodes that typify this disease remains to be demonstrated. Although the medical literature is somewhat conflicting, MELAS patients appear to have reduced vasodilatory capacity, which is suggestive of small vessel dysfunction [36]. Limited evidence suggests that supplementation with l-arginine, a precursor to nitric oxide (NO), may restore this capacity, suggesting a pathological link with this substrate as well as potential for therapy. In 2005, Naini and colleagues proposed that dysfunction of NO production and catabolism was a mechanism underlying the observed angiopathy and nonischemic stroke-like episodes seen in MELAS [37]. In adults, enterocytes of the small intestine are responsible for the bulk of citrulline synthesis, an ATP-dependent process. In MELAS, reduced availability of ATP for citrulline production may lower plasma levels, thus decreasing arginine and NO production. Amid such an environment, it is possible that cytochrome c oxidase (COX; complex IV) activity drives a paradoxical increase in enzymatic activity. This theory is based on the observation that in MELAS, ragged red fibers and blood vessels are typically COX positive, despite seemingly high levels of mutation burden. MELAS patients appear to have a significant amount of residual respiratory chain activity relative to patients with other mitochondrial diseases such as MERRF, where both ragged red fibers and vessel walls are COX negative.

Massive mitochondrial proliferation allows the total COX activity to equal or even surpass that of unaffected individuals. As mentioned earlier, blood vessels in MELAS stain strongly for succinate dehydrogenase, reflective of mitochondrial proliferation and they may also stain strongly for COX [38]. This paradoxical overexpression is a possible cause for the characteristic phenotype seen in MELAS since it may produce localized biochemical derangements. Elevated COX activity may be harmful due to its role in the metabolism of NO and other factors. NO is integral in controlling smooth muscle tone and mediating vasodilation and cerebral perfusion. COX binds NO, potentially displacing oxygen [37]. Supranormal COX levels may result in a relative shortage of NO, leading ultimately to impaired vasodilation and the resultant endothelial dysfunction seen in MELAS. This autoregulation impairment may underlie the development of stroke-like episodes [37]. The low citrulline levels observed in MELAS patients may likewise result from increased conversion of this substrate to arginine to be used as a substrate donor for nitric oxide synthase generation of NO [39].

Alternatively, the overproliferation of mitochondria may be a compensatory mechanism to make up for the potentially abnormal OXPHOS. When this expansion fails to meet the regional metabolic demands of an affected region of tissue, disease is expressed as a stroke-like episode.

Mechanisms of Molecular Pathogenesis

Although the m.3243A>G mutation was identified nearly three decades ago, the molecular consequences of the mutation are still incompletely understood. Muscle cells with the m.3243A>G mutation grown in tissue culture demonstrate respiratory deficiency [40]. King and Attardi developed a cell line called rho-0 that replicates in the absence of mtDNA [41]. The rho-0 cells can be fused with cytoplasts harboring mutant mtDNA to form cybrids. Cybrids with greater than 95% m.3243A>G mtDNA showed decreased rates of protein synthesis, lower levels of steady-state mitochondrial translational products, reduced oxygen consumption, and increased amounts of an unprocessed RNA fragment called RNA-19, which contained the mutant gene [42]. Other investigators have demonstrated that high levels of the mutant tRNA decreased aminoacylation (covalent attachment of leucine to the tRNA) and were associated with hypomodification of the D-stem. These alterations may contribute to decreased protein synthesis [43–45]. An alternative theory also based on cybrid work is that the mutant $tRNA^{Leu\ (UUR)}$ is less efficiently modified at the wobble base due to lack of posttranscriptional methyltaurine modification of the anticodon wobble base [46, 47] This causes leucine codons to be misread as phenylalanine codons [46]. Cultured myoblasts from a patient with the m.3243A>G mutation revealed evidence of both amino acid misincorporation and moderate reduction of protein synthesis [48].

Diagnostic Evaluation

Laboratory evaluation is a logical initial step in the diagnostic work up for suspected mitochondrial cytopathy. Elevated lactate, although the *sine qua non* of most mitochondrial diseases and MELAS in particular, is a nonspecific marker of metabolic derangement of any cause. There are many other potential causes of lactic acidosis including organic acidurias and aminoacidopathies, defects of the citric acid cycle, pyruvate dehydrogenase

complex deficiency, ischemia and hypoxemia, and, of course, laboratory error from improper handling of the specimen. Demonstration of an elevated blood lactate level above 2.2 mmol/L is suggestive of a mitochondrial disorder, but the blood sample should be free flowing, preferably arterial, and analyzed immediately since there will be a spurious elevation if processing is delayed. We typically obtain blood in the resting state but postexercise evaluation and postprandial assessments might be more sensitive. Blood lactate elevation is not specific, and a normal blood lactate does not exclude the diagnosis of MELAS. Lumbar puncture is an invaluable diagnostic tool and should be readily performed. Elevation of CSF lactate above 2.2 mmol/L increases level of suspicion for MELAS. CSF lactate elevation is more sensitive than serum, but if the fluid is bloody, a spurious elevation may occur and as such even CSF lactate elevation cannot be deemed entirely sensitive nor specific.

Muscle Biopsy

The presence of ragged red fibers on muscle biopsy is classically synonymous with MELAS and other mitochondrial cytopathies. Under microscopic examination, affected cells appear red with irregular borders after Gomori Trichrome staining, thus giving the name “ragged red fibers”. This is due to the presence of numerous subsarcolemmal basophilic inclusions, which is indicative of mitochondrial accumulation. On the other hand, hematoxylin and eosin staining shows scattered vacuolated muscle fibers with a clear surrounding rim and the same ragged red fibers stain strongly for succinate dehydrogenase. As mentioned previously, succinate dehydrogenase (complex II) is entirely comprised of nuclear-encoded protein subunits and is therefore a good marker for the compensatory mitochondrial proliferation that seems to occur in MELAS and other mitochondrial cytopathies.

Molecular Diagnostics

The proportion of mutant mtDNA may vary widely depending on the type of tissue sampled [49], which presents both diagnostic and prognostic dilemmas for clinicians. Blood leukocytes have traditionally been the most common specimens analyzed through molecular diagnostics, although individuals may have detectable mutation in muscle cells but undetectable mutant load in

blood [50, 51]. Urine sediment, cheek mucosa, and skin fibroblasts are also frequently assayed. Urine sediment cells and cheek mucosa tend to carry the highest mutation loads and therefore may be more suitable tissues for the diagnosis of a mitochondrial mutation [49].

Several methods have been developed for mutation detection and quantification. These include polymerase chain reaction (PCR) with restriction enzyme digestion followed by gel electrophoresis and band intensity quantification (PCR-restriction fragment length polymorphism) [52], PCR with peptide nucleic acid clamp and sequencing [53], and PCR with subsequent hybridization using allele-specific oligonucleotide probe [54]. The preferred technique is quantitative real-time PCR with allele-specific primers [55]. Other techniques include PCR with a fluorescence-labeled primer followed by restriction enzyme digestion, separation, and detection by capillary electrophoresis [56].

Treatment

There are no curative treatments for mitochondrial disease. Therapeutic options for MELAS and other mitochondrial cytopathies remain anecdotally supported without compelling or definitive evidence of efficacy. A trial assessing the role of dichloroacetate in the treatment of MELAS was terminated prematurely due to a significant incidence of peripheral nerve toxicity within the treatment group [57]. The widespread and unexpected toxicity of this agent contrasted sharply with the significant benefit of dichloroacetate reported in open-label, uncontrolled studies, providing a striking note of caution to those endorsing therapies based on empiric usage and open-label study. Symptomatic management (i.e., addressing cardiac, renal, growth, and nutritional issues and treating seizures) remains the mainstay of therapy. Supplementation with a so-called mitochondrial cocktail is commonly prescribed in basic care for children and adults with mitochondrial diseases.

The “mitochondrial cocktail” strategy is designed to maximize function of the OXPHOS pathway and reduce oxidative stress. While the specific composition of cofactors and supplements varies widely among practitioners, most treatment regimens involve a combination of creatine, coenzyme Q_{10}, and alpha-lipoic acid, in addition to vitamin supplementation of riboflavin, thiamine, vitamin C, vitamin E, and biotin. Improved muscle strength during

aerobic activity following supplementation with creatine monohydrate has been reported in mitochondrial disease patients [58–60]. Studies utilizing coenzyme Q_{10} have been somewhat conflicting, with some reporting benefits [61–64] and other larger studies failing to replicate initial positive findings [65, 66]. Numerous case reports, open trials, and retrospective studies of various combination therapies appear in the literature [67]. However, there is a paucity of randomized controlled trial data demonstrating efficacy of this approach and no consensus regarding appropriate dosing of the respective cofactors, supplements, and vitamins that comprise the cocktail.

Idebenone, an analog of coenzyme Q_{10}, has also gained attention as a potential therapy in mitochondrial disease in general and MELAS in particular [64]. This agent is approved in Canada for the treatment of Friedreich ataxia, a disease of the mitochondria. Frataxin, the defective protein in Friedreich ataxia, is implicated in mitochondrial iron-sulfur metabolism [68].

As mentioned, anecdotal reports have emerged suggesting a possible role for l-arginine in the modulation of the vascular symptoms of MELAS syndrome. Paradoxically increased COX activity and its effect on NO levels may underlie the angiopathy and stroke-like episodes that typify MELAS syndrome. In addition, l-arginine may affect the uptake of glutamate and release of gamma-aminobutyric acid, resulting in increased production of ornithine [69]. Recognizing this potential association, individual case reports [70, 71] and a small case series [72] have reported the value of intravenous l-arginine supplementation in reducing the severity of stroke-like events in an acute setting. Another case report monitored the effects of oral l-arginine taken with idebenone over 27 months suggesting long-term safety and prevention of stroke-like episodes [73]. In a larger prospective trial, though unblinded and unrandomized, l-arginine given as an oral supplement over a period of up to 2 years was noted to significantly improve endothelial function [36], with normalization of flow-mediated systemic arterial vasodilation and improved cerebral blood flow. More recently, a small case control study suggested improvement in aerobic capacity and muscle metabolism by supplementation [74]. Stable isotope infusion techniques have found that citrulline is superior to arginine in increasing NO production, potentially having a greater therapeutic effect in patients with MELAS [75, 76].

Oral taurine supplementation has also come under consideration for the

prevention of stroke-like episodes in MELAS. As discussed, deficiencies in post-transcriptional taurine modification affects wobble pairing and disrupts the ability to decipher codons thereby affecting mitochondrial protein synthesis [77]. The supplementation of taurine improved oxygen consumption in MELAS cybrid cells and prevented new stroke-like episodes in two reported patients [78].

The administration of corticosteroids in acute events to enable recovery has been recommended. IV dexamethasone was given in the case of a 10-year-old girl who presented with acute onset headache, vision changes, and right arm shaking. The function of corticosteroids in acute management is believed to stabilize the blood-brain barrier, reduce tissue edema, and improve perfusion to damaged areas of the brain [79]. Alternatively, regional hyperperfusion during a stroke-like event may lead to neuronal loss in the first place, warranting corticosteroid use to decrease inflammatory processes and prevent further cell damage [80]. Whatever the mechanism, the efficacy and role of this intervention has yet to be clarified.

The ketogenic diet, which utilizes fat rather than glucose as the primary fuel of cell energy production, is traditionally used for the treatment of refractory epilepsy. However, one case report hypothesizes benefits of this diet in MELAS patients through the improved function of respiratory chain complexes, thereby affecting seizure control and decreasing stroke-like episodes [81].

Lastly, prevention of MELAS may become an option in the future by way of preimplantation genetic diagnosis or tRNA gene modification. Female carriers wishing to conceive a healthy child free of inherited mitochondrial disease may undergo preimplantation genetic diagnosis to select for an embryo with mutation percentage below the threshold of clinical expression [82]. In another study, the introduction of recombinant tRNA encoding human mitochondrial leucyl-tRNA synthetase into affected human cybrid cells resulted in improved mitochondrial translation, production of cell respiratory complex subunits, and cellular respiration [83]. These reports target the molecular pathogenesis of MELAS and represent possible therapy in the future. Yet, accessibility and cost are just two among many issues barring the practicality of gene-based intervention at this time.

When to Think of MELAS Syndrome

MELAS should be a consideration in any child or young adult who presents with a stroke-like event, particularly if any of the associated clinical features are present: hearing loss, seizures, short stature, cyclic vomiting, diabetes mellitus, and cardiac conduction abnormalities. Maternal family history of the same should also raise suspicion of a potential mtDNA-mediated disease. While the presence of a pathognomonic stroke-like event should prompt immediate consideration of the diagnosis, it is not uncommon for such episodes to occur well after clinical symptoms clearly suggestive of the diagnosis become present such as seizures and early dementia. Awareness and consideration of mitochondrial disease and MELAS in particular, are vital in making a prompt and accurate diagnosis.

Conclusion

While recent years have seen an expansion in understanding the pathophysiology of MELAS syndrome and the stroke-like episodes associated with this disorder, the central etiology of this disease remains uncertain and is a topic of ongoing speculation. Despite a troubling lack of effective therapy, recent insights into the pathogenesis of MELAS have provided hope and direction for the development of therapy targeting, if not the genetic abnormality, then at least the downstream pathophysiology. There is great optimism that by building on the advances of the last three decades, we are rapidly approaching an era of effective treatment for this devastating disease.

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Footnotes

1 http://www.mitomap.org

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9. Sickle Cell Disease

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Introduction

Sickle cell Disease (SCD) and related hemoglobinopathies are disorders of the red cell, specifically the hemoglobin. These group of blood disorders includes sickle cell anemia (SCA), sickle cell hemoglobin C disease, sickle

cell with beta thalassemia, and the thalassemias. These disorders are generally the result of an abnormality in hemoglobin quality (SCD) or quantity (thalassemias). Individuals with these hemoglobinopathies commonly suffer damage to vital organs such as the central nervous system, the spleen, the kidneys, the lungs, and the heart as a result of microvascular vaso-occlusion, poor tissue oxygenation or chronic oxidative and inflammatory damage from accumulation and/or metabolism of by-products from breakdown of the resulting abnormal red blood cells [1]. Hemoglobinopathies generally present in childhood, and onset of signs and symptoms tend to coincide with class switching of the *globin gene* from fetal (*γ-globin*) to adult (*β-globin*) globin—which is the affected form. The net results is abnormality in quality and quantity of hemoglobin [2]. The clinical phenotypes associated with hemoglobinopathies are syndromic in their characteristics, but the most dramatic phenotypes with far reaching sequelae is stroke and other cerebrovascular diseases. Off all the hemoglobinopathies, sickle cell disease is the most commonly associated with stroke and cerebrovascular diseases.

Pathophysiologically, the mutated hemoglobin (HbS) polymerizes reversibly when deoxygenated to form a gelatinous network of fibrous polymers (tactoids) that stiffen the RBC membrane, increase viscosity, and cause dehydration due to potassium (K^+) leakage and calcium (Ca^{2+}) influx. These changes also give red cells their characteristic sickled shape. Sickled cells lose the pliability needed to traverse small capillaries, and possess altered sticky membranes that are adherent to the endothelium of small vessels. These abnormalities provoke unpredictable episodes of microvascular occlusion, premature RBC destruction and cerebrovascular events due in part to occlusion of the cerebral vasculature [1].

Although the sickle mutation is the same, the clinical and thus phenotypic effect such as cerebrovascular complications are variable. The observed variability in clinical course, even within affected family members, attests to the heterogeneity of influences, including mutational heterogeneity (i.e., sickle haplotype differences) at the HbSS locus, environmental factors, epigenetic modifications, or genetic variability at other unlinked loci [3]. This has prompted the search for other biochemical pathways in addition to the underlying hemoglobinopathy [4]. Sickle cell disease (SCD) is recognized as a systemic inflammatory state [5], and because the risk and severity of stroke and other cerebrovascular diseases has been associated with altered plasma

concentrations of specific inflammatory mediators, many recent studies on stroke and SCD have been focused on isolating specific markers of inflammation that may predispose certain individuals with SCD to stroke [4, 6–8].

Prevalence and Incidence

By studying the natural course of stroke in patients with and without SCD, we know that hematologically normal black Americans have twice the stroke risk of white Americans, and the risk of stroke for a child with SCD is 333 times greater than that of a healthy child without SCD or heart disease, while adults with SCD have a 76 times increased risk for SCD compared those without SCD. In children with abnormal transcranial Doppler (TCD) velocities, defined as >200 cm/s, the risk of stroke is 3000 times greater [9, 10]. A previous study of 29 families with SCD showed that having a child with elevated TCD velocities increased the odds of the sibling having elevated TCD velocities by a factor of 50 [11]. Hence, taken together, SCD is the most significant risk factor for stroke in children in the US [12].

In the SCD population, the risk of stroke is highest during the first decade, most marked between ages 2 and 5 years, reaching an incidence of 1.02% per year [13]. The risk is lowest before age 2 years, presumably due to the protective influence of fetal hemoglobin (HbF). The risk is also lower among individuals with alpha thalassemia occurring concurrently with the homozygous sickle beta-globin mutation [14]. Approximately 11% of all homozygous hemoglobin S (HbSS) patients will have a clinically apparent strokes before the age of 20 years, [9] and 40% of those will experience a second ictus within 3 years of their initial insult [15]. The risk of clinically apparent stroke increases to 24% by age 45 years [9], and another 17–22% of patients with SCD will suffer from silent cerebral infarctions seen only on magnetic resonance imaging (MRI) [16] within their lifetime.

Stroke subtype onset varies with age of SCD patient. The incidence of ischemic stroke, which constitutes about 54% of all strokes in SCD, is bimodal, with the greatest peak occurring in the first decade and a second peak occurring after the age of 30 years. The incidence of hemorrhagic stroke in patients with SCD is highest during their third decade of life [9].

Stroke Risk Stratification Of SCD Patients

Cerebral infarction in SCD is typically, associated with an occlusive vasculopathy. Large vessel stenosis in the distal internal carotid artery and proximal middle and anterior cerebral arteries has been demonstrated on conventional angiogram, computed tomography (CT) angiogram, MR angiogram and, more routinely now, by using transcranial Doppler (TCD) ultrasound, which is both less invasive and less expensive. TCD detects increased flow velocities across the narrowed segments of vessel. Per Bernoulli's law, blood flow velocity is directly related to blood flow and inversely related to the diameter of the vessel [17]. Due to severe, chronic anemia in patients with SCD, their baseline blood flow velocities are already elevated in the absence of stenosis; this strengthens the reasoning that high TCD velocity might represent a maladaptation to anemia [18].

Regular screening of children aged 2–16 with SCA using TCD ultrasonography identifies patients who have an increased risk for developing primary stroke. Children whose time-averaged mean velocity (TAMV), measured in the distal internal carotid artery (ICA) or middle cerebral artery (MCA), is abnormal (defined as TAMV ≥200 cm/s) have approximately a sixfold higher stroke risk than those with normal TCD velocities (<170 cm/s) [17]. While children with abnormal TCD velocities have the highest risk for developing cerebral infarction, stroke also occur in those with conditional TCD velocities (TAMV 170–199 cm/s). The stroke risk among patients with conditional TCD velocities is estimated to be 2–5% per year, compared to 10% per year for children with abnormal velocities who do not get regular blood transfusions [19]. In a recently published case series, it was also demonstrated that a subset of children with SCD with abnormally low TCD velocity have a high risk for cerebrovascular events—overt stroke, TIA or silent infarct [20].

Adams et al. identified a correlation between increased TAMV and stroke risk in children at least 2 years of age, but their study did not include children who were younger than 2 years old. A subsequent trial, the Pediatric Hydroxyurea Phase III Clinical Trial (BABY HUG Trial) investigated the feasibility of obtaining TCD velocities in patients 6–24 months of age, and they were successful in 94% of subjects. However, there are not yet any consensus velocity benchmarks that can identify patients in this age group at increased risk of stroke. Determination of whether the TCD values in this

very young cohort of infants with SCA can be used to predict stroke risk later in childhood will require analysis of follow-up TCD's in this cohort. [21]. This is particularly important, because in a retrospective review of clinical data from a single practice groups, it was shown that despite having normal TCD velocity, about 27% (18/65) of children with MRI and MRA data have had a silent infarct and varying degrees of cerebral vasculopathy [22].

Pathophysiology

Anemia/Hypoxemia

The abnormality in β-globin sub-unit resulting from the sickle mutation, patients with SCD, leads to polymerization of Hb molecule during times of deoxygenation, increasing the likelihood for hemolysis and the attendant consequences of hemolytic anemia. Hemolytic anemia is a process in which intravascular hemolysis releases free hemoglobin and arginase into plasma. The free hemoglobin scavenges nitric oxide (NO), and the arginase depletes the plasma of L-Arginine, the obligate substrate for NO synthase. Both processes severely reducing NO bioavailability [23]. NO in normal vasculature promotes vasodilatation and inhibits platelet aggregation and endothelial adhesion, providing protection from cerebrovascular ischemia.

Because of hemolytic anemia, SCD patients live in a chronically anemic state, and subsequently have decreased oxygen-carrying capacity in serum, making them susceptible to end-organ ischemia with only slight shifts in normal oxygenation status. This leads to a compensatory increase in cerebral blood flow (CBF), in order to offset the relative hypoxic state and avoid ischemia [24]. Quinn et al. investigated the relationship between daytime Hb oxygen saturation measured by pulse oximetry (SpO2) and blood flow velocity measured in the middle cerebral arteries bilaterally with TCD ultrasonography and confirmed that SpO2 significantly inversely correlated with TCD velocity, concluding that Hb oxygen saturation is a determinant of TCD velocity and a risk factor for stroke in children with HbSS [25]. Loss of cerebrovascular O2 responsiveness in SCD patients inhibits the autoregulation required to maintain adequate downstream perfusion in areas affected by significant vasculopathy, and thus confers further increased risk of cerebral infarction. In children who develop abnormal TCD velocities, their velocities depart from this "compensated anemic state," first in a global

manner and then later focal lesions may develop. The trigger of this physiologic change is largely unknown.

Reduction in NO Availability

A reduction in NO-bioavailability leads to impaired cerebrovascular hemodynamics [26]. Reduction of cerebrovascular CO2 responsiveness is thought to play an important role in the pathogenesis of cerebral microangiopathy, and subsequently increased susceptibility of ischemic strokes, however, the mechanism is unknown.

Kim et al. questioned whether or not cerebrovascular responsiveness in patients with SCD is affected by and/or directly related to hemolysis. In a small prospective study, they were able to show that dynamic cerebral autoregulation was impaired in SCD patients, but did not appear to be related to hemolysis directly [27]. Nur et al. replicated these findings in a second prospective study, concluding that even HbSS patients without a history of symptomatic stroke (i.e., having a silent cerebral infarct) had an impaired cerebrovascular CO2 responsiveness, suggesting a reduced cerebrovascular reserve capacity in HbSS patients at their baseline that could play a role in the pathophysiology of stroke [26], but that there was no relationship between markers of hemolytic anemia (LDH and total bilirubin) and cerebrovascular CO2 responsiveness.

Although markers of hemolytic anemia, as well as baseline Hb level were not found to be strongly correlated with impaired cerebrovascular hemodynamics, patients treated with hydroxyurea, which has been shown to limit hemolytic anemia and subsequently maintains higher Hb levels and lower LDH and total bilirubin levels, had a higher cerebrovascular reserve capacity, and subsequently had better cerebrovascular CO2 responsiveness [26]. There is a significant amount of research supporting the importance of hemolysis [28] in SCD in general**,** although controversy [29], i.e., whether hemolysis is overtly or covertly related to stroke risk.

Platelet Aggregation

Altered platelet function might also plays a role in SCD associated stroke. A greater proportion of platelets is activated during steady state in patients with SCD, and this activation accelerates during vaso-occlusive crisis (VOC). The exact inciting mechanism and/or the clinical consequence remains unknown,

but platelet activation is thought to potentially play a role in the development of chronic vascular complications, such as pulmonary arterial hypertension, by secreting mitogenic and vasoactive substances that promote intimal hyperplasia [23]. In patients without SCD, platelet-derived growth factor has been shown to play a fundamental role in the pathogenesis of plexogenic pulmonary hypertension, and pathologic platelet activation likely contributes to the in situ thrombosis seen in pulmonary hypertension [23]. In a recently published study using plasma sample from a biorepository National Institute of Health funded stroke prevention in SCD clinical trial or STOP study, it was shown that patients with SCD who are at risk for stroke have high serum of biomarkers of coagulopathy and thrombin generation. In addition, they also have elevated levels of platelet derived growth factors (PDGF-AA) and brain derived neurotropic factors; both of which correlated with presence of cerebral vasculopathy, measured as high TCD velocity [7, 8].

Endothelial Activation

High levels of biomarkers of endothelial activation, including soluble vascular cell adhesion molecule-1 (sVCAM-1) have been reported in SCD [23]. VCAM-1 regulates the attachment and migration of leukocytes and plays a dominant role in the development of vascular disease in the general population. VCAM-1 is up-regulated in response to sickle erythrocytes and appears to be involved in the pathophysiology of microvascular occlusion in SCD [16]. Histopathology of pulmonary vasculature in patients with pulmonary HTN in patients with or without SCD shows obliterative vascular smooth-muscle proliferation, resulting from vascular stenosis, abnormal endothelium, and in situ thrombosis. VCAM-1 is thought to be involved in this process. As such, it has been postulated that the mechanisms that leads to pulmonary vasculopathy are similar if not identical to the mechanisms that lead to cerebral vasculopathy. Further, in a recently published study using the prior mentioned STOP study biorepository, Hyacinth et al., reported that SCD patients with high TCD and thus a high risk for stroke, had higher serum levels of sVCAM-1, in addition to elevated level of other markers of endothelial activation and thrombogenecity [6].

RBC Dehydration

Hypoxia-induced gelation of HbS deforms the erythrocyte and its membrane

and causes massive cation loss [24] through disruption of the sickled reticulocytes' ability to maintain normal gradients of K+ through activation of Ca2+-activated K+ transport channel (Gardos channel), K+-Cl- co-transport cation channels [30] and the Na+ pump [31]. This leads to cellular dehydration and further structural deformities as well as endothelial activation, increasing adhesive interactions between the sickled cells, endothelial cells, and leukocytes [30]. The concomitant lack of deformability and enhanced stickiness lead to obstructive adhesion of sickle cells to each other and to vascular endothelium. The resultant ischemia from mechanical obstruction, reperfusion injury, and endothelial cell damage leads to leakage of von Willebrand factor, platelet clumping, cytokine release, and attraction of granulocytes, macrophages, T cells and invariant natural killer T (iNKT) cells to areas that become both infarcted and inflamed, in turn causing further hypoxia and acidosis and, consequently further sickling [9].

Role Of Inflammation in Stroke With SCD

Recent studies among children with sickle cell disease at risk for stroke shows elevated serum or plasma levels of pro-inflammatory cytokines such interleukin 1β [4]. Inflammatory biomarkers are thought to be involved in the underlying pathobiological mechanism of stroke in SCD [32].

Inflammatory Vasculopathy

The vessels that constitute the Circle of Willis (CoW) are predisposed to developing a vasculopathy in patients with SCA. The distal ICA and proximal MCA are the most common vessels affected. CoW vasculopathy is thought to be causal, as the strokes tend to be due to thrombosis occurring over the area of vessel wall abnormality. Milbauer et al. postulated that SCD patients who develop CoW disease, and therefore are at increased risk for ischemic stroke, have inherited different polymorphisms affecting endothelial gene expression when compared to SCD patients without CoW disease. They proposed and tested the hypothesis that markers of inflammatory signaling would differ between SCD patients at risk for stroke compared to SCD patients with minimal stroke risk. In a prospective study, they found and reported an exaggerated inflammatory signaling response on the part of the at-risk subjects, and concluded that the biological pathophysiology for CoW

disease most likely involves increased inflammatory signaling [5].

Inflammatory Mediators

Aside from the absence of lipid deposition and plaque formation, many of the histopathologic findings in cerebrovascular lesions in SCA resemble those found in stroke patients in the general population. In fact, atherosclerotic stroke in non-SCD patients, is now believed to be a result of chronic inflammation, involving pathways of immune regulation, thrombosis, and cellular adhesion. These same pathways are likely to contribute to stroke in SCA [16]. Hyacinth et al., recently showed that SCD subjects enrolled in the STOP study with high TCD all have high levels of inflammatory markers compared with control SCD patients without high TCD. While those who received blood transfusion and ultimately experience a lowering of their TCD velocity and did not develop stroke had significantly lower serum levels of inflammatory markers compared with those who did not receive blood transfusion [6].

Vascular Cellular Adhesion Molecule (VCAM)

One particular group of inflammatory mediators of particular interest are the cell adhesion molecules (CAMs), specifically vascular cell adhesion molecule 1 (VCAM-1). VCAM-1 coordinates inflammatory response by recruiting leukocytes and in turn activating lymphocytes, and is postulated to play a critical role in the pathogenesis of HbSS disease. In vitro, sickle erythrocytes adhere to cytokine-stimulated or -transfected VCAM-1 on endothelial or COS cells via VLA-4 (alpha-4 beta-1 integrin) expressed on leucocytes. VCAM-1 binding to VLA-4 is dependent on the presence of endothelial activation [33]; which is abundant in SCD and thus the, conceivable to reason that this process plays a role in SCD associated vasculopathy. Perfusion with sickle erythrocytes induces VCAM-1 expression in cultured endothelial cells [34, 35]. In addition, elevated levels of soluble VCAM-1 have been detected in the plasma of patients with HbSS disease at baseline and during episodes of acute chest syndrome [36] and recently in the presence of high TCD velocity, a marker of stroke risk [6]. This has also been observed in non–HbSS disease patients with acute stroke, as well as in a small series of six adult stroke autopsies, which were notable for high expression of VCAM-1 restricted to areas of brain ischemia.

Furthermore, inhibition of integrin alpha-4beta-1 ($\alpha_4\beta_1$ or VLA-4)a major counter-receptor for VCAM-1, protects against ischemic injury in a rat model of transient cerebral ischemia. Based on these data, Taylor et al. selected VCAM-1 as a candidate gene for study in HbSS disease [3].

Taylor et al. were able to identify a total of 33 single nucleotide polymorphisms (SNPs) by sequencing the entire coding region of the VCAM1 locus, and subsequently analyzed distinct coding regions for healthy individuals and compared them with a population of patients with clinically apparent stroke in HbSS disease conducted in a cohort derived from a single institution in Jamaica (51 symptomatic cases and 51 matched controls) [3].

Of the 10 SNPs analyzed in their pilot study of a Jamaican SS population, one was informative. The wild-type VCAM-1G1238 allele was more common in the stroke group compared with the control group, suggesting that the C variant could be protective against stroke in SS disease. Analysis by allelic frequency showed C1238 alleles to be significantly reduced among stroke cases versus controls. Based on genotype frequencies for the VCAM-1 C1238 allele and an observed 65% reduction in the prevalence of stroke, the estimated proportion of symptomatic stroke cases that might be prevented for carriers of this genetic marker could be as high as 11.9% [3].

While Taylor et al. identified a variant in single nucleotide polymorphisms (SNPs) within the VCAM-1 gene locus that appeared to be protective, Hoppe et al. expanded on this finding by surveying 104 polymorphic markers among 65 candidate atherosclerotic, prothombotic, and pro-inflammatory genes in a well-characterized population of children enrolled in the Cooperative Study of Sickle Cell Disease (CSSCD) [16].

The CSSCD was a national, multicenter study designed to define the natural history of sickle cell disease by following more than 4000 patients with SCD. From that population, 230 patients who had HbSS and MRI-documented asymptomatic and symptomatic cerebral infarction were chosen for DNA analysis. These patients were further subdivided as having either large vessel (LV) or small vessel (SV) disease based on an algorithm that took into consideration their MRI/MRA findings, infarct size, and location. Children in the CSSCD newborn cohort who had a normal MRI at age 10 were included as the “control” subjects [16]. TCD velocities were not measured as part of the CSSCD protocol. Among the 104 polymorphisms that were examined, 57 were sufficiently informative for statistical testing. The multivariate model accounted for 19% of the overall variance ($P <$

0.0001). The IL4R 503*P*, ADRB2, 27*E*, TNF(−308)*A*, VCAM-1(−1594)*C* and LDLR *Nco*I variants revealed distinctive associations when analyzed by stroke subgroup [16].

In the large vessel stroke subgroup, both the IL4R 503*P* variant and HLA-A variation predisposed to stroke, whereas ADRB2 and 27*E* and TNF(-308)*A* were associated with protection from stroke. In the small vessel stroke subgroup, the VCAM-1 (-1594)*C* variant, HLA-DPB1, and HLA homozygosity effects predisposed to stroke, while the LDLR (exon18)*Nco*I variant appeared protective [16].

Hoppe et al. studied SCD patients from the multicenter Stroke Prevention Trial in Sickle Cell Anemia (STOP) with our without large vessel stenosis. Samples were genotyped for 104 polymorphisms among 65 candidate vascular disease genes. Genotypic associations with risk of large vessel stroke were screened using univariate analysis and compared with results from their original study. Joint analysis of the two study populations was done using multivariate logistic regression [16, 37]. Of the SNP associations previously identified in their original study, the TNF (-308)G/A association with large vessel stroke remained significant and the IL4R 503 S/P variant approached significance in the joint analysis of the combined study populations. Homozygosity for the corresponding TNF (-308) G allele was associated with a > 3-fold increase risk of large vessel disease (OR = 3.27; 95% CI 1.6–6.9; $P = 0.006$). Unadjusted analyses also revealed a previously unidentified association between the LTC4S (-444) A/C variant and large vessel stroke risk. The TNF (-308)A allele was again found to be protective against stroke, as was the LTC4S(-444)C allele, and the risk-conferring effect of the IL4R 503P variant only approached significance [37].

Tumor Necrosis Factor

The TNF gene has been linked to increased susceptibility to a variety of conditions characterized by inflammation. Several studies have documented a TNF (-308) allelic association with ischemic stroke, but the results have not been consistent across populations [37].

In their replication study, Hoppe et al. also showed an association of TNF(-308)G/A with large vessel stroke risk in children with SCA . The compelling nature (OR = 3.27; 95% CI 1.6–6.9; $P = 0.006$) of their findings, suggests that the association is unlikely to be spurious. Although they admitted that although they hypothesize that the TNF(-308)G/A SNP itself

has a true effect on large vessel stroke risk, they cannot rule out the possibility that another marker in linkage disequilibrium (LD) with this SNP is causative, and further studies of the LD patterns in this region and haplotype analyses are needed to determine whether it is indeed the TNF locus or other genes in linkage disequilibrium that are responsible for this association [37].

Thus, the proinflammatory TNF gene may directly affect predisposition to stroke in children with SCA, since they show elevated baseline levels of several inflammatory markers, including TNF. In vitro gene expression studies have shown sickled red blood cells, either directly or indirectly, promote endothelial cell upregulation of the TNF gene [38]. Jison et al. demonstrated differential peripheral blood mononuclear cell expression of 112 genes, including IL-15 which induces TNF production, in steady-state SCD patients [39].

Interleukin 4R

Hoppe et al. were able to replicate their initially reported IL4R 503P association with large vessel disease, although the increased risk of large vessel disease associated with the IL4R 503P variant did not reach statistical significance after adjustment for multiple testing. However they comment that the magnitude and direction of this association were similar to the results reported in their original study [37].

Leukotriene C4 Synthase (LTC4S)

The LTC4S (-444)C variant has been associated with protection from large vessel disease [37]. The mechanism of for the role LTC4S gene variant in influencing stroke risk in SCA is less clear, but previous studies have shown that the (-444)C variant upregulates LTC4S mRNA expression, increasing the synthesis of proinflammatory leukotrienes, which has been associated with increased mean carotid artery intimal-medial thickness [40]. Thus while studies suggests a role for this gene variants in protection from large vessel stroke in SCA, the documented effect of the gene product is a direct opposite.

Interleukin-1 Beta (IL-1 Beta)

Animal models of cerebral ischemia have shown a key role for the IL-1

family in regulating blood flow in the presence of cerebral ischemia as well as activating cerebral vascular endothelial cells to produce adhesion molecules and chemokines that increase recruitment of inflammatory cells. Cerebral hypoxia stimulates the production of IL-1 beta from microglia, astrocytes, neurons and endothelial cells, with some contribution from peripheral immune cells. The IL-1 beta secretion prompts cerebral vessel endothelial production of chemokine ligand 2 (CCL2) chemokines as well as increased expression of intercellular adhesion molecule-1 (ICAM-1), E-selectin and P-selectin, in effect promoting acute inflammation [4].

In a study published in 2010 by Asare et al., the association of inflammation with the risk of developing stroke in children with SCD was demonstrated. In this study, they utilized serum samples collected at baseline from participants in the STOP study and measured levels of inflammatory biomarkers. The fully adjusted logistics regression model and subsequent receiver-operator-characteristics (ROC) curve, indicated that baseline serum IL-1β levels was significantly lower among children who developed stroke compared to controls with SCD or with SCD and high TCD velocity. They concluded that IL-1β was not only protective against development of stroke, but was a good predictor of the likelihood of stroke in this sample [4].

There is also evidence to suggest deleterious role for IL-1β in the pathogenesis of cerebral ischemia, albeit in non-SCD settings. For example, experimental transient global cerebral ischemia in rats leads to increased IL-1 beta mRNA and protein and increased brain damage occurs when IL-1 beta is administered to rats prior to inducing cerebral ischemia [41]. Inhibiting IL-1 beta by its naturally occurring competitive inhibitor, interleukin-1 receptor antagonist (IL-1RA), further elucidates its role. Administration of recombinant IL-1RA or over-expression of IL-1RA prior to cerebral ischemia reduces infarct volume, whereas increased damage is found in IL-1RA deficient IL-1RA knockout mice. Inactivation or knockout of interleukin-1 receptor 1(IL-1R1) also reduces cerebral ischemic damage. Conversely, increased IL-1 beta following cerebral insult is associated with more mRNA expression of ceruloplasmin in astrocytes. Ceruloplasmin has potent antioxidant properties and catalyzes the dismutation of free radicals, thus affording protection to the brain. This might explain the protective effect observed by Asare et al., given that the brain SCD patients is hypothesized to be in a state of subclinical ischemia and constantly exposed to IL-1 beta due to the SCD state.

These results support both a beneficial and a deleterious role for IL-1 beta in the pathobiology of stroke in SCD and further mechanistic studies are needed to understand this role and its potential benefits.

In addition to the above, there are polymorphisms in the genes regulating the production of IL-1 beta and other members of the IL-1 family (both ligands and receptors) that may be worth investigating by a genomic study of individuals with impaired TCD, to determine if specific polymorphisms are associated with likelihood of progression to stroke [4].

Modestly increased plasma IL-1 beta concentration has been associated with protection from stroke development in HbSS children with abnormal TCD, and plasma IL-1beta is a good predictor of stroke in HbSS. Using plasma IL-1beta levels in combination with TCD measurements may improve evaluation for stroke risk in HbSS patients, thereby helping to identify those needing intensive prophylactic interventions. These findings need to be confirmed in a larger study and the mechanisms for the IL-1beta protection deserve further investigation [4].

Other Inflammatory Biomakers

Other inflammatory biomarkers have been associated with the development of stroke and other cerebrovascular complications in SCD [42]. In a recently published study using samples from the STOP study biorepository, it was reported that children with SCD who had high TCD at baseline also has elevated serum levels of intercellular adhesion molecule 1 (ICAM-1), myeloperoxidase (MPO) and regulated on activation, normal T cell expressed and secreted (RANTES) also known as chemokine (C-C motif) ligand 5 (CCL5). Subjects from this study who received blood transfusion and thus were prevented from developing stroke at study exit, also had significantly lower levels of these biomarkers except for MPO which was significantly elevated, compared to those who did not receive blood transfusion [6].

Management of SCD and SCD-Related Stroke

Clinical Symptoms and Signs of Stroke in SCD

The clinical symptoms and signs of SCD associated cerebrovascular events depends on the site involved in cerebral infarction, TIAs, intracranial

hemorrhage and/or SCIs. The most recognizable clinical signs is hemiparesis, but there may be associated cognitive and behavioral changes [43] which might present alongside or even after the hemiparesis improves. In SCD, as in non-SCD stroke, symptoms also depends on the size of the lesion(s), which is itself related to the severity of the vasculopathy. Sickle cell disease associated cerebral vasculopathy and resulting stroke can be extensive, with clinically devastating infarctions involving the entire territory of a large artery or could be a smaller, much more subtle lacunar infarcts that may only present with more diffuse symptoms such as neurocognitive dysfunction [44]. Stroke in SCD could also present as hemorrhagic stroke. This stroke sub-type represent a small percentage of strokes in SCD, with a peak incidence at 20 years of age and may be linked to the rupture of a friable moyamoya vessel [45, 46] (Fig. 9.1).

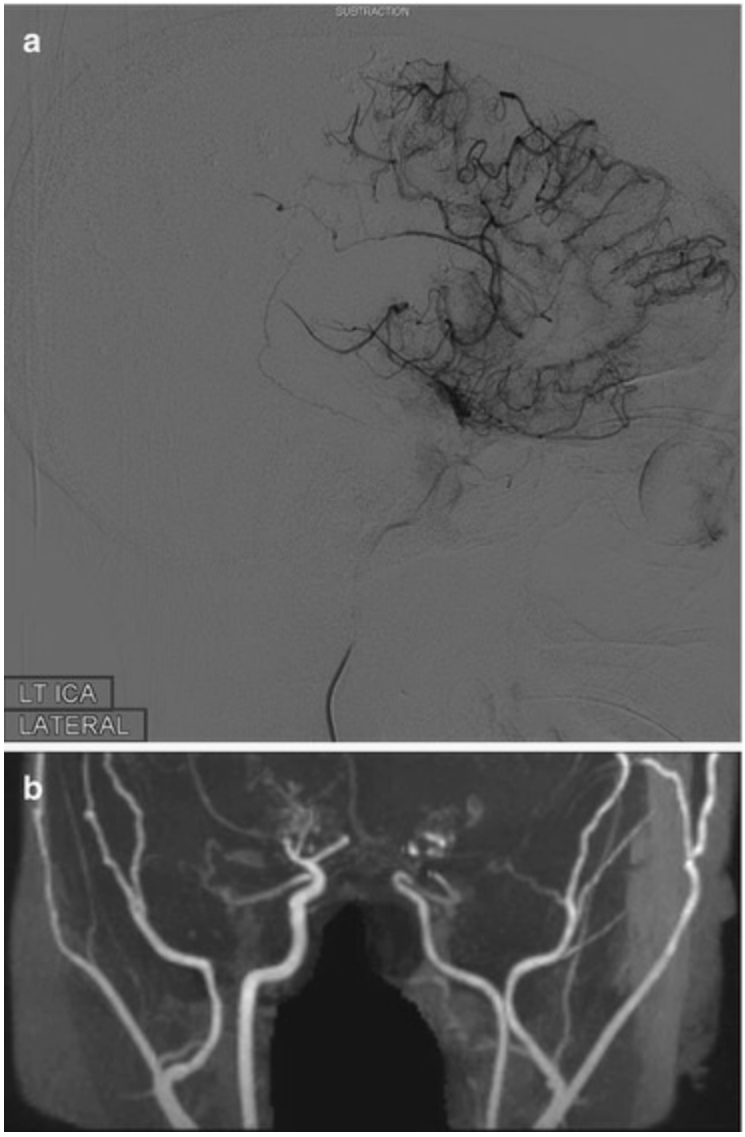

Fig. 9.1 Magnetic resonance angiography (MRA) showing (**a**) stenosis of the right anterior cerebral artery and (**b**) the right middle cerebral artery in a child with sickle cell disease

Work Up of Stroke in SCD

Laboratory Investigations

In the absence of a robust newborn screening programs, stroke or related cerebrovascular complications might be the initial presentation for a child or rare adult with SCD. As a result, the initial laboratory work ups are geared towards establishing a diagnosis of SCD and/or the specific hemoglobinopathy. Diagnosis of SCD is based on the 1975 recommendations of the International Committee for Standardization in Hematology expert

panel on abnormal hemoglobin S and thalassemia, and includes a complete blood count (CBC), hemoglobin electrophoresis at pH 9.2, hemoglobin solubility and sickling test, and quantification of hemoglobin A2, A, S and F; which can be done via simple high performance liquid chromatography (HPLC) or cation exchange HPLC [47, 48]. Hemoglobin electrophoresis at pH 6.0–6.2, globin chain separation, and hemoglobin electrophoresis with isoelectric focusing (IEF) are additional recommended tests if an abnormal hemoglobin is identified on initial testing [49]. Hemoglobin S quantification is important because it guides treatment decisions such as blood transfusion and is also associated with severity of cerebrovascular symptoms and signs [44]. Other laboratory work up that could help in formulating a differential diagnosis for the etiology of stroke might include, evaluation for risk of associated hypercoagulability, vitamin deficiencies and autoimmune diseases.

If a patient with SCD is presenting with a repeat stroke, it might be necessary to evaluate that patient for iron overload, especially when there a history of chronic transfusion for stroke prevention or prevention of other SCD related complications. Further, there is an association between iron overload stroke, thus this could be the etiology of the index event [50]. Liver biopsy is the gold standard for estimating presence or absence of iron overload, but it is invasive. Successful and non-invasive alternatives includes using computerized tomographic (CT) scans or MRI imaging of the liver to monitor liver [51].

Neuroimaging

Initial imaging work up for patients with SCD who presents with a suspected stroke is the same as that of a patient without SCD. A cranial CT scan (Fig. 9.2) is recommended to rule out a hemorrhagic stroke, and is needed to decide whether recombinant tissue plasminogen activator (rtPA) should be administered. When a CT scan is negative and there is a high suspicion of a stroke event, then alternative imaging modalities such as a conventional magnetic resonance imaging (MRI) is recommended. The MRI is more sensitive for detecting parenchymal lesions. Diffusion weighted MRI imaging (DWI), can be used to detect region of ischemia within an hour after clinical manifestation of stroke symptoms and aid early initiation of treatment. Additional advantage of MRI scan is its ability to detect SCIs, which is prevalent in about 20–40% of children with SCD [22, 50, 52]. SCIs are recognized as a 3 mm area of abnormally increased signal intensity on the

intermediate and T2-weighted pulse sequences of MRI [53]. Finally, MR imaging can detect and show areas of cerebral atrophy, which might be a non-specific indicator of chronic brain insult [54, 55] and has been linked to neurocognitive deficit in patients with SCD.

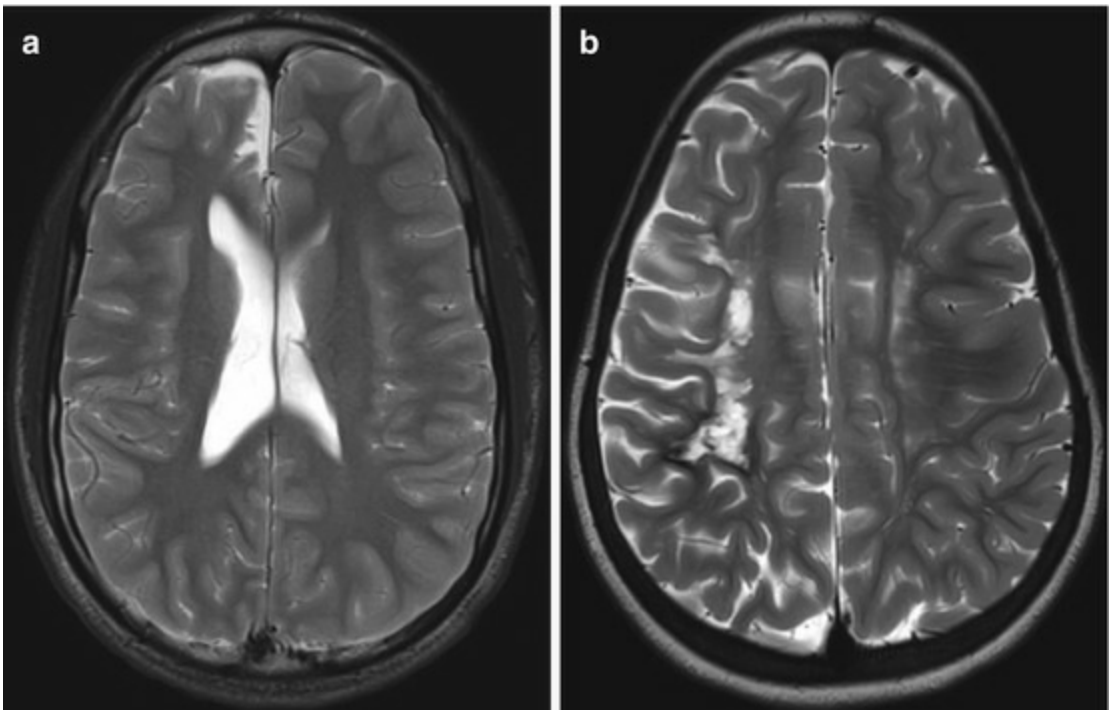

Fig. 9.2 CT scans of patient with SCD and a stroke in the (**a**) anterior cerebral artery and (**b**) middle cerebral artery distribution. There was prior MRA evidence of severe cerebral vasculopathy with stenosis in both cases

Treatment of Stroke in SCD

Treatment of Acute Stroke in SCD

In patients with SCD presenting with acute stroke and symptoms suggestive of a hemorrhagic stroke, initial intensive monitoring of intracranial pressure (ICP), might be required. In addition, treatment of vasospasm with volume expansion and/or nimodipine and aggressive treatment of seizures if any might all be components of the initial treatment. A craniotomy might be required for the evacuation and/or drainage of large intracranial bleeds and to decompress the ICP to prevent herniation. The craniotomy might also provide access needed for clipping or wrapping of the cerebral aneurysm or bleeding moya moya vessels. Other supportive measures includes adequate hydration,

maintenance of normothermia, euglycemia and normotension. Note that hypotension should be avoided in the setting of an acute stroke. Although no clinical data exist on its efficacy in brain hemorrhage it is routine to institute at least temporary exchange transfusion.

Sickle cell disease is not a recognized contraindication for antiplatelet therapy, as such, it is conceivable that where appropriate, antiplatelet therapy could be used. Albeit, the evidence in favor or against use of antiplatelet therapy is still scant. Additional options for management of acute stroke in adults with SCD are laid out in the American Heart Association/American Stroke Association Guidelines on Secondary Stroke Prevention [56] and the National Institutes of Health and National Heart Lungs and Blood Institutes (NIH/NHLBI) guidelines for management and prevention of SCD and SCD complications [57]. No specific recommendations exists, on the use of antiplatelet or anticoagulants agents in children with SCD presenting with an acute stroke. Also, there is no randomized clinical trials (RCTs) of the use of thrombolytics for the treatment of acute ischemic stroke in children with SCD (Fig. 9.3) [56]. Current recommendations for acute management of ischemic stroke in children with SCD involves initial hydration and blood transfusion, preferably exchange blood transfusion because it theoretically avoids the risk of exposure to increased blood viscosity from acute elevation in hematocrit [58, 59]. The following figure (Fig. 9.4), is a flow chart distilling the NIH/NHLBI guidelines for the management of a child with SCD and suspected stroke.

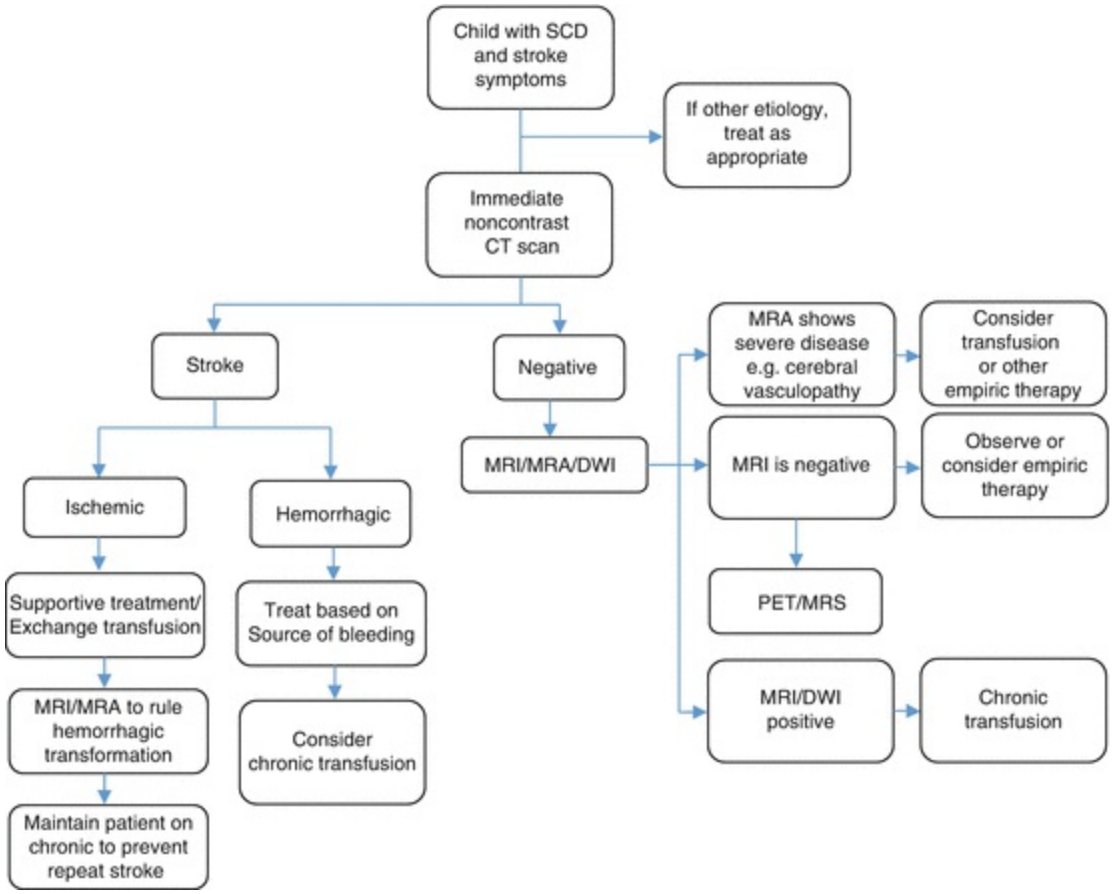

Fig. 9.3 Approach to a child with SCD and suspected stroke

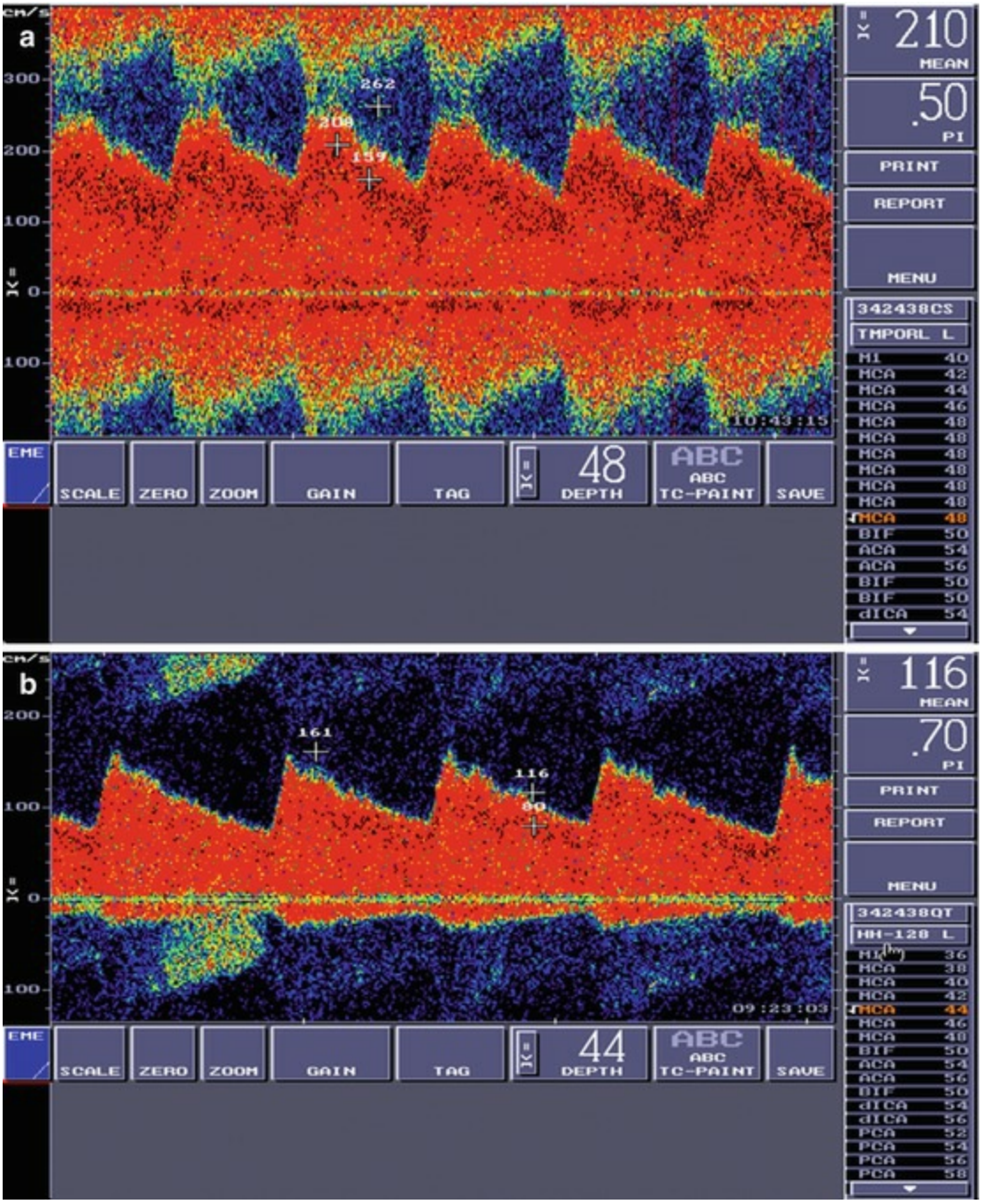

Fig. 9.4 TCD ultrasound velocity waveform from patients with SCD. In (**a**), the patient has high TCD velocity values and thus is at risk for stroke and (**b**) is the same patient after 12 months of chronic blood transfusion therapy and TCD velocity has normalized

In a child with SCD presenting with acute ischemic stroke, a key component of adequate management is blood transfusion. Where possible, exchange rather than simple transfusion should be used recommended [60].

The main goal of blood transfusion during this acute episode is to reduce the %HbS to levels below 30%. Although it should be noted to that this goal is not based on an RCT demonstrating benefit or improved outcome if this goal is met.

Primary and Secondary Prevention of Stroke in SCD

While prevention of stroke in children with SCD has seen significant advances over the last two decades, the same cannot be said for stroke prevention in adults with SCD. In the late 1980s, Adams et al. reported that TCD velocity in the main internal carotid arterial branches were related to the presence of cerebral vasculopathy [61], age and degree of anemia, in children with [18] and without [62] SCD. TCD uses pulsed Doppler to measure the velocity and pulsatility of blood flow within the major intracranial arteries of the Circle of Willis. In children with SCD, there is involvement of the distal intracranial internal carotid artery (ICA) and the proximal portions of the middle (MCA) and anterior (ACA) cerebral arteries [63]. Furthermore, their work revealed that TCD velocity predicted the risk for stroke [17]. Combined with it non-invasive nature, TCD velocity is now the mainstay of screening for stroke risk in children with SCD. In 1992, Adams et al. reported that a TCD velocity based on a Time Average Maximum of the Mean velocity (TAMMV) ≥200 cm/s was associated with significantly increased risk for stroke, RR = 44 (CI = 5.5–346) and as such provided a clinical basis for using TCD velocity as a screening tool for detecting stroke risk in children with SCD [17]. This study paved the way for a large RCT for primary stroke prevention in SCD [19].

In 1995, the STOP trial was started and results published in 1998 indicated that children with SCD having a TCD velocity of ≥200 cm/s, who received chronic blood transfusion in the course of the, had a > 90% reduction in incident stroke, compared to those who did not received chronic blood transfusion. The goal of blood transfusion was to reduce the percentage of sickle hemoglobin to ≤30%. [19]. This study formed the initial basis for the NIH/NHLBI's recommendation of routine TCD screening and subsequent chronic blood transfusion in children with high TCD, as a modality for primary stroke prevention (NHLBI website: http://www.nhlbi.nih.gov/health/dci/Diseases/Sca/SCA_Treatments.html). This has also been echoed, endorsed, and extended by the American Heart Association/American Stroke Association [64].

A subsequent study called STOP II trial, was designed by Adams et al. to evaluate when it is safe to discontinue blood transfusion after normalization of TCD velocity for children with TCD on chronic blood transfusion. The study was halted after only about two-thirds of children were enrolled and randomized. This was because 14 of the 41 children in transfusion halted arm had a reversal of their TCD velocity to high and another 2 had a stroke with the first 4–6 months post randomization. They concluded that blood transfusion for primary stroke prevention in SCD needs to be life-long in order to retain effectiveness [65].

A recently concluded and published phase III clinical trial by Ware et al. [66] reported that hydroxyurea was equally as effective as chronic blood transfusion for stroke primary stroke prevention in children with SCD. In this study, hydroxyurea administered at the maximum tolerated dose, to children with SCD, who were diagnosed with a high TCD velocity and have been on transfusion for at least 12 months and not having evidence of high grade cerebral vasculopathy. The study was terminated early because there was conclusive evidence that hydroxyurea was at least equal to chronic blood transfusion therapy for normalizing high TCD velocity (a surrogate marker for high stroke risk). It is expected that this new evidence will be incorporated into clinical recommendations and guidelines soon. In the meantime, the NIH/NHLBI's recommendation for primary stroke prevention (pre-TWITCH) is summarized in Fig. 9.5 below[1].

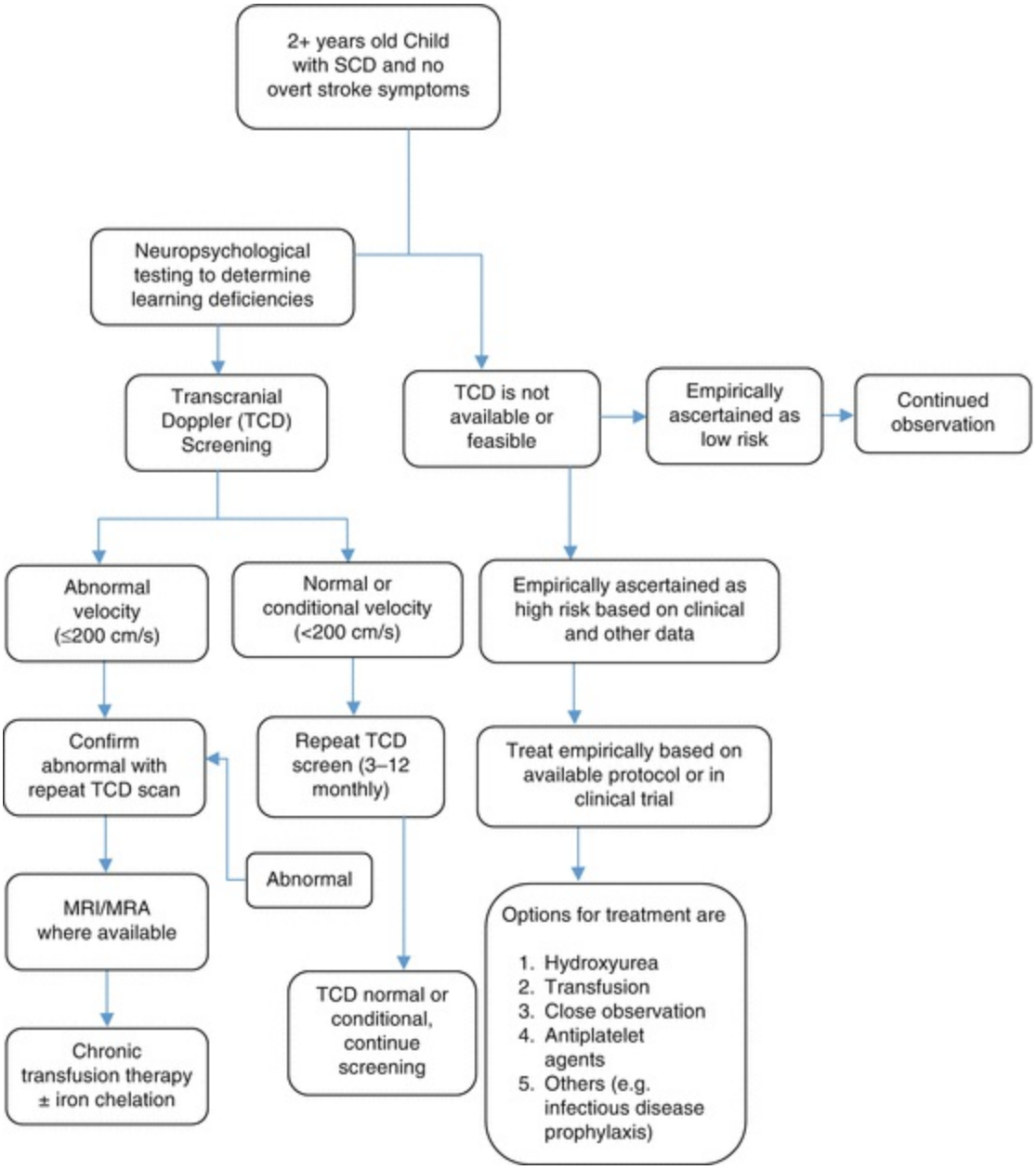

Fig. 9.5 Flow chart summarizing current approach recommended for primary stroke prevention

Other less commonly practiced but available modalities for primary prevention of stroke in SCD includes: allogeneic bone marrow transplantation (BMT) from HLA-identical siblings or haploidentical donors [67] or related and unrelated cord blood transplantation. Additionally, patients with symptomatic moyamoya can be offered direct or indirect bypass procedures such as Encephalo-duro-arterio-synangiosis (EDAS) which permits neovascularization to develop over a larger area of the brain than

observed with direct anastomosis [68]. There is also pial synangiosis which is also used to treat moya moya syndrome in symptomatic patients who have failed alternative medical treatment [69].

Complications Associated with Management of Stroke in SCD and Alternative Strategies

Prevention of stroke in SCD by chronic blood transfusion is associated with complications such as alloimmunization, iron overload, and infections; especially in developing countries with less rigorous donor screening. Erythrocytapheresis, an automated method of red blood cell exchange, is a safe method of controlling Hb S levels while limiting or preventing iron overload in chronically transfused SCD patients, as is phlebotomomy [70]. Further, better and well tolerated iron chelators are now available and have served to reduce the limits on chronic blood transfusion that was posed by the potential for iron overload and associated complications. Finally exchange blood transfusion is another modality which serves both to reduce the risk of iron overload, as well as hyper viscosity and associated complications.

Alloimmunization is another well documented complication of chronic blood transfusion to prevent stroke or other SCD related complications. It is due to exposure of the patient to multiple antigens (usually minor) from transfusion of blood from different donors. It has a rate as high as 47% in adult and 27% in pediatric transfused SCD patients [71]. Alternative strategies to prevent or reduce development of this complication includes; using leuko-depleted packed red blood cells matched for E, C, and Kell antigens, PEG-coating of red blood cells to mask red cell antigens from antibodies [72, 73] and use of artificial blood substitutes, such as perfluorocarbon emulsions and hemoglobin-based substitutes. The latter two options have not been exclusively studied in SCD patients and still remain largely experimental. The use of leukocyte-depleted packed red blood cell for transfusion in SCD has had a significant effect on reducing the transmission of intracellular viruses such as CMV, human lymphotropic virus, EBV and human herpes viruses 6, 7, 8. Furthermore the risk of transmission of HIV, hepatitis B and C, and human T-cell leukemia/lymphoma virus-1 have also dramatically decreased with improved donor selection and screening of banked units in developed countries. Taken together both strategies have significantly reduce blood transfusion related transmission of infection. But

in developing countries, the above mentioned challenges are still prevalent and have limited the use of chronic blood transfusion for stroke prevention in SCD. The results from the study by Ware et al. [66], is thus a welcomed development in the field of primary stroke prevention in SCD, especially in the developing countries.

Prevention of SCD complications in general and stroke in particular can also be done by targeting specific components of the patholobiology of SCD. For instance, induction of hemoglobin F or fetal hemoglobin, to reduce hemoglobin polymerization and RBC sickling, prevention of RBC dehydration resulting in the reduction of the percentage of dense irreversibly sickled cells and associated complications, and use of vasodilatory, anti-inflammatory and recently antiplatelet agents. These alternative strategies have been largely experimental, except for fetal hemoglobin induction, which is the major premise behind treatment of SCD with hydroxycarbamide or hydroxurea.

Targets for New Therapies

Inducers of Fetal Hemoglobin (HbF)

Fetal hemoglobin (HbF) has long been recognized as a prognostic factor of clinical severity, as sustained HbF levels ≥20% are associated with reduced clinical events [74] and low HbF concentration is recognized as a predictor of early mortality [75]. HbF appears to confer protection by preventing HbS from polymerization [30].

Hydroxycarbamide (hydroxyurea) is the only US Food and Drug Administration (FDA)-approved drug for use in patients with SCD. It is a ribonucleotide reductase inhibitor [30], known to stimulate Hb F production. Additional advantages of hydroxycarbamide therapy include its action to lower the white blood cell count, reticulocytes, and platelets, by increasing NO production, improving RBC hydration and decreasing RBC adhesiveness to endothelium [76]. The BABY HUG trial investigated the role of hydroxycarbamide in the preservation of spleen and kidney function, but the results did not show any difference between treatment and placebo groups [77]. Similarly, the SWiTCH study also reported that hydroxycarbamide was not a good substitute for chronic blood transfusion for secondary stroke prevention or prevention of iron overload [70]. As mentioned in an earlier

section, the TWiTCH study published by Ware et al., showed that hydroxycarbamide is an effective alternative to chronic blood transfusion for primary prevention of stroke in a subset of children with SCD who are at risk for stroke. Decitabine, Butyrate, and Erythopoietin have all been shown to increase Hb F production as well, but initial studies involving all three drugs have yet to provide the promise of hydroxycarbamide, either due to poor efficacy, intolerance due to side effects or both.

Prevention of RBC Dehydration

Inhibition of K+-Cl Co-transport

Magnesium is an important regulator of cellular cation transporters, including the KCl co-transporter. Increased intracellular magnesium inhibits K+ efflux from the sickle erythrocyte and consequently prevents RBC dehydration. A phase I clinical trial in children with HbSS who had been stable on hydroxycarbamide for at least 6 months were given oral magnesium pidolate and followed for 24 weeks. A significant reduction of KCl co-transport activity was shown by serum evaluation [78]. A subsequent phase III multicenter clinical trial conducted to investigate whether magnesium infusion will impact frequency of painful events, hospital stay or opioid use, reported no benefit over placebo [79].

Inhibition of the Gardos Channel

The imidazole antimycotic drug clotrimazole was shown to block the Gardos channel and subsequently decrease RBC dehydration. Unfortunately, side effects as severe as transaminitis precluded its long-term clinical use, but a similar compound, ICA-17043 (Senicapoc) was shown to modulate RBC membrane cation permeability by decreasing the Gardos channel activity and the calcium-induced K+ efflux. A phase II clinical trial in which 90 patients with SCD were randomized to high dose study drug, low dose study drug or placebo took place over 12 weeks, and although there was reduced hemolysis, there was no reduction in the frequency of vaso-occlusive painful events, which led to early termination of a phase III clinical trial [76].

Vasodilators

Nitric Oxide/Arginine

Nitric oxide is effective in improving oxygenation in patients with SCD during episodes of acute chest syndrome (ACS). There are currently two phase II, double-blinded, randomized placebo-controlled studies of inhaled NO for acute treatment of pain crisis, but no ongoing studies of NO use in stroke [76].

L-Arginine is the precursor for NO, and is deficient in adults with steady state Hb SS disease. L-Arginine decreases further during times of VOC, a finding attributable to the increase in serum arginase that occurs during increased hemolysis. At their baseline, patients with SCD have higher plasma arginase levels, with the highest activity found in patients with pulmonary hypertension. Several small trials have shown higher NO levels during times of VOC when oral L-Arginine was given as a supplement, however, a multicenter, double-blinded, placebo controlled, phase II study of children with SCD who received oral arginine for 3 months showed no increase in arginine level or change in NO level, Gardos channel activity or RBC hydration status. A phase II trial to determine if oral arginine will increase NO in SCD patients during ACS reported a negative finding indicating no significant improvement in NO in SCD patients with ACS compared to those without ACS or healthy controls [80].

Anti-inflammatory Agents

Statins

Independent of their lipid-lowering effects, statins have been shown to prevent blood vessel damage by several mechanisms, including through up-regulation of endothelial NO. Given that NOx metabolism is altered in SCD, statins may provide a beneficial role in this disease. Simvastatin was shown to improve NOx levels significantly in both moderate and doses. In addition to decreasing inflammation and leukocyte adhesion to the activated endothelium [81, 82]. It is not yet clear whether there will be a role for statins in stroke prevention as no study has been done to demonstrate or refute this.

Corticosteroids

Steroids have been shown to have short-term beneficial effects in patients

with painful crises and ACS, but the increased risk of rebound painful events has been reported. There is currently a phase III randomized trial of high dose methylprednisolone followed by prednisone taper for treatment of vaso-occlusive crisis in SCD.

Future Directions

Because several different mechanisms are involved in the pathophysiology of SCD and subsequently cerebrovascular disease, combination therapy with drugs aimed at specific targets could, in theory, provide the most benefit. Combining an agent that induces Hb F formation like hydroxycarbamide with a potent vasodilator like inhaled NO and magnesium pidolate to minimize erythrocyte dehydration would appear to provide additive protection in times of VOC, but each individual responds to each intervention in their own way. The differential response by SCD patients to hydroxycarbamide with variable degrees of Hb F production prompted an investigation into the potential contribution of genetics that promote or inhibit a specific individual from responding as expected to the drug. Several SNPs were found in association with the degree of Hb F production following 2 years of hydroxycarbamide therapy in adults with SCD. This finding suggests that optimal therapy would vary amongst individuals, based not only on their specific phenotype, but also their underlying genotype and epigenetic processing of that genome [76].

Conclusion

Sickle cell disease is a monogenetic disease, but with a great deal of phenotypic variability. This variability is thought to be the result of each individual's "interpretation" of their underlying genome or epigenetics. Nearly half of all patients with SCD will have a stroke and/or silent cerebral infarct, leading to subsequent morbidity such as physical disability and cognitive decline within their lifetime. Because of the prevalence of cerebrovascular disease in this population, further investigation into the underlying pathophysiology and subsequent treatment is not only appropriate, but is warranted, and investigations into therapies based on the underlying genetics and epigenetics are ongoing and hopefully there is "a light at the end of this tunnel". Furthermore, with improvement in clinical care, the need to investigate the interaction of this gene variant with other gene variants and

also environmental factors is becoming important as more patients with SCD achieve adulthood. Finally there is also the need to investigate the interaction of hemizygous state with other know cerebrovascular and cardiovascular risk factors, given that the prevalence of this state is usually five to ten times more that the homozygous state.

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Footnotes

It is expected that the results TWITCH trial results will modify these recommendations in the future.

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10. Other Monogenetic Stroke Disorders

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Introduction

As described throughout this book, most strokes occur secondary to the interaction of multiple genes in combination with both lifestyle and environmental factors, thereby making stroke a prototypical complex disease. While this may be true for most forms of ischemic and hemorrhagic stroke, there are several established monogenetic disorders (i.e., transmitted via Mendelian inheritance) that may present with stroke. In some situations, stroke can be the predominant clinical feature, while in others, stroke can occur infrequently. In this chapter, we will describe such disorders focusing on clinical manifestations (Table 10.1) and then present details regarding the established genetic and diagnostic testing (Table 10.2) that are available. Given the disparate relationships between these disorders and stroke, the disorders have been classified in Tables 10.1 and 10.2 based upon their predominant etiologic mechanisms, including large arterial diseases, small vessel diseases, hematological diseases, mitochondrial diseases, and connective tissue disorders. The disorders are presented in the same order in

Tables 10.1 and 10.2. For completeness and ease of reference, we have also included several monogenetic disorders in our tables that have dedicated chapters elsewhere in this book, including MELAS syndrome (see Chap. 8), CADASIL syndrome (see Chap. 6), and sickle cell disease (see Chap. 9).

Table 10.1 Mendelian disorders: history and findings

	History		**System based findings**		
Disease type	**Patient and family history**	**Age of onset, predilection**	**General appearance**	**HEENT**	**CVS/re**
Large arterial disease					
Homocystinuria OMIM: 236200	– Seizures – Arterial and venous thrombotic events – Osteoporosis – PVD – Psychiatric disorders	– Occasional failure to thrive in infancy – High risk of thrombotic events in childhood	– Marfanoid habitus—tall, thin body habitus – Kypho-scoliosis – Pectus excavatum	– Sparse brittle hair – Crowded teeth – Myopias – Glaucoma – Ectopia lentis – Dislocation of lenses – Elevated palate	– Mitra – MI – Inguin
Familial hyper cholesterolemia (Type II-a) OMIM: 143890	–Early MI –PVD –Hereditary dyslipidemia	– CAD after 30 years in heterozygotes, childhood in homozygotes	– At birth—web xanthomas between first and second digits	– Corneal arcus (by 3rd decade) – Xanthelasma – Bruit—atherosclerosis	– CAD
Tangier disease (Familial HDL deficiency, Type I) OMIM: 205400	– Extremity pain – Asymmetric sensory deficits or abnormalities – Hereditary dyslipidemia – Early MI	– Infancy or childhood with pharyngeal findings	– Adenopathy	– Orange tonsils: cholesterol laden	– Hepo – Intest
MoyaMoya disease; Spontaneous Occlusion of the Circle of Willis—Several Forms MYMY1 OMIM: 252350 MYMY2 OMIM: 607151	– Sudden onset of hemiplegia in childhood – Epileptic seizures starting in childhood – MYMY4 short stature: hypergonadotropic hypogonadism	– Juvenile form typically presents with transient motor deficits following occlusion of major intracerebral vessel(s) – Adult presentations usually result from collapse of collateralization	MYMY4 short stature	MYMY4 facial dysmorphism	

MYMY3: 608796 MYMY4 OMIM: 300845 MYMY5 OMIM: 614042 MYMY6 OMIM: 615750					
Small vessel disease					
CADASIL—Cerebral Autosomal Dominant; Subcortical Infarcts and Leukoencephaly OMIM: 125310	– Migraines with prolonged aura – Familial hemiplegic migraine – Early onset dementia – Manic episodes – Depression – Seizures – Recurrent subcortical infarcts	– Early adulthood – Migraines by age 30 – First stroke by age 45	– Lumbar spondylosis		
CARASIL OMIM: 600142 (2, 3, 8)	– Early gait disturbances: Due to lower extremity spasticity – Recurrent lacunar infarcts – Back pain – Alopecia: progressive baldness – Progressive mental and motor deterioration	– Male to Female 3.2:1 – Strokes starting in 30’s – Dementia in 30–50’s	– Spondylosis deformans – Premature alopecia (age 20)	Alopecia; optic neuritis	Normot
Fabry disease OMIM: 301500	– Anhidrosis – Periodic fever – Lancinating pain in hands and feet: often initial symptom, sometimes induced by heat or exercise – Cardiomegaly	– Children and young adults with paresthesias – Stroke occurs in adults	– Retarded growth – Delayed puberty	– Corneal opacity – Tortuous retinal conjunctive vessels – Crystalline deposits in conjunctiva – Oral, conjunctival lesions (vascular)	– Cardi – MI – Mild disease – Episo – Renal – Cardi defects – Hype

	with MI – Arthritis				cardion – Renal
BRAIN small vessel disease with hemorrhage (COL4A1) OMIM 607595 (2, 4, 8)	– Perinatal hemorrhage with porencephaly – Infantile hemiparesis – Migraine with aura – Developmental delay – ICH from minor trauma – Lacunar Stroke, subcortical bleeds – Migraine with aura – Seizure – Intracranial aneurysms – Visual loss – Mental retardation – Dementia	– Early adulthood strokes average age 36 – Infantile hemiparesis		– Cataracts; retinal vessel tortuosity – Retinal hemorrhages – Glaucoma – Axenfeld-Rieger anomaly: developmental abnormalities in the anterior chamber angle, iris, and trabecular meshwork	– Renal – Renal – Mitra – Supra arrhyth
Hereditary Angiopathy with Nephropathy, Aneurysms and Muscle Cramps (HANAC) OMIM: 611773	– Mutation cluster in 31 amino acid region of the COL4A1 protein encompassing integrin binding sites			– Retinal artery tortuosities	– Hema – Bilate – Supra arrhyth
Vasculopathy, Retinal, with Cerebral Leukodystrophy; RVCL (formerly TREX1 and HERNS Spectrum) OMIM: 192315 (1,2,3)	– Pseudotumors strokes – Seizures – Migraines – Progressive visual impairment – Progressive dementia – Apraxia – Dysarthria – Hemiparesis	– TIA and strokes: 4th or 5th decade of life – Headaches – Behavioral |abnormalities – Death within 5–10 years of clinical presentation		– Retina: capillary dropouts in macula – Telangiectasias – Loss of central vision – Retinopathy: Neovascularization of the optic disc, retinal hemorrhages and macular edema	– Eleva phospha – Liver regener – Renal disease – GI ble – Anem

Early Onset Stroke and Vasculopathy Associated with Mutations in ADA2 (Polyarteritis Nodosa, Childhood-Onset; PAN; ADA2 Deficiency) OMIM: 615688	– Recurrent fevers – Early onset stroke – Livedo racemose – Marked elevation of acute phase reactants in setting of fever – Recurrent infections in setting of mild immunodeficiency	– Onset of stroke before age 5 – Strokes normally during inflammatory episodes		– Diverse Ophthalmologic involvement: central retinal artery occlusions, optic nerve atrophy, diplopia, strabismus	– Hepat
Hematologic diseases					
Sickle cell disease OMIM: 603903	– Acute pain crisis with physical exertion – Unexplained fevers – Abdominal, bone, and chest pain – Large or small vessel occlusive disease – Intracerebral epidural or subdural hemorrhages – Subarachnoid Hemorrhages	– Black children – Peak incidence: ages 2–5 years – Age < 20 years old Ischemic > Hemorrhagic stroke – Age >20 years old Hemorrhage>Ischemic – High recurrence risk, 2/3 have stroke within 2 years of index stroke	– Slow growth – Jaundice	– Proliferative retinopathy – Scleral icterus	– Acute – Asple autospl – Cor P – Painl – Chole – Vaso- – Comp anemia
Protein C deficiency OMIM: 176860 Protein S Deficiency OMIM: 176880	– DVT – Recurrent thrombotic events – Warfarin induced skin necrosis – Purpura Fulminalis Neonatalis	– Young adults – Occasional late onset with homozygosity with Protein C deficiency		– Neonatal vitreous hemorrhages	– Pulm – Intraa thromb
Factor V Leiden Mutation OMIM: 227400	– Cerebral venous thrombosis – DVT	– Young adults			
Mitochondria based disease					
MELAS—Mitochondrial	– Seizures	– Infancy: failure to thrive	– Short stature	– Opthalmoplegia	– Cardi (hypert

Encephalopathy Lactic Acidosis and Stroke OMIM:540000	– Episodic vomiting – Visual disturbances – Episodic migraines – Deafness – Maternally inherited diabetes	– Children or young adults: stroke (often before age 40)		– Pigmentary retinal—degeneration – Bilateral cataracts – Hearing loss – Eyelid ptosis	– Cardi defects: White S – Progr dysfunc – Diabe – Recu
Connective tissue disorders					
Ehlers-Danlos Syndrome (Type IV) OMIM: 130050	– Cerebral aneurysms – Arterial dissections – Spontaneous rupture of aneurysms – Easy bruising: vascular fragility – Excessive scarring s/p surgery	– Death usually by age 40–50 years secondary to dissecting aneurysms	– Short stature – Alopecia of scalp – Gingival recession	– Pinched, thin nose – Thin lips – Lobeless ears – Keratoconus – Peridontal disease – Early loss of teeth – Horner's Syndrome with carotid dissection	– MVP – ASD/ – Aorti – Spont pneumo – Color – Bladd – Uteri
Marfan Syndrome OMIM: 154700	– Cerebral aneurysms – Internal carotid artery dissection – Premature arthritis	– Typically death by age 40–50 years secondary to dissecting aneurysms	– Tall, thin body habitus – Pectus exvacutum – Bossing of frontal eminences – Prominent supra-orbital ridge – Scoliosis	– Ectopia lentis – Retinal detachment – Early cataracts and glaucoma – Lens sublaxation – Micrognathia – Horner's Syndrome with carotid dissection	– Aorti – MVP – Aorti insuffic – CHF – Recu hernias
Fibromuscular dysplasia OMIM: 135580	– Headaches – Myocardial infarction – Tinnitus and/or vertigo – Transient retinal or cerebral ischemia – Dissection: carotid aneurysms – SAH	– Female > Male – Typically middle aged females – White > Blacks	– Neck pain: carotidynia	– Carotid bruits – Transient retinal ischemia – Carotidynia – Horner's Syndrome with carotid dissection	– Renal hyperte

Pseudoxanthoma Elasticum AD Form OMIM: 177850 AR Form OMIM: 264800	– HTN – Angina – CAD, MI – Gradual vision loss – Epistaxis – Hematuria – Arterial Dissections – GI Hemorrhages – PVD		– Pectus deformities	– Angiod retinal streaks – Macular degeneration – Retinal hemorrhages – High arched palate – Yellowish lip mucosal nodules – Horner's syndrome with carotid dissection	– MVP – Gastr – HTN

Table 10.2 Mendelian disorders: other features and diagnostic tests

Disease type/OMIM number	**Associated abnormal findings and laboratory tests**	**Inheritance and chromosomal location**	**Resulting defect/basis for disease**	**Availability of genetic test**	**Ancillar tests**
Large arterial disease					
Homocystinuria OMIM: 236200	– Homocystine is elevated in blood, CSF and urine – Methionine elevated in blood and urine – Treatment with Vitamin B6	AR, 21q22.3	Deficiency of cystathione beta-synthase	Yes, DNA	–Urine/s homocys –Urine/s methion
Familial hyper—cholesterolemia (Type II-a) OMIM: 143890	– Serum: elevated LDL, elevated cholesterol	AD, 19p13.2	Abnormal LDL receptor	Yes, DNA	–Fasting
Tangier disease (Familial HDL deficiency, Type I) OMIM: 205400	– Serum: low HDL, low LDL, low cholesterol, elevated triglycerides, low phospholipids, abnormal chylomicron remnants	AR, 9q22-q31	– HDL—Apo-Gln-I very low – Severe atherosclerosis – Thymus and reticuloendothelial cells filled with cholesterol esters	No	Fasting Denerva on EMG
MoyaMoya disease; Spontaneous Occlusion of the Circle of Willis—Several Forms		MYMY1: AR, 3p26-p24.2 MYMY2: 17q25.3 MYMY3:	MYMY1: MYMY2: RNF213 gene MYMY3: MYMY4:		–Bilater carotid a –Conver angiogra "puff of

OMIM: 252350		8q23 MYMY4: XLR, Xq28 MYMY5: 10q23.31 MYMY6: AR, 4q32.1	MYMY5: ACTA2 gene MYMY6: GUCY1A3		
Small vessel disease					
CADASIL—Cerebral Autosomal Dominant; Subcortical Infarcts and Leukoencephaly OMIM: 125310	MRI with multiple subcortical infarcts, both clinically evident and silent	AD, 19p13.2-p13.1 Notch 3 gene	–Codes for large transmembrane protein needed for vascular smooth muscle differentiation and development –Non-amyloid eosinophillic material in small vessel walls and reduplicated internal elastic lamella	Yes, DNA	MRI—C subcorti matter cl may ext tempora –MRI cl clinical s –O'Sulli T2 hype the whit anterior –Other c findings capsule collosun abnorma –Microb
CARASIL OMIM: 600142 (2,3)	MRI with leukoencephalopathy, lacunar infarcts, spinal anomalies, and alopecia	AR; 10q25; reduced HTRA1 protease activity or loss of protein	–Elevated TGF-B signaling via disinhibition –Cleaves pro-domain pro-TGF-beta-1	Yes, DNA	MRI—D matter d multiple infarcts
Fabry disease OMIM: 301500	–Proteinuria –Lipid laden macrophages in bone marrow –Therapy: Recombinant GLA enzyme replacement therapy (no known benefit in stroke though)	–X-linked, Xq21.33-q22 –Complete form in males: Males more severely affected –Incomplete form in female carriers	–Alpha-galactosidase A deficiency –Leads to accumulation of ceramide trihexidose in peripheral nerves and blood vessels –Glycosphingolipids accumulate in vascular endothelial smooth muscle, autonomic	–Prenatal diagnosis available –Measurement of leukocyte GLA activity –Molecular genetics	–Skin bi of skin f

			and dorsal root ganglion cells		
Brain small vessel disease with hemorrhage (COL4A1) OMIM: 607595 (2,4,8)	MRI with mostly bilateral and symmetric WM hyperintensities; lacunar infarcts, dilated perivascular spaces, cerebral microbleeds (deep WM, deep gray nuclei, brain stem, cerebellum); Elevated CPK; Hematuria	AD; Chromosome 13q24; COL4A1; Missense mutations involving glycine residues in triple helical domain	Type IV collagen alpha 1 chain (structure of basement membrane)	Yes, DNA	MRI—D leukoenc with dee involver posterio perivent subcorti and mic
Hereditary Angiopathy with Nephropathy, Aneurysms and Muscle Cramps (HANAC) OMIM: 611773	Elevated CPK	AD, Chromosome 13q34	–Mutation cluster in 31 amino acid region of the COL4A1 protein encompassing integrin binding sites	Yes-DNA	
Vasculopathy, Retinal, with Cerebral Leukodystrophy; RVCL (formerly TREX1 and HERNS Spectrum[a]) OMIM: 192315 (1, 2, 3)	MRI: fronto-parietal contrast enhancing subcortical lesions with surrounding edema; elevated protein in CSF; elevated alkaline phosphatase –Can treat retinal changes with intravitreal bevacizumab	AD; 3p21.31	–TREX1 gene; Heterozygous mutation in carboxyl-terminus, disruption of transmembrane domain –Encodes DNA exonuclease of which a frameshift mutations prevents the normal translocation into the nucleus	Yes, DNA	MRI
Early Onset Stroke and Vasculopathy Associated with Mutations in ADA2 (Polyarteritis Nodosa, Childhood-Onset; PAN; ADA2 Deficiency) OMIM: 615688	–MRI: Lacunar infarcts	–Autosomal recessive vs. spontaneous?, 22q11.1	–Loss of function mutation in CERC1 which encodes ADA2 protein –ADA2 is likely growth factor for endothelial and leukocyte development and differentiation		

Hematologic diseases					
Sickle cell disease OMIM: 603903	–Aplastic crisis induced with Parvo B-19 virus	AR, 11p15.5 Missense mutation,	Defective Beta-chain hemoglobin molecule	Yes, DNA Can also screen using	Follow-u Transcra studies r
	–Prophylactic treatment and acute treatment with exchange transfusion	valine for glutamte in position 6 of beta hemoglobin chain		electrophoresis or chromatography tests	–High ri blood flo >200 cm
Protein C deficiency OMIM: 176860 Protein S deficiency OMIM: 176880	–Vitamin K antagonists may worsen –Acquired deficiencies may occur with pregnancy, liver disease, DIC, oral contraceptive use, warfarin use and following surgery	–Protien C—AD, 2q13-q14 –Protien S—AD, 3p11.1-q11.2	Deficiency of functional proteins	Yes, DNA	–Protein –No hep greater t
Factor V Leiden Mutation OMIM: 227400	Prolonged bleeding time Prolonged clotting time Prolonged one-stage prothrombin time, corrected by rabbit plasma	AR, 1q23 Point mutation G to A nucleotide 1691R506Q protein mutation, glutamine for arginine at residue 506	Abnormal Factor V molecule	Yes, DNA	–Protein –No hep greater t –Prefera warfarin
Mitochondria based disease					
MELAS: Mitochondrial Encephalopathy Lactic Acidosis and Stroke OMIM:540000	–Elevated serum lactic acid and pyruvic acid –Ragged Red fibers on muscle biopsy –Progressive renal dysfunction –Therapeutic considerations: CoQ10, Levocarnitine, L-arginine, B-vitamins –AVOID valproate, statins	– Mitochondrial –Defect in transfer RNA for leucine (A3243G and T3271C) –Additional mutations affect respiratory chain enzymes	Abnormal leucine processing	Yes, Mitochondria DNA	–Serum pyruvic elevated –Marked with exe –Muscle –Patholo red fiber fibers –MRI—infarcts vascular generally cortex w deep wh

Connective tissue disorders					
Ehlers-Danlos Syndrome (Type IV) OMIM: 130050	Premature delivery because of cervical insufficiency or membrane fragility	AD, 2q31 Collagen III, alpha-1 gene —COL3A1	Type III collagen abnormal	Yes, DNA	Echocar
Marfan Syndrome OMIM: 154700	Dilated aortic root Coarctation of aorta	AD, 15q21.1 Fibrillin 1 gene	Defective Fibrillin 1	Yes, DNA—linkage and mutation based tests available	Protein l immuno Echocar
Fibromuscular Dysplasia OMIM: 135580	MRA—"String of beads" in carotid arteries @ cervical levels C1and C2	AD, location unknown	Degradation of elastic tissue of vessel wall	No	Carotid ultrasou
Pseudoxanthoma Elasticum AD Form OMIM: 177850 AR Form OMIM: 264800	Females—estrogen, pregnancy, puberty may increase skin lesions	AD and AR (rare) forms,16p13.1	Defective transmembrane protein ABCC6 (ATP-binding cassette subfamily C, member 6 gene), substrate and function unknown	Research only, DNA	Skin bio fragmen fibers

AD autosomal dominant, *AR* autosomal recessive, *ASD* atrial septal defect, *ATP* adenosine triphosphate, *CAD* coronary artery disease, *CHF* congestive heart failure, *CSF* cerebrospinal fluid, *CPK* creatine phosphate kinase, *CSF* cerebral spinal fluid, *DIC* disseminated intravascular coagulation, *DNA* deoxyribonucleic acid, *DVT* deep venous thrombosis, *EMG* electromyogram, *FMD* fibromuscular dysplasia, *GI* gastrointestinal, *HDL* high-density lipoprotein, *HTN* hypertension, *ICA* Internal carotid artery, *ICH* Intracerebral hemorrhage, *LDL* low-density lipoprotein, *MI* myocardial infarction, *MRI* magnetic resonance imaging, *MVP* mitral valve prolapse, *PVD* peripheral vascular disease, *RNA* ribonucleic acid, *SAH* subarachnoid hemorrhage, *VSD* ventricular septal defect, *WM* white matter

[a]TREX Spectrum includes HERNS (hereditary endotheliopathy, retinopathy, nephropathy and strokes), CRV (cerebroretinal vasculopathy), HVR (hereditary vascular retinopathy), RVCL (Retinal Vasculopathy and Cerebral Leukodystrophy)

We also emphasize that an outstanding reference for such disorders is *Online Mendelian Inheritance in Man* (OMIM).[1] This database catalogues all known diseases with a genetic component and—when possible—links them

to the relevant genes in the human genome and provides references for further research and tools for genomic analysis of a catalogued gene. OMIM is one of the databases housed in the US National Center for Biotechnology Information (NCBI) and is included in its search menus.[2] Another federally funded online database, Gene Test-Gene Clinics, provides an international directory of genetic testing laboratories and genetics clinics. We encourage readers to utilize these websites and our listed references to attain additional information if a monogenetic form of stroke is suspected.

In summary, this chapter has been designed as a concise initial reference for readers regarding important monogenetic stroke disorders. This chapter emphasizes established clinically relevant information, as well as introducing several concepts important for interpreting the continually evolving literature on stroke genetics. Lastly, we close with a brief summary of responsible genetic testing.

Definition of Terms

Several terms used throughout this chapter require definition:

Allele—Alternative forms of a gene or marker locus due to changes at the level of DNA.

Autosomal dominant—A disease inheritance pattern that requires only one mutated copy of the gene in a single autosomal chromosome (1–22). Only one copy is necessary for a person to be affected.

Autosomal recessive—A disease inheritance pattern that requires two mutated copy of the gene in a single autosomal chromosome (1–22). Two copies are necessary for a person to be affected.

Complex trait—A trait with a genetic component that is not strictly Mendelian (dominant, recessive, sex-linked). Complex traits may involve the interaction of two or more genes to produce a phenotype or may involve gene-environment interactions.

Genotype—The observed alleles at a genetic locus for an individual. For an autosomal locus, a genotype is composed of two alleles, one transmitted maternally and the other transmitted paternally.

Mendelian trait—A trait that is controlled by a single locus and shows a simple Mendelian inheritance pattern. Such disorders are inherited according to Mendel’s laws.

Phenotype—The observed manifestations of a genotype. The phenotype

may be an observed trait, a particular type of clinical event, or may be expressed physiologically.

Polymorphism—Genetic loci at which there are two or more alleles that are each present at a frequency of at least 1% in the population.

X-linked dominant—A disease inheritance pattern that requires only one mutated copy of a gene on the X chromosome; only one copy is necessary for a person to be affected. Males and females are both affected in these disorders, with males typically being more severely affected than females. The sons of a man with an X-linked dominant disorder will all be unaffected (since they receive their father's Y chromosome), and his daughters will all inherit the condition. A woman with an X-linked dominant disorder has a 50% chance of having an affected fetus with each pregnancy.

X-linked recessive—A disease inheritance pattern in which a mutation in a gene on the X chromosome causes the phenotype to be expressed (1) in males (who are necessarily hemizygous for the gene mutation because they have only one X chromosome) and (2) in females who are homozygous for the gene mutation (i.e., they have a copy of the gene mutation on each of their two X chromosomes). The chance of passing on the disorder differs between men and women; the sons of a man with an X-linked recessive disorder will not be affected, and his daughters will carry one copy of the mutated gene. A woman who is a carrier of an X-linked recessive disorder (X^RX^r) has a 50% chance of having sons who are affected and a 50% chance of having daughters who carry one copy of the mutated gene and are therefore carriers.

Monogenetic or Mendelian Disorders

Rare Mendelian disorders arising from single-gene defects have been described in which stroke is a prominent presenting feature. It should be emphasized that these are not "stroke genes" but rather mutations that may have stroke as an accompanying manifestation. There are several reasons to recognize monogenetic conditions associated with stroke. First, there may be specific treatments that can alter the course of the disease (e.g., transfusion therapy and sickle cell disease); such treatments are often more effective if the disease is identified early. Second, natural history or prognostic information may be available, including the possibility of anticipating problems in other organ systems (e.g., Fabry disease and renal failure).

Finally, diagnosis and counseling may be of benefit to family members of the affected case, potentially leading to earlier diagnosis and amelioration of the disease.

The search for monogenetic stroke etiologies begins with the history and physical examination. As mentioned, we have chosen to illustrate the established monogenetic disorders associated with stroke as categorized by the most common mechanism and etiological causes (i.e., large arterial diseases, small vessel diseases, hematological diseases, mitochondrial diseases, and connective tissue disorders). Table 10.1 [1–114] highlights clues to these disorders from the history and examination. Table 10.2 [1–114] describes other features of these disorders including associated laboratory findings, inheritance patterns, the resulting pathophysiologic defect, and the availability of diagnostic testing. We suggest that readers concerned for a monogenetically suspected case of stroke read through the tables to evaluate for similarities with their case. Utilizing the patient's history and exam findings, note other organ system abnormalities such as the eyes (e.g., *COL4A1*, *TREX*, MELAS), the kidneys (e.g., Fabry disease), and the heart (e.g., familial hypercholesterolemia, connective tissue disorders). Further, the appearance of the stroke(s) on imaging can also be used to further isolate the diagnosis. For example, looking for patterns of strictly small infarcts with white matter changes involving the bilateral extreme capsules and anterior temporal poles might imply CADASIL. As another example, strokes that appear to fluctuate with time and/or do not conform to vascular territories might imply MELAS. Additionally, vessel imaging including magnetic resonance angiography (MRA) or computed tomography angiography (CTA) can also assist with diagnosis; for example, evidence of the pathognomonic "string of beads" sign in the internal carotid artery as seen with fibromuscular dysplasia and vertebrobasilar dolicoectasia as often seen in Marfan syndrome. Magnetic resonance venography (MRV) demonstrating cerebral venous thrombosis may imply a hypercoagulable state such as a factor V Leiden mutation. While we have attempted to make our tables as comprehensive as possible, we also suggest reviewing our references for each condition, and we recommend these other excellent review articles [115–122], as well as the OMIM reference for the specific disease.

Lastly, a genetic cause should be considered in any young stroke patient who lacks established vascular risk factors or whose initial workup is negative for an etiologic cause. Clinicians should always ask about a familial

history of stroke (see Chap. 10). A family history of stroke, particularly at an early age, is probably the strongest indicator of a potential underlying genetic etiology. As such, to maximize the likelihood of detecting a familial condition, a structured approach to the family history is needed. This should include recording the family pedigree in detail, including, at a minimum, all first-degree relatives (i.e., parents, siblings, and children). The major medical conditions of all family members should be recorded as well as age at onset for relevant medical conditions and age at death. Depending on the context, this information from the pedigree should be included for second- and third-degree relatives. A pedigree diagram helps highlight patterns of transmission (e.g., an autosomal dominant pattern versus a maternal transmission pattern as seen in mitochondrial disorders).

Responsible Genetic Testing

Here, we address the questions of when genetic testing is indicated and what is the appropriate context for genetic testing. Genetic tests include analyses not only of DNA or RNA for the purpose of detecting a genetic condition but also analyses of proteins and certain metabolites for the same purpose. Unlike other types of laboratory tests, genetic tests provide information not only about the tested person but also about his or her relatives or descendants. The most appropriate criteria to be used to evaluate the risks and benefits of genetic tests and how these criteria should apply to different categories of genetic tests have been the subject of much societal scrutiny and, in the United States, the focus of several relatively recently instituted federal policies. It has been proposed that the decision to use a particular genetic test in a particular individual should include consideration of analytical validity, clinical validity, and clinical utility, including social consequences.

Analytical validity refers to whether the test is reliable and valid in measuring what it purports to measure. Clinical validity refers to the accuracy of the test in diagnosing or predicting risk of disease and is measured by sensitivity and specificity, which are test characteristics, and predictive value, which is also a function of the prevalence of the disease in the population. Clinical utility refers to outcomes associated with a positive or negative test result.

Here, we also emphasize that genetic testing may have unintended social consequences affecting both the patient and their family through what is

termed as genetic discrimination. Genetic discrimination occurs when people are treated differently by their employer or insurance company because they have a gene mutation that causes or increases the risk of an inherited disorder. Several countries have laws that help protect people against genetic discrimination; however, genetic testing remains a fast-moving field, and these laws do not necessarily cover every situation. In the United States, it was widely recognized that there was a need for federal legislation to limit risks pertaining to discrimination in employment or health insurance on the basis of genetic information. As such, in 2008, the *Genetic Information Nondiscrimination Act* (GINA) was signed into law [123, 124]. GINA protects individuals from the misuse of genetic information in health insurance and employment and was created to remove barriers to the appropriate use of genetic services by the public.

Under GINA, group and individual health insurers cannot:

Use a person's genetic information to set eligibility requirements or establish premium or contribution amounts

Request or require that a person undergo a genetic test

Under GINA, employers *cannot*:

Use a person's genetic information in decisions about hiring, firing, job assignments, or promotions

Request, require, or purchase genetic information about an employee or family member

Types of Genetic Information Protected by GINA:

Family medical history

Carrier testing; i.e., sickle cell anemia and other conditions

Prenatal genetic testing; i.e., amniocentesis, chorionic villus sampling, and other techniques

Susceptibility and predictive testing; e.g., BRCA testing for risk of breast or ovarian cancer, testing for Huntington disease, or hereditary nonpolyposis colorectal cancer (HNPCC) testing for risk of colon cancer

Analysis of tumors or other assessments of genes, mutations, or chromosomal changes

It is important to note that GINA regulates health insurers and employers, not health-care professionals. The law does not require that health-care professionals counsel patients about GINA. Doing so, however, might help your patients feel more comfortable about providing family history information, taking a genetic test, or participating in genetic research. The

law should not keep practitioners from taking a comprehensive family history; rather it protects patients from having that information misused. More recently the Equal Employment Opportunity Commission (EEOC) amended various GINA regulations providing further clarification on acceptable workplace wellness programs with these new guidelines going into effect in July 2016. The new amendments require that (1) employee wellness programs are voluntary; (2) employers cannot deny health care coverage for non-participation, or (3) take adverse employment actions against or coerce employees who do not participate in wellness programs. Additionally, the new GINA regulations cover spousal participation in wellness programs and employers may not ask employees or covered dependents to agree to permit the sale of their genetic information in exchange for participation in wellness plans. [125]

Consideration of the aforementioned factors implies the need to individualize the decision for genetic testing. It should be apparent that the clinical validity, clinical utility, and social consequences of a test could vary greatly depending on whether it is performed for diagnostic or prognostic purposes, individual testing, or population screening; whether a treatment is available for the condition; and whether the genotype has a high or low probability of being associated with the disease phenotype. While there is currently no formal mandate, there is a consensus [126] that genetic education and counseling, as well as written informed consent, should accompany certain types of “high-scrutiny” genetic testing. “High-scrutiny” tests include those that have a relatively low clinical validity and utility but pose a high risk of adverse social consequence. For example, a test whose purpose is predictive, which detects a variant with a low probability of being associated with disease, and for which there is no proven intervention, would fall into this category. It follows from this line of reasoning that the obligation to ensure that the patient is well informed and participates in the decision to proceed with genetic testing depends on where the test falls on the spectrum from low to high scrutiny. An online and freely available e-learning course by the Centers for Disease Control and Prevention reviews the application of the Clinical Laboratory Improvement Amendments (CLIA) requirements to molecular genetic testing and quality assurance measures for molecular genetic testing as consistent with good laboratory practices [127].

Several examples are now mentioned illustrating these considerations with respect to genetic screening for stroke prevention. One obvious example

is the value of screening young African-American stroke patients for sickle cell disease, this is widely accepted because this information directly influences treatment of the initial stroke and strategies for preventing recurrent stroke [53–60]. In contrast, the issues relating to factor V Leiden mutation testing are more complex. A consensus statement by the American College of Medical Genetics recommends that factor V Leiden mutation testing be performed in any person with venous thrombosis in an unusual site, including cerebral vein thrombosis [75]. Although the finding of heterozygosity for this mutation (lifetime risk of venous thrombosis is approximately 10%) does not currently change management or prophylaxis for most patients, the finding of homozygosity (lifetime risk of venous thrombosis >80%) would dictate consideration of lifelong antithrombotic prophylaxis. While more controversial [75], another benefit of testing for factor V Leiden mutation in cerebral venous thrombosis is that relatives of those possessing one or more of the mutant alleles could choose to be screened. Knowledge of factor V Leiden status in asymptomatic relatives could influence a woman's decision to use oral contraceptive or could lead to antithrombotic prophylaxis during periods of increased risk, such as the postpartum period. Routine testing is not currently recommended for young patients with arterial stroke, even those with a family history [75], although testing could play a role in the evaluation of persons with suspected paradoxical embolism. In contrast to stroke attributable to a Mendelian trait, most candidate genes for stroke have a low probability of being associated with disease and do not have proven interventions. For these reasons, testing for such candidate stroke genes would fall into the category of high-scrutiny genetic tests at this time.

One final consideration that warrants emphasis is that the costs of genetic testing are not always covered by insurance, and that these costs are often quite high. As such, we suggest practitioners discuss this issue with the patient and their family 'pre-test', such that there is time to attain insurance authorization for the test. Most insurance companies have prespecified testing policies that typically emphasize that genetic testing be medically necessary to establish a molecular diagnosis of an inheritable disease, emphasizing that the patient is:

Displaying clinical features, or is at direct risk of inheriting the mutation in question (presymptomatic).

That the result of the test will directly impact the treatment being

delivered.

After history, physical examination, pedigree analysis, genetic counseling, and completion of conventional diagnostic studies, a definitive diagnosis remains uncertain, and a specific diagnosis is suspected.

Conclusion

While technological and research advances are accelerating our ability to understand the interplay between genetics and the environment in the pathogenesis of stroke, there are several established monogenetic disorders that cause stroke. Such disorders should be considered in any young patient whose initial workup for stroke is negative. Monogenetic disorders should also be considered in situations in which multiple family members are affected, notably this can be across several disparate organ systems. Through the use of a detailed medical and family history and physical exam, practitioners can determine if an established monogenetic disease seems likely and then weigh the options regarding genetic testing. If a monogenetic form of stroke is suspected, referring a patient for genetic counseling should be considered. As with any stroke, the ultimate goals of these efforts are disease prevention and treatment optimization.

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Footnotes

1 http://www.omim.org/

2 http://www.ncbi.nlm.nih.gov/omim

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11. White Matter Disease

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Introduction

Cerebral white matter (WM) plays a fundamental role in transmitting electrical signals between the neurons; thus, any disorder affecting structural or functional integrity of the WM is bound to present with diverse neurological manifestations. The range of neurological dysfunction varies greatly from relentless failure to thrive in a spectrum of pediatric leukodystrophies to subtle cognitive decline in the elderly with leukoaraiosis. Pathophysiology of these disorders is heterogeneous, but invariably there is a known genetic contribution ranging from monogenetic disorders such as metachromatic and globoid cell leukodystrophies, adrenoleukodystrophy, leukoencephalopathy with vanishing WM disease in the pediatric population to common genetic risk factors in the elderly. In this chapter, we will explore the neurogenetics of sporadic WM disease caused by cerebrovascular pathology, which has been linked to risk of ischemic and hemorrhagic strokes. This subtype of WM disease, also known as WM lesions (WMLs),

WM hyperintensity (WMH), or leukoaraiosis, has been described in the context of epidemiological studies of aging adults with subtle, but progressive, signs of neurological dysfunction and manifest clinical cerebrovascular disease. This chapter will discuss how genetic analysis applies to a spectrum of cerebrovascular WM diseases, outline the current state of knowledge, and delineate the potential implications for future research and neurological practice in the new era of genetic discovery.

Epidemiology, Neuroimaging, and Clinicopathological Correlates of White Matter Disease

WM changes, visible on T2-weighted fluid attenuation inversion recovery (FLAIR) magnetic resonance imaging (MRI) as hyperintensities or as areas of hypoattenuation on computed tomography (leukoaraiosis), are known to be the most common manifestations of cerebrovascular disease in the elderly (Fig. 11.1). A community-based study in Austria found that 70% of individuals aged 50–75 years had some degree of WMH [1], while the figure for hypertensive siblings in the US (mean age 65 years) was 73% [2]. In the Rotterdam Scan Study, only 5% of 1075 individuals aged 60–90 years lacked any subcortical or periventricular WMLs [3]. In all of these populations, the volume of these WMHs increased markedly with advancing age. While each of these studies varied slightly in its methods for measuring lesion burden, the consistent demonstration that WMH is both common in elderly populations and increases in prevalence and severity with age [4] suggests that these lesions develop at least in part as a function of the aging process [5]. Independent of normal aging, higher WMH volume was associated with worse performance on visuospatial memory and organization, visual scanning, motor speed, and new learning in 1820 stroke- and dementia-free subjects from the Framingham Heart Study [6]. Additionally, greater progression in WMH volume has been linked to a higher risk of multiple falls [7]. Understanding the underpinnings of WMH progression, and thereby developing preventative measures, may be integral to preventing morbidity and mortality in the elderly.

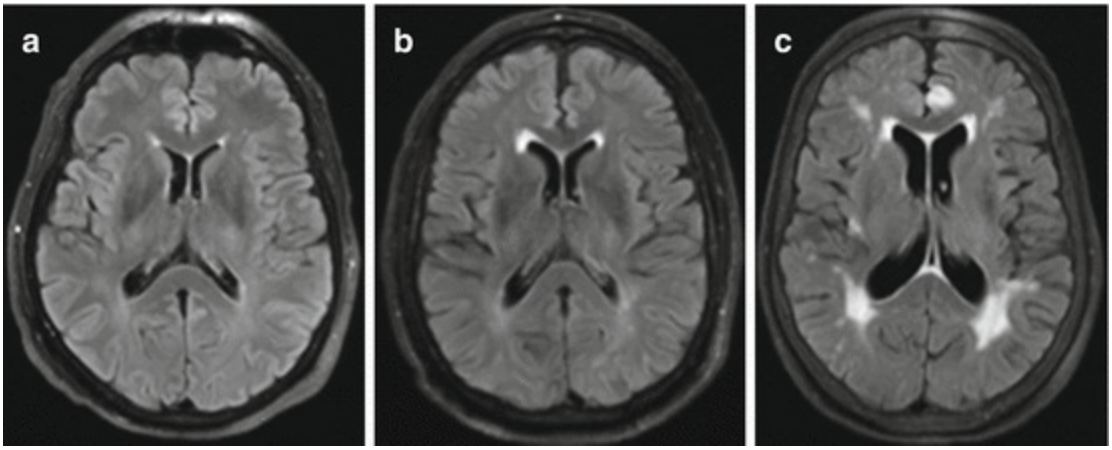

Fig. 11.1 White matter hyperintensity on 3T MRI. The burden of underlying cerebrovascular disease in adults can be visualized using T2 FLAIR MRI, which demonstrates mild (**a**), moderate (**b**), and severe (**c**) leukoaraiosis involving both periventricular and subcortical areas of the white matter. Severity and topographical distribution of WMH varies depending on pathophysiology of the primary process; however, the small cerebral vessel pathology is often implicated in both sporadic and hereditary syndromes (Courtesy of Dr. Rost)

However, the pathophysiology of WMH is poorly understood, and it is unclear whether WMH represents one or multiple disease processes. Accumulating evidence suggests a final common pathway of chronic ischemic injury to the WM, as evidenced by neuropathological studies [8] and, furthermore, by strong epidemiological links between WMH and known vascular risk factors, including hypertension (HTN) [9], especially higher systolic blood pressure [10], carotid [11] and intracranial atherosclerosis [12], homocysteine levels [13, 14], diabetes [15], impaired kidney function [16] and cigarette smoking [17]. Progression of WMH has similarly been associated with smoking and elevated levels of inflammatory markers, in addition to age, HTN, and preexisting WMH volume [18–21]. A study of 972 individuals in the Atherosclerosis Risk in Communities (ARIC) study demonstrated a dose-dependent effect of smoking on WMH progression, highlighting the role of preventable vascular risk factors in modifying disease risk [22]. Aside from vascular risk factors and age, early-life processes also appear to significantly contribute WMH at old age. In 277 dementia-free participants from the Aberdeen Birth Cohort, childhood socioeconomic status has been inversely correlated with WMH independent of hypertension [23].

Notably, current population-based studies go beyond quantifying macrostructural changes of WMH, and are starting to focus on microstructural modifications including structural and functional integrity of

cerebral WM. Alterations in normal appearing WM (NAWM) precede *de novo* WMH lesions and expansion of existing WMH. This has been demonstrated in 689 subjects from the Rotterdam Scan Study, where diffusion tensor imaging (DTI) was used to determine microstructural changes in NAWM [24]. Compared to conventional imaging techniques, DTI metrics such as fractional anisotropy and mean diffusivity allow for assessment of microstructure that are not assessable otherwise. Independent of aging and macrostructural WM changes, microstructural changes in NAWM are worse in subjects with vascular risk factors like hypertension, smoking, and diabetes mellitus [25]. Disturbed structural and functional integrity of NAWM is associated with reduced kidney function [26] and higher overall mortality, specifically secondary to cardiovascular diseases [27]. Likewise, functional disruptions in WM tracts have been associated with systolic blood pressure [28] and ensuing cognitive decline, [29] raising the question if addressing vascular risk factors may modify progression of WMH and thus WMH-related morbidity.

Severity of WMH has been studied in population-based cohorts, using both semi-quantitative visual grading and semi-automated volumetric methods [1, 9, 14, 17, 30]. In these studies, advancing age, the presence of cardiovascular disease, HTN, and cigarette smoking were independent predictors of severity of WMH [17, 30, 31]. After adjusting for age and sex, the Framingham Stroke Risk Score, a composite measure of multiple stroke risk factors that strongly predicts future cerebrovascular events [32], strongly correlated with volume of WMH, even in healthy individuals ≤55 years of age [17]. In addition, other population-based studies showed that homocysteine [14] and chronic kidney disease [33, 34] were independent predictors of the WMH severity in otherwise healthy individuals. The importance of WMH is reflected in the multitude of age-related co-morbidities that are mediated by the presence and progression WMH, including: cognitive abilities [35] including executive functioning, verbal fluency and visuospatial perception [36], cognitive decline [37], gait [7], urinary incontinence [38] and risk of stroke [39].

Pathologic and epidemiologic evidence links chronic ischemia, impaired cerebrovascular hemodynamics and vascular risk factors to incidence and progression of MRI-detectable WMH. Several underlying pathological processes have been linked to the presence of WMH—including myelin attenuation, axonal loss, oligodendrocyte loss, astrocytic gliosis, and

arteriolar sclerosis [40–43]. However, inherited diseases like cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) [44, 45], Alzheimer's disease, or cerebral amyloid angiopathy (CAA) [40, 46, 47] likewise display extensive WMH lesions. In addition to these structural changes, functional WM network integrity in 426 patients with small vessel disease without dementia (aged 50–85 years) was significantly associated with WMH burden and cognitive performance, highlighting the importance of both structural and functional integrity of WM in the elderly [48]. Despite this plethora of potential pathophysiological factors, accumulating data support a role for chronic ischemia as the common mechanism leading to WMH [5]. As part of the population-based Cognitive Function and Ageing Neuropathology Study, 207 donated brains were examined using postmortem MRI and histopathology [49]. Authors compared areas of WMH and normal WM and found significantly increased arteriolar sclerosis, capillary endothelial and microglial activation, elevated immune system reactivity for hypoxia-inducible factor (HIF) 1 and 2, and increased expression of the hypoxia-related proteins MMP-7 and neuroglobin [49]. Further evidence for the role of endothelial dysfunction in WMH was uncovered in an investigation of 15 inflammatory biomarkers and ischemic and hemorrhagic features of small vessel disease in 1763 stroke-free participants of the Framingham Offspring Cohort. Higher levels of osteoprotegerin, ICAM-1, Lp-PLA_2 mass, and myeloperoxidase were observed in patients with silent cerebral infarcts and greater WMH burden. In contrast, higher levels of tumor necrosis factor receptor 2 and myeloperoxidase were associated with cerebral microbleeds, suggesting a differential role of inflammation in chronic ischemic and hemorrhagic cerebrovascular lesions [50].

WMH contributes to risk of future symptomatic ischemic stroke [51], deterioration in gait [52], and dementia [53] and correlates with cognitive dysfunction [54], and late-life depression [55]. WMH may also determine the severity of cerebral injury in acute ischemic stroke, serving as a risk factor for symptomatic hemorrhage following thrombolysis [31]. Furthermore, WMH predicts infarct growth [56] and poor post-stroke outcomes [56–59].

In contrast to studies of normal aging adults, hospital-based studies of leukoaraiosis in patients with stroke demonstrated that the risk factors for WMH severity do not appear to fully overlap with those previously reported for population-based cohorts [60, 61], suggesting the possibility of greater

heterogeneity between the types of WMH than previously suspected [62]. WMH burden appears significantly greater in patients with clinical manifestation of stroke, and it is strongly linked to small vessel (lacunar) stroke subtype [63–67] (Fig. 11.2), including lacunar infarct evolution [67]; however, the diffuse abnormality of small cerebral blood vessels that manifests as WMH may differ from the diffuse abnormality that manifests as small vessel (SV) stroke [68, 69]. These clinical-pathologic observations are further confirmed by emerging genetic research, which demonstrates that genetic contribution to WMH burden in stroke patients may differ from that to the risk of lacunar infarct [70]. Furthermore, there is a possibility that the processes underlying WMH burden accumulation in stroke patients may differ from those within the broader population and may not be simply mediated by traditional vascular risk factors. Among 809 acute ischemic stroke patients with WMH volume (WMHv) quantified on admission MRI, elevated levels of plasma homocysteine (Hcy) and a marker of chronic hyperglycemia (HgbA1c) were independent predictors of pre-existing WMH burden. Because Hcy and HgbA1c have been previously linked to endothelial dysfunction related to oxidative stress, the association between these metabolic blood markers and WMH burden in acute ischemic stroke suggests that the degree of endothelial dysfunction may be greater in patients with increased WMHV, and may in part explain the relationship between WMHV and poor post-stroke outcomes [71]. Furthermore, different vascular risk factors may exert their effect on WMHv across the lifespan of patients presenting with acute ischemic stroke. Among 1008 acute ischemic stroke patients with WMHV examined at the extremes of their age of presentation, history of tobacco use was a strong independent predictor of WMH burden in patients with early-onset stroke, whereas age is no longer associated with WMHv in acute ischemic stroke patients older than 75 years of age. These findings suggest not only that the major risk factors for WMH vary across age groups, but also that these risk factors may be modifiable [72].

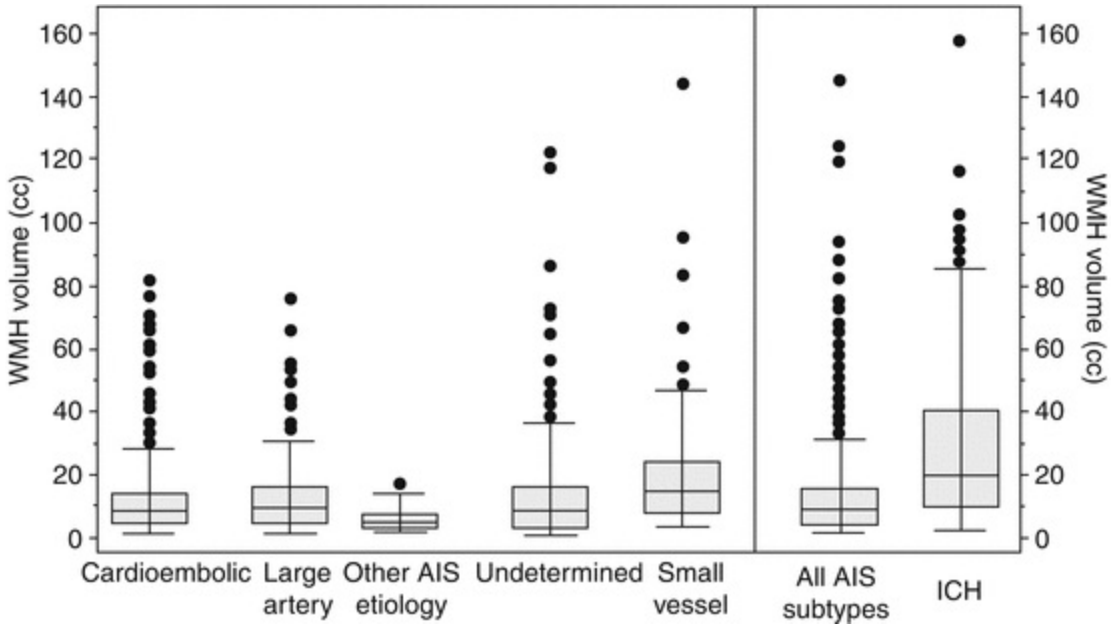

Fig. 11.2 Box plot graphic representation of WMH volume distribution by acute ischemic stroke (*AIS*) subtype and intracerebral hemorrhage (*ICH*). *AIS* acute ischemic stroke, *cc* cubic centimeters, *ICH* intracerebral hemorrhage, *WMH* white matter hyperintensity (Reprinted with permission from Rost et al. [63])

Several epidemiologic models have been proposed to elucidate the relationship between MR-detectable WMH and clinical manifestations of cerebrovascular disease (Fig. 11.3). While the biology of WMH is complex, it may predispose to stroke and other manifestations of chronic cerebrovascular dysfunction through the common final pathophysiological pathways that are yet to be determined and are likely to be affected by common genetic variants. Since traditional vascular risk factors failed to account for variability of WMH–accounting for only 1.42% of WMH variance in stroke-free individuals and 0.1% of WMH variance in acute ischemic stroke patients–genetic risk factors may explain the phenotypic variability in WMH [73]. In light of African American race as a risk factor for extensive deep WMH burden, differential genetic background may account for differences in WMH burden [74]. In summary, WMH plays a dual role, as both a marker of cerebral ischemia and a risk factor for clinical manifestations of cerebrovascular disease.

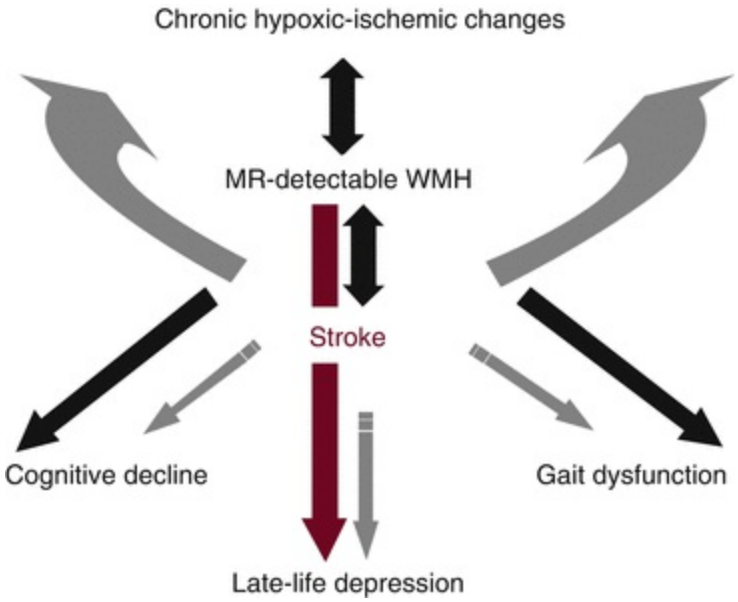

Fig. 11.3 Proposed mechanisms of cerebrovascular white matter disease involving cerebral ischemia, WMH, and its clinical manifestations

Genetic Architecture of Cerebrovascular White Matter Disease

WMH Heritability and Results of the Linkage and Candidate Gene Studies

MRI-detectable WMH is the most heritable cerebrovascular phenotype in the elderly. In contrast to other cerebrovascular phenotypes and despite its association with environmental risk factors such as tobacco use, WMH demonstrates substantial heritability across multiple populations. When comparing 74 monozygotic and 71 dizygotic American male twin pairs aged 68–79 years at time of MRI, WMH heritability was estimated at 0.71 (95% CI 0.66 to 0.76), adjusting for age and head size [75]. Similarly, the heritability estimate of 0.67 (adjusted for sex, age, systolic blood pressure, and brain volume) was obtained from an analysis of 210 hypertensive non-Hispanic white Americans from the GENOA-Rochester study [2], and the heritability estimate of 0.55 (adjusted for age, sex, and brain volume) was obtained from 1330 stroke- and dementia-free individuals from the Framingham Heart Study [76]. In the similarly designed San Antonio Family Heart Study, heritability estimates of subcortical, periventricular, and whole-

brain WMH were consistent (0.66, 0.73, and 0.72, respectively) [77]. With an estimate of 0.76 heritability of overall WMH, 0.64 for periventricular and 0.77 for deep WMH, the Older Australian Stroke Study of 92 monozygotic and 68 dizygotic pairs confirm previous estimates. Voxel-wise heritability estimates indicate regional differences in heritability, the highest being for deep WMH lesions [78]. An estimate of WMH heritability in 2336 ischemic stroke patients demonstrated a higher heritability in hypertensive ($h = 0.45$, $P = 7.99 \times 10^{-5}$) than normotensive ($h = 0.13$, $P = 0.13$) subjects [79]. The overall heritability of WMH suggests a large genetic component to the individual expression of WMH volume and makes it an ideal target for gene discovery.

Despite high heritability, linkage studies have not identified genetic risk variants in either stroke or WMH. Candidate gene studies have reported associations of a handful of polymorphisms in different genes with WMH [80–86], but none of these findings has been convincingly replicated. Association of polymorphisms in renin-angiotensin system with WMH burden failed to replicate this previously reported locus and demonstrated a nominal association for rs4362 (*ACE* gene, $P = 0.01$) and rs699 (*AGT* gene, $P = 0.03$) in males only [87]. While genetic variants in the ACE gene have not consistently been linked to cerebrovascular WMH lesions, studies suggest a modifying effect of ACE insertion/deletion polymorphisms on WMH burden in Alzheimer disease [88]. A comprehensive list of genetic polymorphisms previously reported in association with MRI evidence of WM lesions has been previously outlined [89] (Table 11.1), and in its current form, the list is a testament to the substantial effort of multiple researchers to understand the genetic architecture of WMH. However, it is also an example of the methodological failure that has resulted from applying simple rules of association to a complex phenotype. With exception of perhaps APOE allele contributions, the probability of any single genetic locus contributing significantly to the variability in WMH burden in the general population is very small. Any genetic contribution to sporadic leukoaraiosis is likely to be due to a large number of genetic variants with individually small effect, the model that is emerging for common diseases such as stroke and other cerebrovascular disease phenotypes [90]. Family-based linkage studies for WMH have not yielded consistent results. In the Framingham cohort [91], DeStefano et al. reported a 4-centimorgan peak with a maximum multipoint logarithm of odds (LOD) score of 3.7 on chromosome 4. However, a

genome-wide search for linkage in the GENOA-Rochester sibships neither replicated this finding nor identified any other chromosomal regions with significant evidence for linkage [92]. More recent analysis of the overlap between WMH and blood pressure-related phenotypes demonstrated a novel locus on chromosome 1 in whole-genome linkage analysis of the San Antonio Family Heart Study [93]. However, these findings will require independent confirmation prior to drawing conclusions about this locus. Family-based linkage analysis has been highly successful in rare, monogenic, or oligogenic diseases [94, 95]. In contrast, linkage studies of complex diseases have yielded few statistically significant results and, even where successful, have only led to identification of causal variants in a handful of cases. The limited success of linkage in the presence of substantial heritability suggests that there are few, if any, individual loci that carry alleles of major effect. However, alleles that are common can have a substantial population effect without giving a strong linkage signal [96, 97].

Table 11.1 Genetic polymorphisms associated with white matter lesions identified by linkage and candidate gene studies

Gene	Polymorphism	*P*/OR	*n*	Sample	Association
Apolipoprotein E (*APOE*)					
	APOE E4	$P < 0.03$	396	Population[a]	CVD[b]
	APOE E4	$P < 0.005$	92	Population	Depression[b]
	APOE E4	$P = 0.016$	971	Population	Hypertension[b]
	APOE E2/E3	OR = 3.0; $P = 0.01$	280	Population	Positive[c]
	APOE E4	NS	93/583	CI/control	None[d]
	APOE E4	NS	55/66	AD/control	None
	APOE genotype	NS	215/20	Dementia[e]/control	None
Angiotensinogen (*AGT*)					
	AGT promoter-20:c	$P = 0.017$	410	Population	Positive
	AGT M235 T	$P < 0.001$	267	Population	Positive
	AGT M235 T	$P = 0.008$	>1000	Population	Positive
Angiotensin II receptor type 1 (*AGTR1*)					
	AGTR1 A1166C	$P < 0.005$	134	Population	Positive
	AGTR1 A1166C	$P < 0.05$	93	Hypertensive	Negative[c]
	AGTR1 A1166C	NS	129/27	Ischemic stroke/population	None

	AGTR1 A1166C	NS	510	Acute BI	None
Angiotensin I-converting enzyme (*ACE*)					
	ACE D/D genotype	$P < 0.05$	182	Dementia	Positive
	ACE D allele	OR = 2.95; $P < 0.01$	129/27	Ischemic stroke/population	Positive
	ACE D/D genotype	$P < 0.0005$	229	LA combined with infarcts	Positive
	ACE D/D genotype	OR = 4.44; $P = 0.02$	60	Hypertensive	Positive
	ACE D allele	NS	134	Population	None
	ACE D allele	NS	93	Hypertensive	None
Aldosterone synthase (*CYP11B2*)					
	CYP11B2 TT genotype	OR = 4.6; $P = 0.009$	829	Population	Positive
Methylenetetrahydrofolate reductase (*MTHFR*)					
	MTHFR A1298C	$P = 0.001$	68	PCSNL receiving MTX	Positive
	MTHFR C677T	$P = 0.017$	178/85	Depressed/nondepressed	Positive
Brain-derived neurotrophic factor (*BDNF*)					
	BDNF V66M	$P = 0.044$	199/113	Depressed/nondepressed	Positive
Paraoxonase (*PON1*)					
	PON1 L55M LL genotype	OR = 2.65; $P = 0.004$	264	Population	Positive
	PON1 Q191R	OR = 6; $P = 0.02$	104/113	ONFH/control	Positive
Nitric oxide synthase (*NOS3*)					
	NOS3 G894T	$P < 0.05$	93	Hypertensive	Positive
	NOS3 G894T	NS	300/600	SVD/control	None

Adapted with permission from Assareh et al. [89]
NS not significant, *CVD* cardiovascular diseases, *CI* cerebral infarction, *AD* Alzheimer's disease, *BI* brain infarction, *LA* leukoaraiosis, *PCSNL* primary CNS lymphoma, *MTX* methotrexate, *ONFH* osteonecrosis of the femoral head, *SVD* small vessel disease
[a]Population: community sample
[b]Association observed only in interaction with stated medical conditions
[c]Positive association: the genotype mentioned or the mutant allele/amino acid is associated with more WMLs. Negative association: genotype mentioned or the mutant allele/amino acid is associated with less WMLs

[d]Non-Caucasian population
[e]Dementia: Alzheimer's disease (AD), non-AD dementia, or mixed neuropsychiatric disorder

Since linkage and candidate-gene studies had diminished power due to small sample size, a meta-analysis of 43 case-control candidate gene studies analyzing 7 genes in 6314 WMH cases and 15,462 controls detected a decreased risk of WMH in *CYP11B2* T(−344)C carriers in a fixed-effects meta-analysis and *MTHFR* C677Y carriers in a Mendelian randomization study with homocysteine levels and WMH burden. However, both loci could not be validated in replication [98]. The *MTHFR* C677T locus has also been interrogated in 5153 ischemic stroke cases of European ancestry (1359 lacunar stroke, 1824 large artery stroke, and 1970 cardioembolic stroke) and 14,448 controls and demonstrated and association of *MTHFR* C677Y with lacunar stroke. The stroke subtype-specific association may account for the failure of homocysteine lowering measures as a secondary preventative strategy for stroke recurrence [99].

Newer candidate gene approaches examine common variation in genes causing monogenic WMH-related disorders to establish if common variation in these genes contributes to sporadic WMH lesion burden. Mutations in the *NOTCH3* gene cause CADASIL. In 888 participants from the population-based Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) study, four common SNPs (rs1043994, rs10404382, rs10423702 and rs1043997) were significantly associated with the presence and progression of WMH in the setting of hypertension. This finding was validated in a stroke-free, hypertensive replication cohort (n = 4773) from CHARGE (P = 0.04) [100]. However, in a recent study investigating ischemic stroke cases, common *NOTCH3* variants were neither associated with lacunar stroke status (1350 lacunar stroke cases and 7397 controls) nor with WMH volume (3670 ischemic stroke patients) [101]. Common variation in *COL4A1/COL4A2*, which has been recognized as a monogenic disorder causing cerebral small vessel disease, has also been linked to hemorrhagic stroke. In particular, 3 SNPs (rs9521732, rs9521733, rs9515199) have been identified from a cohort of 1545 intracerebral hemorrhage cases and 1485 controls. While these loci were found to have the same direction of effect in lacunar ischemic stroke (12,389 cases and 62,004 controls), WMH burden in ischemic stroke (2733 cases) and WMH burden in community-dwelling

subjects (9361 cases), they failed to pass a Bonferroni-corrected significance threshold [102].

Single-Gene Disorders Associated with White Matter Disease

For several monogenic cerebrovascular phenotypes, WMH is a major clinical feature (Table 11.2, Fig. 11.4). These disorders usually have a well-described and predictable pattern of inheritance, and the genetic variants (alleles) responsible for disease rarely occur in the general population (minor allele frequency <5–10%). However, the effects of these genetic variants are strong, and their presence is both necessary and sufficient to cause disease [104].

Table 11.2 Single-gene disorders associated with cerebral white matter disease

Disease	Genetic basis	Clinical spectrum	Diagnostic tools
CADASIL	*NOTCH3* (AD)	Extensive WMH, small vessel and territorial strokes, depression, migraine headaches with aura, cognitive decline, cerebral microbleeds, rare ICH	MRI, skin biopsy (for granular osmophilic inclusions), mutation screen
CARASIL	*HTRA1* (AR)	Extensive WMH, small vessel and territorial strokes, early-onset motor impairment, cognitice decline, early-onset diffuse alopecia	Mutation screen
Familial CAA	*APP* (AD), *cystatin C*, *BRI*, *transthyretin*	Extensive WMH, cognitive decline and dementia, cerebral microbleeds, lobar ICH, rare cerebral infarcts	Family history, clinical features, MRI, experimental PIB-PET scan
HANAC	*COL4A1*, *COL4A2* (familial and sporadic)	Extensive WMH, retinal arteriolar tortuosity and retinal hemorrhages, neonatal stroke and porencephaly, ICH, cerebral microbleeds, nephropathy, intracranial aneurysms	MRI features, ocular fundus examination, renal function testing, mutation screen
Fabry's disease	*GLA* (X-linked)	Cataracts, small fiber neuropathy, WMH, stroke, angiokeratomas, renal and cardiac failure	Alpha-galactosidase activity, mutation screen, lyso-Gb3
Homocystinuria	*CBS* (AR)	Premature atherosclerosis, WMH, lens dislocation, Marfan-like features, stroke, mental retardation	Plasma and urine levels of homocysteine and methionine, mutation screen
MELAS	Multiple mitochondrial (maternal	Mitochondrial myopathy, encephalopathy, lactic acidosis, strokes; extensive WMH, developmental delay, sensorineural	MRI and MR spectroscopy, muscle biopsy, CSF for lactate-

	inheritance)	hearing loss, seizures	to-pyruvate ratio; mutation screen of mtDNA
HDLS	*CSF1R* (AD)	Extensive WMH, personality and behavioral changes, dementia, depression, parkinsonism, and seizures	MRI, clinical features, family history, mutation screening
RVCL/HERNS	*TREX1*	WMH, progressive visual loss, nephropathy, stroke	Clinical features, family history, mutation screening
Axenfeld-Rieger Syndrome	*FOXC1, PITX2*	WMH, subcortical infarcts, retinal arteriolar tortuosity, cerebellar malformations	Clinical features, family history, mutation screening

Adapted from Goldstein LB [142]
AD autosomal dominant, *APP* amyloid precursor protein, *AR* autosomal recessive, *CAA* cerebral amyloid angiopathy, *CADASIL* cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy, *CARASIL* cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy, *CBS* cystathionine β-synthase, *CSF* cerebrospinal fluid, *CSF1R* colony stimulating factor 1 receptor, *COL4A* collagen type IV A, *GAL* α-galactosidase, *HANAC* hereditary angiopathy with nephropathy, aneurysm, and muscle cramps, *HDLS* hereditary diffuse leukoencephalopathy with spheroids, *HERNS* hereditary endotheliopathy with retinopathy, nephropathy, and stroke, *ICH* intracranial hemorrhage, *lyso-Gb3* globotriaosylsphingosine, *MELAS* mitochondrial encephalopathy with lactic acidosis and stroke-like episodes, *MRI* magnetic resonance imaging, *mtDNA* mitochondrial DNA, *PIB-PET* Pittsburgh compound B positron emission tomography, *RVCL* retinal vasculopathy with cerebral leukodystrophy

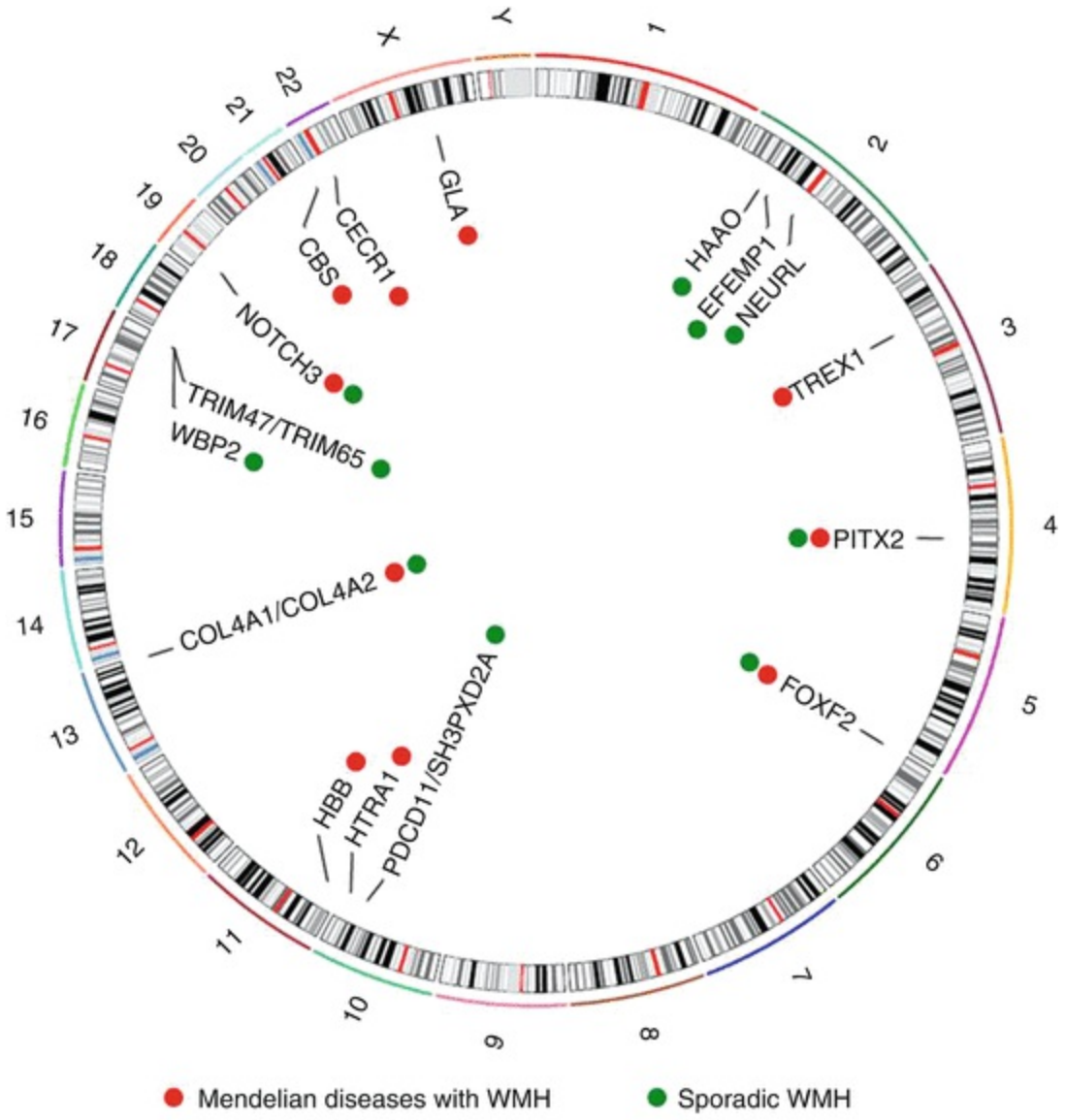

Fig. 11.4 Summary of known loci causing rare and common forms of WMH. Adapted from Haffner et al. [103]

CADASIL (see Chapter 6) is a prototypical cerebrovascular disorder linked to a single genetic locus causing extensive small cerebral vessel disease [105], which, among many clinical symptoms, manifests with extensive MRI-detectable leukoaraiosis [106]. The *NOTCH3* gene (chromosome 19p13.1) mutations that span exons 3 through 6 [107] are responsible for most CADASIL cases among individuals of European origin [108]. The proposed mechanisms of disease in CADASIL involve a gain or loss of cysteine residues in the extracellular domain of the Notch3 protein, leading to cerebral vascular smooth muscle dysfunction and resulting in

small, predominantly cerebral vessel disease [109]. Characteristic MRI patterns of WMH topography and distribution have been described in CADASIL, involving the anterior temporal lobes, the frontal lobes, and the periventricular WM [109, 110]. In recent years, understanding that the genetic architecture of single-gene disorders is likely to be more complex than previously thought [44, 109, 111] came from finding shared genetic contributions between single-gene and sporadic presentations of the same phenotypes [112]. For example, the presence and severity of WMH behave as complex traits in CADASIL [113, 114]. This has been further substantiated by a GWAS of 466 subjects investigating the genetic architecture WMH burden in CADASIL. Due to different genetic backgrounds, the cohorts were analyzed in two clusters. While no single SNP crossed genome-wide significance, the phenotypic variance explained by all SNPs was 0.85 (SE = 0.21) and a polygenic score derived from cluster 1 was significantly associated with WMH burden in cluster 2, indicating that common variants play an important role in disease modification even in monogenic disorders [115]. An analysis of *APOE* in the same cohort detected a disease modifying effect of *APOE ε2* on WMH volume [116].

Similarly, an epidemiologic investigation of WMH burden in 223 patients with Fabry disease, an X-linked lysosomal storage disorder, identified cardiomyopathy and enzyme replacement therapy status as independent determinants of WMHv in patients in their fourth decade of life. This point in time may be integral for disease progression and also raises the question whether WMHv in Fabry disease is under both environmental and genetic influence and acts as a complex trait [117].

Mitochondrial encephalopathy with lactic acidosis and stroke-like episodes (MELAS) has been a focus of intense investigations aiming to understand the effect of mitochondrial genetics on cerebrovascular disease [118] (see Chapter 8). It is a maternally inherited disorder, which presents with recurrent cerebral infarctions and which is thought to be caused by a spectrum of mutations in the mitochondrial DNA (mtDNA), including the alanine-to-glycine transition (A3243G) [119]. Radiographic and neuropathologic correlation studies of WMH in MELAS demonstrate evidence of myelin loss and fibrous gliosis corresponding to the areas of leukoaraiosis on brain scans, not unlike those in sporadic WMH [120].

Another gene linked to an autosomal dominant syndrome named hereditary diffuse leukoencephalopathy with spheroids (HDLS) [121] is an

exciting development for those trying to disentangle the genetic architecture of leukoaraiosis. This familial disorder has been characterized by a variable spectrum of clinical presentation including cognitive, behavioral, and motor deterioration occurring in the fourth or fifth decades of life, progressing to death within less than 10 years [122, 123]. Pathognomonic MRI findings of extensive and relentlessly progressive leukoaraiosis in HDLS [124] correspond to the neuropathological changes that involve predominantly the frontal and parietal WM, with evolving cortical atrophy affecting these lobes (Fig. 11.5), and widespread loss of myelin sheaths and axonal destruction, axonal spheroids, gliosis, and autofluorescent lipid-laden macrophages [121]. Using combined genome-wide linkage analysis and exome sequencing in 14 families with HDLS, Rademakers et al. identified 14 different mutations affecting the tyrosine kinase domain of the colony stimulating factor 1 receptor (*CSF1R*); furthermore, *de novo* occurrence of the *CSF1R* mutation and additional *CSF1R* mutation in an individual diagnosed with corticobasal syndrome were identified using exome sequencing techniques [121]. Partial loss of CSF1R function is now implicated in the HDLS pathophysiology based on its crucial role in mediation of microglial proliferation and differentiation in the brain [118]. While the role of microglial dysfunction in development and progression of sporadic leukoaraiosis is unknown, these findings may help further elucidate the complex mechanisms of cerebral WM disease**.** Understanding the genetic contribution to WMH in brain disorders with fundamentally different underlying disease pathology, ranging from the neuroinflammatory (i.e. Multiple Sclerosis [125]) and neurodegenerative (i.e. Parkinson Disease [126]) spectrum, may further help to elucidate the pathophysiology of WMH.

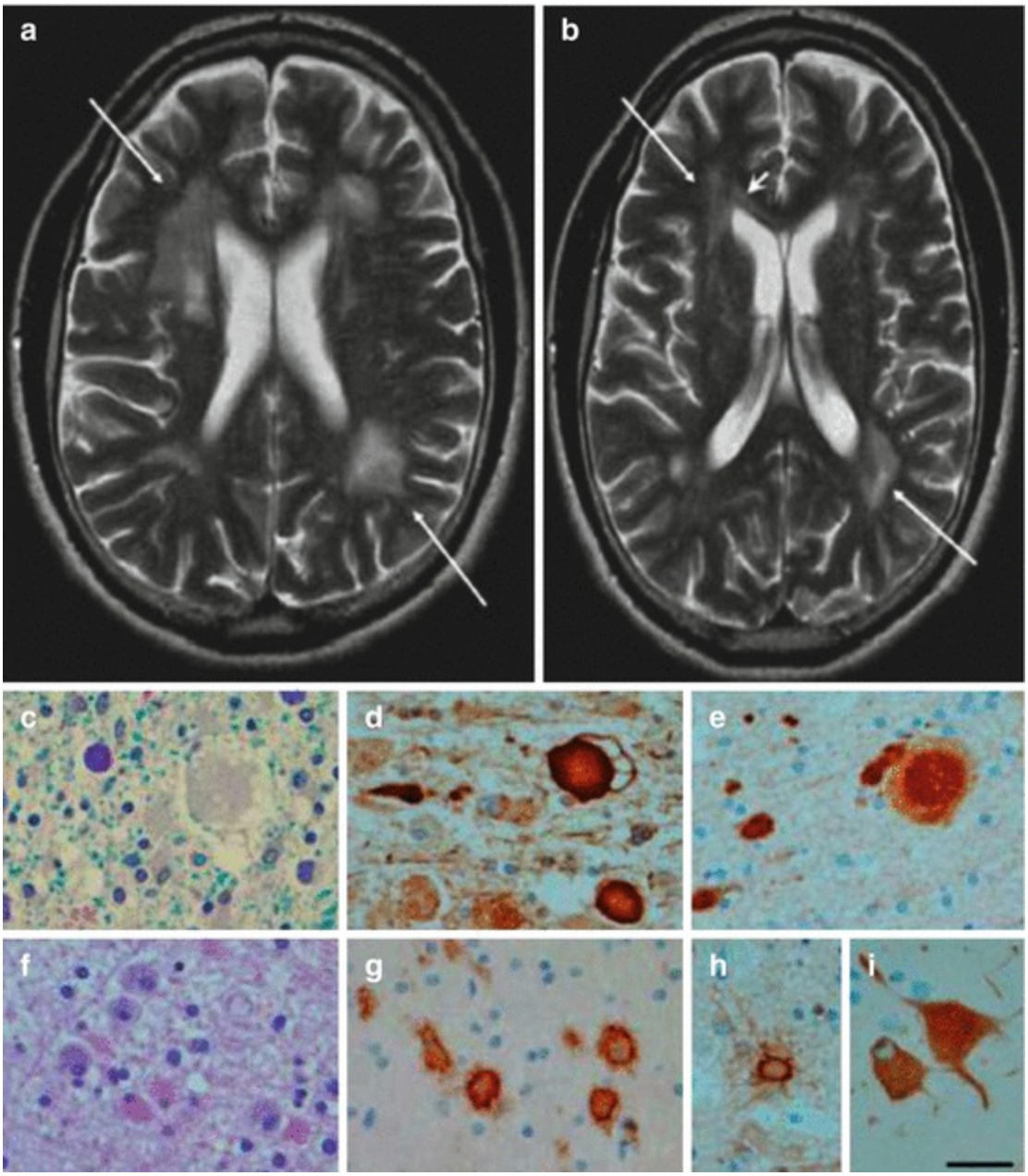

Fig. 11.5 Neuroimaging and neuropathological findings in HDLS. (**a**, **b**) Axial T2-weighted magnetic resonance images showed localized hyperintense foci in both frontal and parietal lobes (*long arrows*), involving the periventricular, deep and subcortical white matter but sparing the subcortical U-fibers. Hyperintense focus in the right forceps minor (*small arrow*) was seen. (**c**) Myelin loss in frontal white matter with a pigmented macrophage and a pale vacuolated axonal spheroid (*Luxol fast blue*). (**d**) Spheroids with phosphorylated neurofilament immunohistochemistry. (**e**) Spheroids with amyloid precursor protein immunohistochemistry. (**f**) Pigmented macrophages and reactive astrocytes (hematoxylin and eosin). (**g**) White matter macrophages with HLA-DR (human leukocyte antigen, DR epitope region) immunohistochemistry. (**h**) Bizarre white matter astrocytes. (**i**) Ballooned cortical neurons with α-B-crystallin immunohistochemistry. Scale bar (**c**–**i**), 30 μm (Reprinted with permission from Rademakers et al. [121])

Additionally, new genes continue to be mapped to hereditary forms of small vessel disease, including the recent discovery of *FOXC1* and *PITX2* that have been linked to cerebral SVD [127]. Both genes have been described previously in the context of the Axenfeld-Rieger Syndrome (ARS), a rare disease affecting the formation of anterior eye structures with concurrent extraocular manifestations [128, 129], though the link to WMH and stroke has only been recognized recently [127]. Interestingly, rs6843082-G near *PITX2* has been identified as a risk locus for cardioembolic stroke [130], a finding that has been recently confirmed in a meta-analysis of 18 population-based studies encompassing a total of 84,961 participants [131], highlighting again the importance of common variants in genes classically causing monogenic disorders.

Complex Disease Genetics and Cerebral White Matter Disease

The role of common DNA variants in complex cerebrovascular phenotypes is a focus of intense investigations by stroke geneticists. Given high estimates of heritability and a very limited contribution to observed WMH variability by the known monogenic disorders associated with cerebrovascular WM disease, pursuing the genetic underpinnings of WMH using genome-wide association studies (GWAS) offers the potential for gene discovery and a high-yield translational approach to study of complex diseases, as it has been demonstrated in other fields [132]. Significant obstacles to GWAS-linked discoveries of common variants in WMH are the apparent heterogeneity of the disease as well as the possibly of even greater complexity of the genetic architecture of cerebrovascular disease than previously suspected [133, 134]. However, these challenges to discovery can be overcome through the use of sophisticated tools of MRI analysis and larger sample sizes to increase the power of the GWAS approach [133]. An example of such an approach is demonstrated by the investigation of common mitochondrial genetic variants in a large, multicenter, mitochondrial GWAS of 2284 ischemic stroke cases and 1728 controls from the International Stroke Genetics Consortium, all of whom were genotyped for 64 common mtDNA variants [135]. A genetic score comprised of the sum of contributions from individual variants across the mitochondrial genome showed association in meta-analysis with ischemic stroke (OR = 1.13, $P < 0.0001$) as well as with the volume of WMH in 792

nested case individuals with ischemic stroke ($P = 0.037$). In this analysis, low frequency of identifiable mutations, haploid nature of mitochondrial genome limiting analytic approach to genetic modeling, and low effect sizes of individual variants, the challenges specific to mitochondrial genetics, have been overcome by the GWAS approach and creative use of computational methods [135].

A significant breakthrough in understanding the genetic architecture of WMH in healthy, aging adults was the discovery of a locus on chromosome 17q25 encompassing six known genes (*WBP2*, *TRIM65*, *TRIM47*, *MRPL38*, *FBF1*, and *ACOX1*) [136]. In this study, a remarkable effort to overcome the limitations of phenotype heterogeneity and lack of statistical power proved effective when a meta-analysis of genome-wide associations with WMH burden was tested in seven individual community-based cohorts of 9361 stroke-free individuals of European descent. Furthermore, significant findings were replicated in 3024 individuals from two additional cohorts [136]. Even though the role of the chromosome 17q25 locus in pathophysiology of WMH is not currently understood, the implications of this study's feasibility are far more reaching at this stage than identifying a specific pathway responsible for a small proportion of variability in this phenotype.

More recently, a study of 7773 older subjects from CHARGE reported WMH lesion progression in 1085 individuals, however GWA could not detect genetic variants passing genome-wide significance, though four suggestive loci have been reported: 10q24.32 (rs10883817, $P = 1.46 \times 10{-}6$, intron in CNNM2 gene); 12q13.13 (rs4761974, $P = 8.71 \times 10{-}7$, intron in SLC4A8); 20p12.1 (rs6135309, $P = 3.69 \times 10{-}6$, intron in MACROD2 gene); and 4p15.31 (rs7664442, $P = 2.26 \times 10{-}6$, intron in an uncharacterized RNA gene (LOC105374524)). Heritability has been estimated in a subset of patients enrolled into the Framingham Heart Study (n = 1376), and, at 6.5%, the heritability of WMH lesion progression is much lower than estimates for overall WMH burden, indicating that environmental and vascular risk factors may be instrumental in preventing lesion progression [137].

In a hypothesis-driven approach, 126 differentially expressed genes from a rat model for SVD were selected for gene-based analysis in 621 subjects from the Lothian Birth Cohort 1936 (LBC1936) and replication was undertaken in 9361 subjects from CHARGE. Of ten genes passing a nominal significance threshold in LBC1936, *XPNPEP1* ($P = 6.7 \times 10{-}5$) and *FARP1*

(*P* = 0.024) were replicated in CHARGE. Reverse look-up of hits previously associated with WMH volume in CHARGE, three loci (rs3744028, P = 0.000511 (*TRIM65)*, rs1055129, P = 3.34 × 10−5 (*TRIM47)*, rs1052053, P = 0.048 (*PMF1)*) could be replicated in LBC1936 [138].

Finally, a multi-ethnic meta-analysis of 21,079 stroke- and dementia-free subjects from 29 population-based cohorts of European (n = 17,936), African (n = 1943), Asian (n = 405) and Hispanic (n = 795) ancestry, with WMH volume quantified in a semi-automated fashion or by visual grading scales, identified two new loci: chr1q22 (rs2984613, intron in PMF1 − BGLAP, P = 2.0×10^{-8}) and chr2p16 (rs78857879, intron in EFEMP1, P = 1.5×10^{-8}). In addition, the locus on chromosome 17q25 was validated in the 17,936 subjects of European ancestry (rs7214628, nearest gene TRIM65, P = 2.7×10^{-19}) and two novel loci on chromosome 10q24.33 (highest SNP rs7894407, intron in PDCD11, P = 1.6×10^{-9}) and chromosome 2p21 (P = 4.4×10^{-8}) were identified in the European ancestry subset (a comprehensive list of SNPs is listed in Table 11.3). Interestingly, the reported loci overlap with loci reported for intracranial hemorrhage (chromosome 1q22), Alzheimer disease (chromosomes 2p21 and 10q24), neuro-inflammation (chromosome 2p21) and glioma (chromosome 10q24 and 2p16), highlighting the potentially shared genetic contributions across these diseases with development and severity WMH burden [139].

Table 11.3 Genetic polymorphisms associated with white matter lesions identified by GWAS*

Locus	Polymorphism	Nearest gene/ Transcript	*P*/OR	*n*	Cohort	Phenotype
17q25 (Fornage et al. [136]						
	rs3744028	*TRIM65*	$P = 4.0 \times 10^{-9}$	9361	EA Population	WMHv
	rs9894383	*TRIM47*	$P = 5.3 \times 10^{-9}$	9361	EA Population	WMHv
	rs11869977	*WBP2*	$P = 5.7 \times 10^{-9}$	9361	EA Population	WMHv
	rs936393	*WBP2*	$P = 6.8 \times 10^{-9}$	9361	EA Population	WMHv
	rs3744017	*TRIM47*	$P = 7.3 \times 10^{-9}$	9361	EA Population	WMHv
	rs1055129	*TRIM47*	$P = 4.1 \times$	9361	EA Population	WMHv

			10^{-8}			
17q25 (Adib-Samii et al. [70])						
	rs3744028	*TRIM65*	$P = 0.003$	2588	EA AIS cases	WMHv
	rs9894383	*TRIM47*	$P = 0.00064$	2588	EA AIS cases	WMHv
	rs11869977	*WBP2*	$P = 0.00069$	2588	EA AIS cases	WMHv
	rs936393	*WBP2*	$P = 0.0012$	2588	EA AIS cases	WMHv
	rs3744017	*TRIM47*	$P = 0.0032$	2588	EA AIS cases	WMHv
	rs1055129*	*TRIM47*	$P = 0.015$	2588	EA AIS cases	WMHv
1q22 (Verhaaren et al. [139])						
	rs2984613	*PMF1 − BGLAP*	$P = 2.0 \times 10^{-8}$	21,079	Multiethnic Population	WMHv
2p16 (Verhaaren et al. [139)						
	rs78857879	*EFEMP1*	$P = 1.5 \times 10^{-8}$	21,079	Multiethnic Population	WMHv
2p21 (Verhaaren et al. [139])						
	rs11679640	*HAAO*	$P = 4.4 \times 10^{-8}$	17,936	EA Population	WMHv
10q24 (Verhaaren et al. [139])						
	rs72848980	*NEURL*	$P = 2.6 \times 10^{-9}$	21,079	Multiethnic Population	WMHv
	rs7894407	*PDCD11*	$P = 2.6 \times 10^{-8}$	21,079	Multiethnic Population	WMHv
	rs12357919	*SH3PXD2A*	$P = 1.5 \times 10^{-8}$	21,079	Multiethnic Population	WMHv
	rs7909791	*SH3PXD2A*	$P = 2.9 \times 10^{-9}$	21,079	Multiethnic Population	WMHv
5q23 (Traylor et al. [140])						
	rs17148926	*LOC10050584*	$P = 0.001$	3760	EA AIS cases	WMHv
13q34 (Traylor et al. [140])						
	rs9515201	*COL4A2*	$P = 7.0 \times 10^{-4}$	3760	EA AIS cases	WMHv
14q32(Traylor et al. [140])						
	rs941898	*EVL*	$P = 2.3 \times 10^{-4}$	3760	EA AIS cases	WMHv

17q21 (Traylor et al. [140])						
	rs962888	*C1QL1*	P = 0.0021	3760	EA AIS cases	WMHv
2q33 (Traylor et al. [140])						
	rs72934505	*NBEAL1*	$P = 2.2 \times 10^{-8}$	3760/17,936	EA AIS cases/EA stroke-free population	WMHv
10q24 (Traylor et al. [140])						
	rs7909791	*SH3PXD2A*	$P = 1.7 \times 10^{-8}$	3760/17,936	EA AIS cases/EA stroke-free population	WMHv
13q34 (Traylor et al. [140])						
	Rs9515201	*COL4A2*	$P = 6.9 \times 10^{-9}$	3760/17,936	EA AIS cases/EA stroke-free population	WMHv
14q32 (Traylor et al. [140])						
	rs941898	*EVL*	$P = 4.0 \times$ 10–8	3760/17,936	EA AIS cases/EA stroke-free population	WMHv
17 q21 (Traylor et al. [140])						
	rs962888	*C1QL1*	$P = 1.1 \times 10^{-8}$	3760/17,936	EA AIS cases/EA stroke-free population	WMHv
17q25 (Traylor et al. [140])						
	rs7214628	*TRIM65*	$P = 2.4 \times 10^{-15}$	3760/17,936	EA AIS cases/EA stroke-free population	WMHv
6p25 (CHARGE, SiGN and ISCG [131])						
	rs12204590	*FOXF2*	P = 0.0025	21,079	Multiethnic Population	WMHv
10q24.32 (Hofer et al. [137])						
	rs10883817**	*CNNM2*	$P = 1.46 \times 10^{-6}$	1085	EA Population	WML Progression
12q13.13						
	rs4761974**	*SLC4A8*	$P = 8.71 \times 10^{-7}$	1085	EA Population	WML Progression
20p12.1						
	rs6135309**	*MACROD2-AS1*	$P = 3.69 \times 10^{-6}$	1085	EA Population	WML Progression
4p15.31						
	rs7664442**	*LOC105374524*	$P = 2.26 \times 10^{-6}$	1085	EA Population	WML Progression

AIS acute ischemic stroke, *EA* European Ancestry, *WML* white matter lesion,

WMHv white matter hyperintensity volume, *all association were positive in direction-of-effect ** suggestive loci, not genome-wide significant

The studies discussed so far primarily focused on WMH in population-based studies as discovery cohorts. Since WMH burden is a significant contributor to stroke risk and severity, understanding the genetic architecture of WMH in acute ischemic stroke is of utmost importance. The first major locus for WMH in population-based studies–17q25–has been replicated in ischemic stroke, but was not associated with lacunar stroke status in particular. Overall, 6 SNPs were associated with WMH burden in ischemic stroke (see Fig. 11.6: Linkage map, Table 11.3) A polygenic risk score, examining to which extent the genetic contribution to WMH burden was shared in AIS subjects, demonstrated a dose-dependent effect where patients with a higher GRS had worse WMH burden [70].

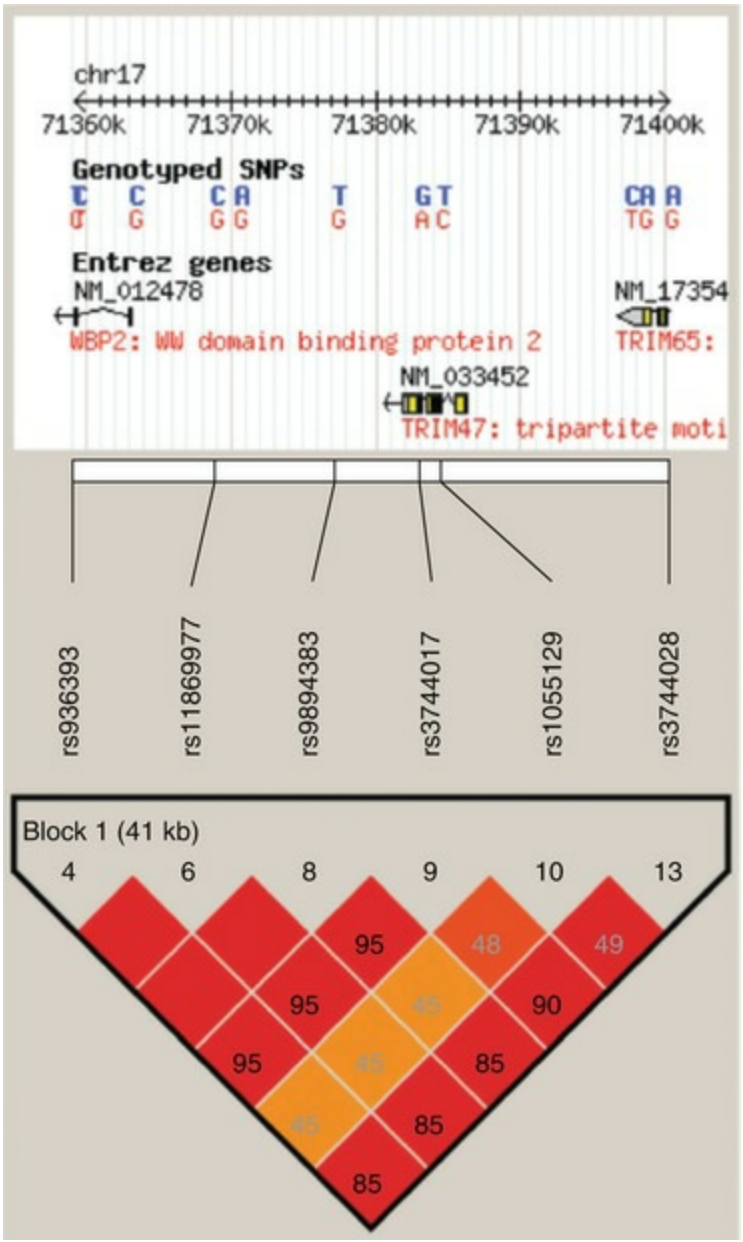

Fig. 11.6 Map of the linkage disequilibrium at the 17q25 locus. SNPs are highly correlated (r^2 in percentiles between 85 and 95) with exception of rs1055129, which is moderately correlated with all other SNPs at this locus (r^2 = 45–48) (Reprinted with permission from Dr. Rost [70])

The contribution of common variants associated with WMH burden to risk of lacunar stroke has also been analyzed recently in 4176 stroke cases (1373 lacunar stroke cases, 1331 cardioembolic stroke cases and 1472 large vessel stroke cases). A genetic risk score of 15 known SNPs associated with WMH burden in community studies was constructed and was significantly associated with lacunar stroke status. Albeit not significant after Bonferroni-

correction, lacunar stroke cases with WMH (n = 568, OR [95% CI] = 1.15 [1.05–1.26], P = 0.003) had a marginally stronger association than those without WMH (n = 787, OR [95% CI] = 1.11 [1.02–1.21], P = 0.019) [141].

The genetic underpinnings of WMH burden in ischemic stroke has been investigated in a meta-analysis of 3760 ischemic stroke patients of European ancestry from ten study populations from the United States, Europe, and Australia [140]. Even though no single SNP reached genome-wide significance, the analysis of 15 significant and suggestive hits derived from a previous investigation of WMHv in stroke-free adults of European ancestry [139] identified four SNPs passing the Bonferroni-corrected significance threshold (rs17148926, P = 0.001, LOC10050584; rs941898, P = 2.3×10^{-4}, EVL; rs962888, P = 0.0021; rs9515201, P = 7.0×10^{-4}). Since WMH in stroke-free subjects and the ischemic stroke population share a genetic background, a meta-analysis between WMHv in ischemic stroke cases and the population-based samples was undertaken. Overall, 6 SNPs were associated with WMHv in both cohorts, 4 of which were novel associations at the genome-wide significant level (rs72934505, p = 2.2×10^{-8}, *NBEAL1*; rs941898, P = 4.0×10^{-8}, *EVL*; rs962888, P = 1.1 × 10–8, *C1QL1*; rs9515201, p = 6.9×10^{-9}, *COL4A2*) [140]. Interestingly, the SNP in COL4A2 was shown to be in strong linkage dysequilibrium with SNPs in the COL4A2 gene associated with sporadic cerebral small vessel disease [102].

In the largest-to-date analysis of 18 population-based cohort studies, 4348 stroke cases and 80,613 controls were meta-analyzed. SNPs passing a pre-specified nominal significance threshold of $p < 5 \times 10^{-6}$ were taken forward for validation in cross-sectional case-control studies (SiGN, METASTROKE, HVH1, CADISP; 19,816 cases and 50,988 controls). In the combined meta-analysis a novel locus on chromosome 6p25 (rs12204590, near FOXF2) passed the genome-wide significance threshold for the risk of all stroke (OR 1.08, P = 1.48×10^{-8}). This locus was also associated with increased WMH burden (p = 0.0025) in stroke-free adults (n = 21,079). Notably, patients with segmental deletions of FOXF2 present with periventricular and deep WMH. The pathophysiologic role of FOXF2 in WMH and stroke were further confirmed in a conditional FOXF2 knockout model, with mice deleterious for FOXF2 displaying stroke, reactive gliosis and microhemorrhages. Functional analysis of FOXF2 orthologs in a zebrafish (FOXF2A and FOXF2B) provided further insight, as both orthologs are expressed in zebrafish

pericytes and knockout of either led to defects in maturation of pericytes, further substantiating the role of FOXF2 in stroke and WMH [131].

Conclusion

MRI-detectable WMH is a highly heritable and widely prevalent manifestation of cerebrovascular disease in the elderly that is also strongly linked to progressive functional disability and risk of stroke. In subjects with stroke, WMH severity is associated with cerebral infarct growth, poor functional outcomes, and greater risk of stroke recurrence. The genetics of leukoaraiosis are complex, sharing both the features of monogenic and polygenic disorders. Understanding the genetic architecture of the cerebrovascular WM disease may revolutionize our current approach to prevention and treatment of stroke, dementia, and functional decline of the elderly. Genetic discoveries offer a promise of novel interventions and drug targets that may have a potential to deter the growing burden of cerebrovascular disease worldwide.

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12. Genetics of Carotid Disease

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Introduction

Many issues related to carotid disease and stroke remain unresolved despite the recognition of risk factors and development of effective stroke prevention strategies. Who is at greatest risk of developing carotid atherosclerosis? Among those with carotid atherosclerosis, who is at highest risk to have a stroke? Who is likely to benefit from carotid revascularization? Should endarterectomy or stenting be used for revascularization? Who should be managed with intensive medical therapy without revascularization? Are there predictors of response to these medical therapies? Genetic investigation has potential to help answer these questions. In the last few years we have seen genetic advances leading to a better understanding of carotid disease and response to therapy. This chapter will focus on carotid atherosclerotic disease and its relationship to ischemic stroke. Other chapters in this volume will address other forms of carotid pathology related to stroke, such as carotid dissection.

Epidemiology

Carotid disease causes a substantial proportion of the public health burden from ischemic stroke worldwide and accounts for disproportionate disability and death following ischemic stroke [1]. Stroke related to carotid disease carries a high risk of early recurrence and demonstrates both sex and race/ethnic variation [2–5]. Population-based studies estimate that between 16% and 36% of ischemic strokes are due to large artery atherosclerosis [6, 7], and carotid disease accounts for ~2/3 of all large artery cases or about 10% of all ischemic strokes [6, 7]. This proportion differs by sex and race/ethnicity [8–11], and ancestry/genetics may explain some of this racial variation [12]. Large artery atherosclerotic stroke predicts disability, acute mortality, and longer-term mortality [13]. Transient ischemic attack (TIA) or stroke associated with large artery atherosclerosis carries the highest risk of early stroke [7, 14]. More than one quarter of untreated patients presenting with a symptomatic carotid stenosis have recurrent stroke or TIA within the first 2 weeks [15].

The therapeutic benefit of carotid endarterectomy (CEA) for stroke prevention in symptomatic disease is well-established [16, 17]. Although the magnitude of benefit for CEA in asymptomatic patients is much more modest, the data support use of CEA in selected populations with asymptomatic carotid disease [16] although the value of screening for asymptomatic carotid stenosis has been challenged given no reduction in stroke burden [18]. The data on carotid artery stenting (CAS) are less consistent, but the Carotid Revascularization Endarterectomy versus Stenting Trial (CREST) found the therapeutic benefit for CEA and CAS to be comparable overall, with a relative advantage for each modality in specific populations [19–21]. A recent comparative effectiveness analysis suggests that Medicare beneficiaries undergoing CAS and CEA had comparable outcomes although several patient- and provider-specific variables influenced the results [22].

The number of carotid revascularization procedures, defined as having either CEA or CAS, declined 30% in the USA between 2002 and 2010 [23]. However, in the same time period, CAS accounted for a growing proportion of total procedures with dramatic differences by region and specialty [23]. Rates of CEA and CAS are lower in the United Kingdom than other European [24] or many of the British Commonwealth countries [25]. Data

from other countries demonstrate a much lower utilization of either modality compared to the rates in the USA, especially for asymptomatic individuals [25]. According to some commentators, between 70% and 90% of all CAS in the USA are performed in asymptomatic individuals [23, 26]. In recent years, this proportion has risen in part due to broadened coverage for the procedure by Medicare in the USA [27] despite strong caution against such coverage [28]. Utilization within the USA varies widely [23], but without clear data to determine what optimal use would be [29] or even who should have asymptomatic carotid imaging [30]. Results from the ongoing two-armed CREST 2 trial (ClinicalTrials.gov Identifier: NCT02089217) comparing medical management to CEA or CAS should offer guidance on the merits of aggressive modern medical management versus each intervention. Using clinical and genetic factors to predict who would benefit most from revascularization, and by which approach, remains an as yet unattained goal.

Phenotypes of Carotid Disease

Investigation into genetic risks for carotid artery disease and resultant ischemic stroke has relied on several intermediate “phenotypes,” including carotid intimal-medial thickness, carotid artery plaque burden, degree of stenosis, and whether the carotid plaque is symptomatic or asymptomatic.

Carotid Intimal-Medial Thickness as a Phenotype

Carotid intimal-medial thickness (IMT) is strongly correlated with cerebrovascular and cardiovascular disease. As a quantitative trait readily measured noninvasively that develops long before overt stroke, carotid IMT is an appealing intermediate phenotype or surrogate marker of carotid disease. Estimates for the heritability of carotid IMT have varied. Early estimates suggested 66–75% of the variability attributable to genetic factors [31], whereas more recent studies have found a modest genetic contribution of 32–45% of the adjusted variability [32, 33]. This association may differ by race/ethnicity and risk factor profile [34]. The residual variation is attributable to known vascular risk factors such as cigarette smoking, diabetes mellitus, hypertension, dyslipidemia, and especially age. However, the adequacy of IMT as an intermediate phenotype has been challenged with data suggesting that, while closely related, IMT and carotid atherosclerosis are

distinct entities with distinct genetic determinants [35]. Further, common and internal carotid IMT may serve as markers for different risks and may have unique genetic risk profiles. Thus, while data using IMT as a marker of carotid disease may provide important leads, ultimately any associations made with IMT will need to be assessed independently in carotid disease.

Early linkage data from the Framingham Heart Study Offspring Cohort found strong evidence of linkage between a distal locus on the short arm of chromosome 12 and carotid IMT as a quantitative trait (QTL) [36]. In the same study, a variant in the scavenger receptor class B type I (*SCARB1*) gene on distal 12q, which encodes a protein that mediates the bidirectional transfer of cholesterol between cells and high-density lipoprotein (HDL) cholesterol, also associated with lower IMT. However, the association of IMT with *SCARB1* did not account for the observed linkage peak. Other studies have reported significant genetic associations with *SCARB1* and IMT [37] and myocardial infarction in a multiethnic population [38].

Using a candidate gene approach, others have found that polymorphisms in the peroxisome proliferator-activated receptor gamma (*PPARG*) gene influence carotid IMT and carotid atherosclerosis. The A12 allele of the proline-12-alanine polymorphism (*P12A*) has been associated with lower IMT in three different populations, but it was not associated with carotid plaque volume [39–41]. Data evaluating the effect of P12A on general ischemic stroke risk are conflicting, and no analysis to date has looked specifically at stroke related to carotid disease [42, 43]. In the Progressione Lesione Intimale Carotidea (PLIC) study, individuals with the D299G allele of the toll-like receptor 4 (*TLR4*) gene had lower levels of inflammatory mediators such as interleukin-6 and fibrinogen, and were more susceptible to severe bacterial infections, but had smaller IMT and a lower risk of carotid atherosclerosis [44].

An initial genome-wide association study (GWAS) including IMT failed to identify any single nucleotide polymorphisms (SNPs) that reached genome-wide significance among participants in the Framingham Heart Study (FHS) Offspring Cohort for subclinical markers of atherosclerosis. The top candidate for maximum internal carotid artery IMT, rs1376877 ($p = 3.8 \times 10^{-7}$), is located in the Abelson interactor 2 gene (*ABI2*) [45] and has yet to be replicated in other studies.

Subsequent larger scale efforts by the Cohorts for Heart and Aging Research in Genomic Epidemiology consortium (CHARGE) consortium and

including FHS identify three regions associated with common carotid IMT in both the discovery and the validation cohorts, but none associated with internal carotid IMT [46]. Cross-phenotype analyses showed that one of the IMT GWAS candidates, located in the apolipoprotein C-I gene (*APOC1*), was associated with coronary disease, but none of the coronary disease GWAS candidates were associated with IMT. Neither the zinc fingers and homeoboxes 2 (*ZHX2*) gene nor the PIN2/TERF1 interacting, telomerase inhibitor 1 (*PINX1*) gene was associated with coronary disease.

An independent replication of four loci identified in a genome-wide association meta-analysis from the CHARGE consortium including the three significant (*ZHX2*, *APOC1*, *PINX1*) loci and a fourth locus with suggestive evidence (*SLC17A4*) for association found similar direction and effect size for all four. Fine mapping around each lead SNP found 27 variants within these loci with larger effect sizes and several with potential functional consequences [47]. No analyses of ischemic stroke or other cerebrovascular phenotypes were undertaken for any of the SNPs, although several of the SNPs have been implicated in cardiovascular health and disease [48]. *SLC17A4* and *PIK3CG* demonstrated a modest association with several measures of subclinical carotid atherosclerosis [49]. Several studies have implicated specific GWAS hits such as *BCAR1*, *LEKR1*, and *GALNT10* in observed sex differences in IMT [50], and stroke gene-by-environment interactions with smoking status [51].

A study using four population-based cohorts undertook a different approach by testing GWAS-identified coronary artery disease loci for association with IMT and presence of plaque in the carotid artery bulb [52]. They meta-analyzed 45 SNPs identified as coronary disease-associated loci in four independent population-based studies and combined them to calculate a genetic pre-disposition score for coronary artery disease. Using intima-media thickness measured by B-mode ultrasonography at the far wall of the carotid bulb and common carotid artery and plaque presence, investigators tested for an association between the genetic pre-disposition score, prevalent coronary disease, and three indices of carotid atherosclerosis, adjusting for sex, age, and Framingham risk factors. The genetic predisposition score was associated with prevalent coronary disease and IMT with plaque presence at the far wall of the carotid bulb. Each additional risk allele added into the genetic pre-disposition score was associated with a 0.24% increase in IMT and a 2.8% increased odds of plaque presence. Investigators found no association of the

genetic predisposition score with IMT of the common carotid artery.

Another cross-investigation of coronary artery loci from GWAS studies found no association between these loci and IMT supporting the potential for independent pathophysiologies [53]. Similarly, a multi-ethnic investigation of 66 SNPs identified in GWAS of both symptomatic and subclinical cardiovascular phenotypes found evidence of definite but incomplete shared architecture for IMT and other subclinical atherosclerotic phenotypes [54]. Genetic analyses of IMT in non-European populations are limited and findings to date await further replication [55, 56]. Investigations going in the other direction, testing IMT associated loci in coronary and cerebrovascular disease, have found similar incomplete overlap with only one of six loci associated with coronary disease and none with ischemic stroke or abdominal aortic aneurysms [57]. Their genetic risk score for IMT SNPs showed effect in other vascular phenotypes arguing against pleiotropic effects. A promising application of exome sequencing using a trans-ethnic approach recently found a missense mutation in APOE ε2 associated with reduced cIMT in both European and African ancestry individuals. A growing number of trans-ethnic investigations are yielding new insights into genetic risk for carotid disease and stroke. The forthcoming trans-ethnic MEGASTROKE effort promises to substantially increase the number of GWAS ‘hits’ for stroke including carotid disease.

Taken together, these results suggest that carotid anatomy is an important consideration for both phenotypic and genotypic characterizations of carotid disease (i.e., common carotid versus bifurcation versus distal ICA likely have distinct pathophysiological profiles). Further, these findings demonstrate that clinical cardiovascular disease, atherosclerotic cerebrovascular disease, and IMT share genetic mechanisms and warrant careful investigation of shared and distinct genetic contributions to these phenotypes.

Carotid Plaque Burden as a Phenotype

Intuitively, measurements of carotid atherosclerotic plaque have many of the characteristics of an optimal intermediate phenotype for stroke caused by atherosclerotic carotid disease. The connection to the pathogenic pathway is logical, and both plaque burden and progression can be readily imaged noninvasively and quantitatively. Carotid plaque is present in a much larger segment of the population than carotid stroke so it can be readily studied in cohort studies with greater power. However, the clinical relevance of

subclinical carotid atherosclerosis remains unclear, particularly when below the threshold for revascularization or when characterized by non-standardized morphological traits such as ulceration.

Initial family based linkage studies found strong evidence for heritability of carotid plaque burden, identifying multiple loci (Chr11p15, Chr14q32, and Chr15q23) with the strongest evidence for variants in the Sex Determining Region Y-Box 6 (*SOX6*) gene [58]. However, a more recent follow-up study was unable to replicate the association for carotid plaque or other carotid phenotypes [59]. The large-scale GWAS discussed previously for IMT also included carotid plaque volume as a phenotype. The study identified two regions associated with internal carotid plaque volume in both the discovery and the validation cohorts [46]. The SNPs in these two regions, near the phosphoinositide-3-kinase catalytic, gamma polypeptide (*PIK3CG*) and the endothelin receptor type A (*EDNRA*) genes, were associated with coronary artery risk in cross-phenotype analyses in the expected direction based on their association with carotid atherosclerosis (Table 12.1). However, similar to the IMT analysis, none of the coronary disease GWAS candidates were associated with carotid plaque burden. Again, no analyses of stroke or other cerebrovascular phenotypes were undertaken in the initial study, and subsequent efforts have failed to show significant association with ischemic stroke or other vascular phenotypes [57] (Table 12.2).

Table 12.1 cIMT/plaque associated SNPs reported by the literature and the association results for Second Manifestations of ARTerial disease cohort (SMART). Reproduced from Hemerich et al. [58]

Reported by literature									**This study**		
Plaque associated variants											
Locus	*SNP*	*Chr*	*Alleles*	*EAF*	*HWE*	*OR (95%CI)*	*p*	*EAF*	*OR (95%CI)*	*p (cIMT)*	*p (plaq*
EDNRA	rs1878406	4	T/C	0.13	0.5535	1.22 (1.15–1.29)	6.90×10^{-12}	0.16	1.11 (0.98–1.26)	**0.01**	**0.05**
PIK3CG	rs17398575	7	A/G	0.25	0.01141	1.18 (1.12–1.23)	2.30×10^{-12}	0.24	1.17 (1.04–1.30)	**0.009**	**0.00**
cIMT associated variants											
Locus	*SNP*	*Chr*	*Alleles*	*EAF*		*ß (95%CI)*	*p*	*EAF*	*ß (95%CI)*	*p (cIMT)*	*p (plaq*
PINX1	rs6601530	8	G/A	0.45	0.4141	0.0078	1.70×10^{-8}	0.46	0.0009	0.404	0.17

ZHX2	rs11781551	8	A/G	0.48	0.5388	−0.0078	2.40×10^{-11}	0.45	−0.0071	**0.033**	0.133
BCAR1-CFDP1-TMEM170A	rs4888378	16	A/G	0.43	0.003173	−0.0045	7.25×10^{-6}	0.39	−0.006873	**0.039**	0.496

SNP single-nucleotide polymorphism, *Alleles* effect and non-effect alleles, *EAF* effect allele frequency. Results listed in bold are nominally significant ($p = 0.05$). *HWE* Hardy–Weinberg Equilibrium

Table 12.2 Single SNP associations with ischemic stroke, AAA, CAD, PAD, and ABI in SMART. Reproduced from Hemerich et al. [58]

		IS *N* = 1764		**AAA *N* = 640**		**CAD *N* = 3743**		**PAD *N* = 1726**		**ABI *N* = 7953**	
SNP	Alleles	*OR (95%CI)*	*p*	*OR (95%CI)*	*p*	*OR (95%CI)*	*p*	*OR (95%CI)*	*p*	*ß (95%CI)*	*p*
rs1878406	T/C	1.02 (0.88–1.19)	0.791	1.10 (0.88–1.39)	0.407	1.24 (1.08–1.42)	**0.002**	1.14 (0.98–1.34)	0.094	−0.001 (−0.01–0.01)	0.886
rs17398575	A/G	1.07 (0.95–1.21)	0.260	1.03 (0.85–1.25)	0.743	1.01 (0.90–1.13)	0.905	1.15 (1.01–1.30)	0.035	−0.003 (−0.01–0.00)	0.314
rs6601530	G/A	1.04 (0.93–1.16)	0.523	1.06 (0.89–1.25)	0.514	1.11 (1.00–1.22)	0.040	1.09 (0.97–1.22)	0.132	−0.008 (−0.01–0.00)	0.006
rs11781551	A/G	1.00 (0.90–1.11)	0.975	1.12 (0.95–1.33)	0.170	1.03 (0.94–1.14)	0.515	1.06 (0.95–1.19)	0.307	0.000 (−0.01–0.01)	0.923
rs4888378	A/G	1.00 (0.90–1.11)	0.966	1.13 (0.95–1.33)	0.160	0.97 (0.88–1.07)	0.569	0.97 (0.86–1.08)	0.546	0.003 (0.00–0.01)	0.213

IS ischaemic stroke, *AAA* abdominal aortic aneurysm, *CAD* coronary artery disease, *PAD* peripheral artery disease, *ABI* ankle-brachial index, *SNP* single-nucleotide polymorphism
N refers to the number of cases. Controls = 1981
Bold values signifies that the Bonferroni corrected threshold defined for the five tested vascular beds (IS, AAA, CAD, PAD, and ABI) was $p < 2 \times 10^{-3}$

Notably, the directions of the associations were consistent for common carotid IMT and carotid plaque volume in the other carotid phenotypes. Additionally, the three regions associated with coronary disease (*APOC1*,

PIK3CG, *EDNRA*) were significantly associated with all three carotid phenotypes (carotid plaque volume, internal carotid and common carotid IMT), suggesting these genes may have broad effects on the accumulation of atherosclerosis. Again, the lack of specific investigation into stroke symptoms or large vessel atherosclerotic stroke makes it difficult to gauge how these variants might actually affect stroke risk. Most studies in non-European populations have been small and limited to preliminary results [60, 61]. The Population Architecture using Genomics and Epidemiology (PAGE) study found a novel association for an intronic SNP (rs780094) in the Glucokinase Regulatory Protein gene (*GCKR)* with carotid plaque in Native Americans, but no other race/ethnic group [62]. This SNP had been previously associated with metabolic traits and carotid phenotypes in other race/ethnic groups [63–65].

Mechanistic studies have considered genetic determinants of lipid levels [66]. However, no consistent associations with plaque presence or composition (calcification, collagen, cellular content, atheroma size, plaque density and intraplaque hemorrhage) have been found [67, 68].

Stroke Due to Large Artery Disease as the Phenotype

The genetic research of large artery atherosclerotic stroke can claim several successes. Investigation of ischemic stroke related to carotid disease is complicated because the most broadly used subtype classification systems do not differentiate extracranial from intracranial atherosclerosis or anterior from posterior circulation involvement [69, 70]. Whether genetic determinants are shared across or differ within the categories of large artery atherosclerotic stroke has yet to be explored. Yet, one might hypothesize genetic variation in the anatomical distributions of the cerebrovasculature, particularly given distinct physiologic and structural variation in the arteries (e.g., lack of external elastic lamina intracranially, autonomic circulatory differences between anterior and posterior circulation).

Leukotriene Pathway

The earliest large-scale genomic investigations of ischemic stroke implicated a locus near phosphodiesterase 4D (PDE4D) for carotid and cardioembolic stroke [71]. Replication attempts have been largely negative or inconsistent

prompting cautious interpretation. The second locus to emerge, near the 5-lipoxygenase-associated protein (*ALOX5AP*) gene [72], has sustained interest in this gene and the related leukotriene pathway, despite lacking robust replication. Associations of variants in *ALOX5* and *ALOX5AP* with IMT and carotid plaque have been noted in individuals with diabetes [73]. Expression of mRNA and protein levels for both *ALOX5* and *ALOX5AP* were increased in human carotid atherosclerotic plaques compared to controls and ALOX5 levels correlated with plaque instability based on clinical symptoms [74]. Moreover, the *ALOX5AP* variant has been associated with ischemic stroke risk [75]. An interesting interaction with diet has been noted whereby high levels of dietary arachidonic acid enhance the association of the *ALOX5* variant genotypes with higher IMT, and diets rich in $n-3$ fatty acids reversed the effect [76]. Additionally, serum markers of inflammation were increased twofold among carriers of two variant alleles as compared with that among carriers of the common allele. Another gene coding for leukotriene A4 hydrolase (*LTA4H*) in this pathway found a similar genotype-by-dietary interaction [77]. The story remains unclear, with several studies failing to show genetic variation associated with carotid phenotypes or stroke and prompts investigation into other mechanisms, such as epigenetic modification or environmental factors [67, 78].

Leveraging GWAS Findings from Other Diseases

A variant on chromosome 9p21 shows consistent and independent association with increased risk for coronary artery disease (CAD) and is anticipated to be crucial to the development of genetic risk profiling and the potential for personalized medicine [79]. The association was first reported with MI [80–82] and the variants identified on chromosome 9p21 fell into what was initially thought to be an intergenic region with few plausible candidates. Subsequent investigations have focused on a large antisense noncoding RNA called ANRIL and two cyclin-dependent kinase inhibitor genes (*CDKN2A* and *CDKN2B*). The situation is further complicated because numerous phenotypes in addition to atherosclerotic disease including diabetes, intracranial aneurysms, aortic aneurysms, multiple cancers, and migraine without aura have shown associations with this region [83, 84]. Furthermore, data are emerging that suggest that there may be two or more clusters of phenotypes within the region relating to different pathways [85, 86]. The

underlying mechanisms related to cell proliferation, remodeling, or regulation of epigenetic modification remain to be sorted out [87–89].

The shared risk factors for heart disease and stroke prompted early investigation into this region for an association with ischemic stroke overall [90] and a link with carotid plaque occurrence and progression [91]. While some data have challenged the association with stroke, the effect of sample size and phenotypic heterogeneity likely accounts for these negative findings [92]. Investigation into other non-European populations has found nominal association for all ischemic stroke in African Americans [93]. A meta-analysis confirmed the association with ischemic stroke broadly defined but noted differences by race/ethnicity [94]. Using a unique pathological database, investigators found a strong association between risk variants of the 9p21 locus and directly measured macroscopic cerebral infarcts on neuropathological examination [95].

Focusing on large artery atherosclerotic disease, a meta-analysis by the International Stroke Genetics Consortium (ISGC) identified six SNPs associated with large vessel atherosclerotic stroke even after controlling for demographics, prevalent coronary atherosclerotic heart disease, and other vascular risk factors [96]. The effect size was modest (a pooled odds ratio of 1.21) and appeared to be independent of myocardial infarction. The significance with large artery disease-associated stroke was substantially greater than for all ischemic strokes and approached the effect size observed in coronary disease [92]. The SNP most strongly associated with large-vessel stroke (rs1537378-C) differed from the lead coronary disease SNP (rs10757278-G) located more than 70 kb away. A subsequent study found the 9p21.3 locus had the strongest signal in a combined meta-analysis of coronary disease and large artery atherosclerosis [97]. The possibility remains that this locus may influence large vessel disease risk through alternative mechanisms, such as vascular remodeling or repair rather than through atherosclerosis *per se*. This is especially interesting, given divergence of lead SNPs by phenotypes and the association of this locus with other non-atherosclerotic arteriopathies such as intracranial aneurysms [92]. The data on large artery atherosclerotic stroke add to the complex story emerging regarding the chromosome 9p21 region.

One of the hurdles facing genetics research is demonstration of clinical utility of the findings. On this front, there are some data suggesting variants on 9p21 can augment risk stratification and inform preventive strategies in

systemic atherosclerotic disease. An analysis of individuals of European descent from the Atherosclerotic Risk in Communities (ARIC) study found that 9p21 genetic information plus carotid IMT and plaque data performed better than the IMT and plaque data alone for predicting coronary heart disease risk [98]. Other data suggest that adding 9p21 genetic information to the Framingham Risk Score can improve the targeting of prevention strategies [99]. For example adding 9p21 genotypic information increased the number identified as high risk from 21% to 27% in th0 between 70 and 80. Others have verified the association of 9p21 with atherosclerosis but feel the clinical utility of a genetic test is likely to be limited [100]. Clinical application of 9p21 genotypic data is not yet ready for use in routine clinical practice [101], but understanding the mechanisms underlying risk provides important clues into susceptibility and targets for treatment and prevention of atherosclerotic diseases [83].

Novel Large Vessel Stroke Risk Genes Identified by GWAS

HDAC9

The Wellcome Trust Case-Control Consortium 2 (WTCCC2) stroke working group conducted a genome-wide association study of ischemic stroke and its subtypes using a discovery cohort of 3548 stroke cases and 5972 common controls [102]. Results were replicated in an independent group of 5859 cases and 6281 controls. In addition to replicating associations with several previously implicated loci, this study found a novel association for large artery stroke and the histone deacetylase 9 (*HDAC9*) gene on chromosome 7p21.1, with consistent findings in both the discovery and the replication sets (Fig. 12.1). This gene has been implicated in effector T cell-mediated immunity [103] and regulates chromatin structure and gene transcription [104]. The SNP most strongly associated with large artery disease had an adjusted odds ratio of 1.42 (95% confidence interval = 1.28–1.57) [102]. The association of *HDAC9* with large artery atherosclerotic stroke has been replicated in multiple analyses, including the initial METASTROKE GWAS meta-analysis [105] and the subsequent National Institute of Neurological Disorders and Stroke (NINDS) Stroke Genetics Network (SiGN) GWAS [106].

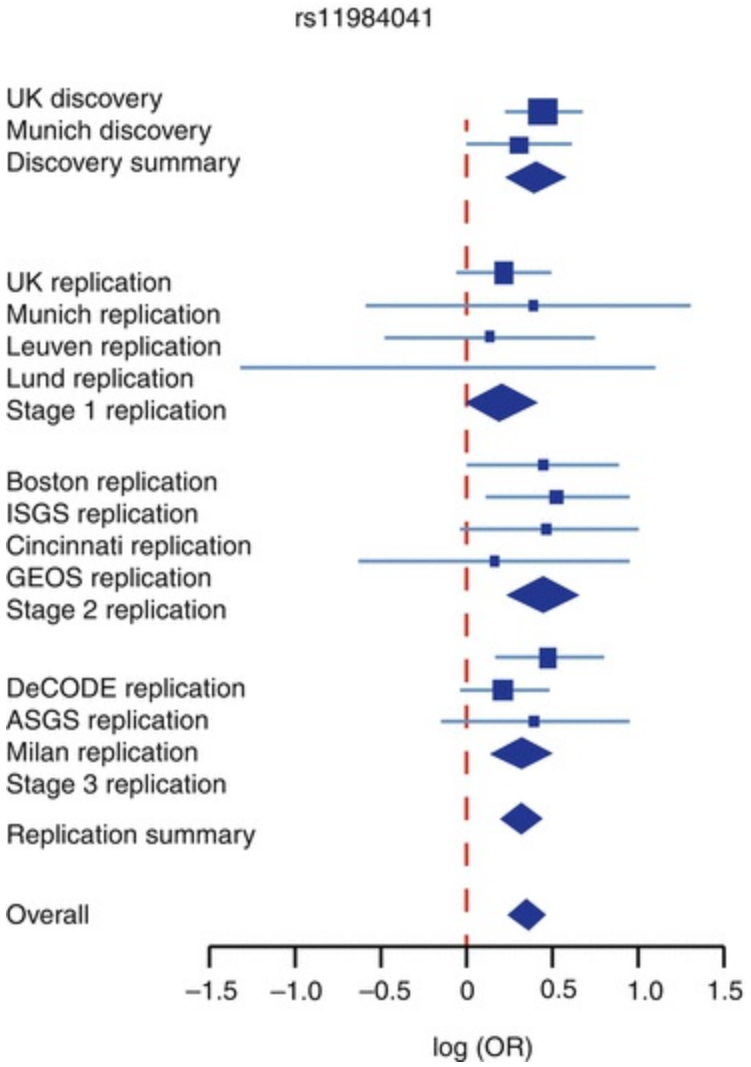


Fig. 12.1 Forest plot for the associations between rs11984041 and large-vessel stroke in discovery and replication collections. The blue lines show the 95% confidence intervals of the log (OR) for each cohort, with the area of each square proportional to the inverse of the standard error. The diamonds indicate the 95% confidence interval for the discovery summary (Reprinted with permission from The International Stroke Genetics Consortium (*ISGC*), the Wellcome Trust Case Control Consortium 2 (*WTCCC2*). Bellenguez et al. [102])

The mechanism by which *HDAC9* variants might increase atherosclerotic stroke risk remains incompletely characterized. *HDAC9* mutations have been previously associated with blood pressure in a study of Nigerian families [107]. The HDAC9 protein has been shown to protect neurons from apoptosis [108], raising the possibility of alteration of ischemic vulnerability, although why this would preferentially affect patients with large artery stroke versus other types of ischemic stroke is unclear. An elegant study of allele-specific expression in human peripheral blood mononuclear cells found that risk

alleles in the *HDAC9* region exert their effect by increasing HDAC9 expression [109]. Furthermore the same group showed the Apolipoprotein E/*HDAC9* double knockout mice have less advanced carotid atheroma than Apolipoprotein E knockout/*HDAC9* wild-type animals. They and several groups have suggested that HDAC inhibitors are appealing candidates for prevention of large artery atherosclerotic stroke [110, 111].

Examining gene expression between stable ($n = 6$) and unstable ($n = 16$) plaques obtained at endarterectomy [112], demonstrated that expression of *TWIST1*, a gene very close to the GWAS locus implicating *HDAC9* in large artery stroke [102], was higher in stable plaques. *HDAC9* expression appeared to follow the opposite pattern, although the finding was not significant. Importantly, the definitions of stable or unstable were based solely on histopathological characteristics, since all patients were clinically asymptomatic. These data suggest that both *HDAC9* and its neighbor *TWIST1* may play a role in plaque progression and possibly in opposite directions. Interestingly, the distribution of risk alleles did not influence *HDAC9* and *TWIST1* gene expression.

6p21

The Australian Stroke Genetics Collaborative (ASGC) identified another novel locus for large artery atherosclerotic stroke [113]. They studied 1162 ischemic stroke cases, of which 421 were large artery atherosclerosis-associated stroke and 1244 controls. They identified a novel susceptibility locus on chromosome 6p21.1 (rs556621) with an odds ratio of 1.62 ($p = 3.9 \times 10^{-8}$). Data from the WTCCC stroke working group, the ISGC, and the METASTROKE consortia confirmed the association of rs556621 with large artery atherosclerosis, with no evidence of between-study heterogeneity [113]. Subsequent efforts have yet to robustly and independently replicate this association. For example, in the NINDS-SiGN GWAS, following the first stage of analysis, there was only weak evidence of the association between 6p21 and large artery atherosclerosis and subsequent analysis in the independent samples failed to detect this association [106].

MMP12

Using a weighting strategy that takes into account the expected age-associated risk for stroke, members of the ISGC identified a novel association

between a locus near *MMP12* and large artery atherosclerotic stroke in the Wellcome Trust Case-Control Consortium II project stroke population and replicated in the METASTROKE consortium [114]. They found the strongest association for rs660599 ($p = 2.5 \times 10^{-7}$ in the discovery, $p = 0.0048$ in the replication set with an OR (95% CI) = 1.18 (1.05–1.32), and $p = 2.6 \times 10^{-8}$ in the combined meta-analysis). The *MMP12* gene product was overexpressed in carotid plaques compared to normal artery tissue with a 335.6 fold-change ($p = 1.2 \times 10^{-15}$). This finding awaits replication attempts.

TSPAN2

The NINDS-SiGN and ISGC more recently conducted a two-stage GWAS with 16,851 ischemic stroke cases and 32,473 controls recruited between 1989 and 2012, constituting the largest and most comprehensive GWAS of stroke and its subtypes to date [106]. In the second stage, *in silico* association analysis of the top SNPs identified from the first stage GWAS was performed in a set of independent samples of 20,941 cases and 36,4736 controls. Ischemic stroke subtypes were classified by the Causative Classification of Stroke (CCS), as well as the TOAST subtype classification system, into five phenotypes (all stroke, cardioembolic, large artery atherosclerosis, small artery occlusion, and undetermined). Both stages were then analyzed together, including 37,893 cases and 397,209 controls, to identify loci exceeding the threshold for genome wide significance. In the joint analysis, SNPs in two novel loci exceeded genome-wide significance. Four common SNPs near the *TSPAN2* locus on chromosome 1 were associated with large artery atherosclerotic stroke. The lead SNP in the associated locus was rs12122341 (odds ratio for the G allele 1.19, 95% CI 1.12–1.26, $p = 1.3 \times 10^{-9}$). *TSPAN2* is the gene encoding tetraspanin-2, belonging to a family of proteins mediating signal transduction to regulate cell development, activation, growth, and motility. The gene is highly expressed in arterial tissue and whole blood cells, and *TSPAN2* knockout mice experience increased neuroinflammation. The lead SNP identified is located in an intergenic region upstream of *TSPAN2*, and is thought have a role in the regulation of *TSPAN2* [106] (Fig. 12.2).

PTCSC3

Investigators from Taiwan identified 5 genome-wide significant SNPS in or

near the papillary thyroid carcinoma susceptibility candidate 3 (*PTCSC3*) gene as a novel large artery atherosclerotic stroke locus in Han Chinese despite a modest sample size (444 individuals with LAA stroke and 1727 stroke-free controls) [115]. This finding was replicated in a comparably sized independent Han Chinese sample. They strongly replicated the *HDAC9* locus in this population as well. It will be interesting to see whether this holds up to replication efforts and what trans-ethnic analyses teach us about the specificity or generalizability of this finding.

Other Loci

The SiGN effort also identified a novel locus with a genome wide association with all ischemic stroke, but only in a small sample of African ancestry. The finding of an association of rs74475935 in *ABCC1* on chromosome 16 must therefore be interpreted with caution. Lesser evidence for an association of large artery stroke with *CDKN2B-ASI* and *ABO* was noted when analysis was restricted to only samples not used for the initial discovery [106].

A recent follow-up GWAS meta-analysis of 10,307 Caucasian cases of ischemic stroke and 19,326 Caucasian controls by the METASTROKE collaboration identified several low-frequency variants of interest. Though not reaching genome-wide significance, *GUCY1A3* showed suggestive association with large artery atherosclerosis ($p = 8.25 \times 10^{-6}$), and has previously been reported as a risk gene for early onset myocardial infarction. Additional variants with p values less than 1×10^{-4} were noted for association between *NACC2* and an intergenic locus near *KCNN2* and large artery atherosclerotic stroke. Further investigations will be needed to understand the potential role of these low-frequency variants in the heritability of stroke subtypes [105].

A collaboration among the METASTROKE, CARDIoGRAM, C4D, and International Stroke Genetics Consortiums sought to perform a genome-wide analysis to evaluate the extent of shared genetic determination of ischemic stroke, particularly large artery atherosclerotic stroke, and coronary artery disease. The locus with the strongest signal for ischemic stroke identified in this joint meta-analysis was chr12q24/*SH2B3*, which had not been previously reported as associated with stroke phenotype, but also demonstrated a strong signal indicating its role as a major susceptibility locus for cardiovascular disease [97]. Genetic variants at the *ABO* locus, which has been previously

associated with venous thromboembolism, low density lipoprotein, and von Willebrand factor, revealed the second strongest signal for ischemic stroke. Loci associated with both CAD and the large artery ischemic stroke phenotype included the previously described 9p21.3, *HDAC9*, and several loci not previously reported as associated with ischemic stroke. Among these were *EDNRA*, which had been associated with IMT and carotid artery atherosclerosis, thus suggesting that this locus might promote early atherogenesis [97].

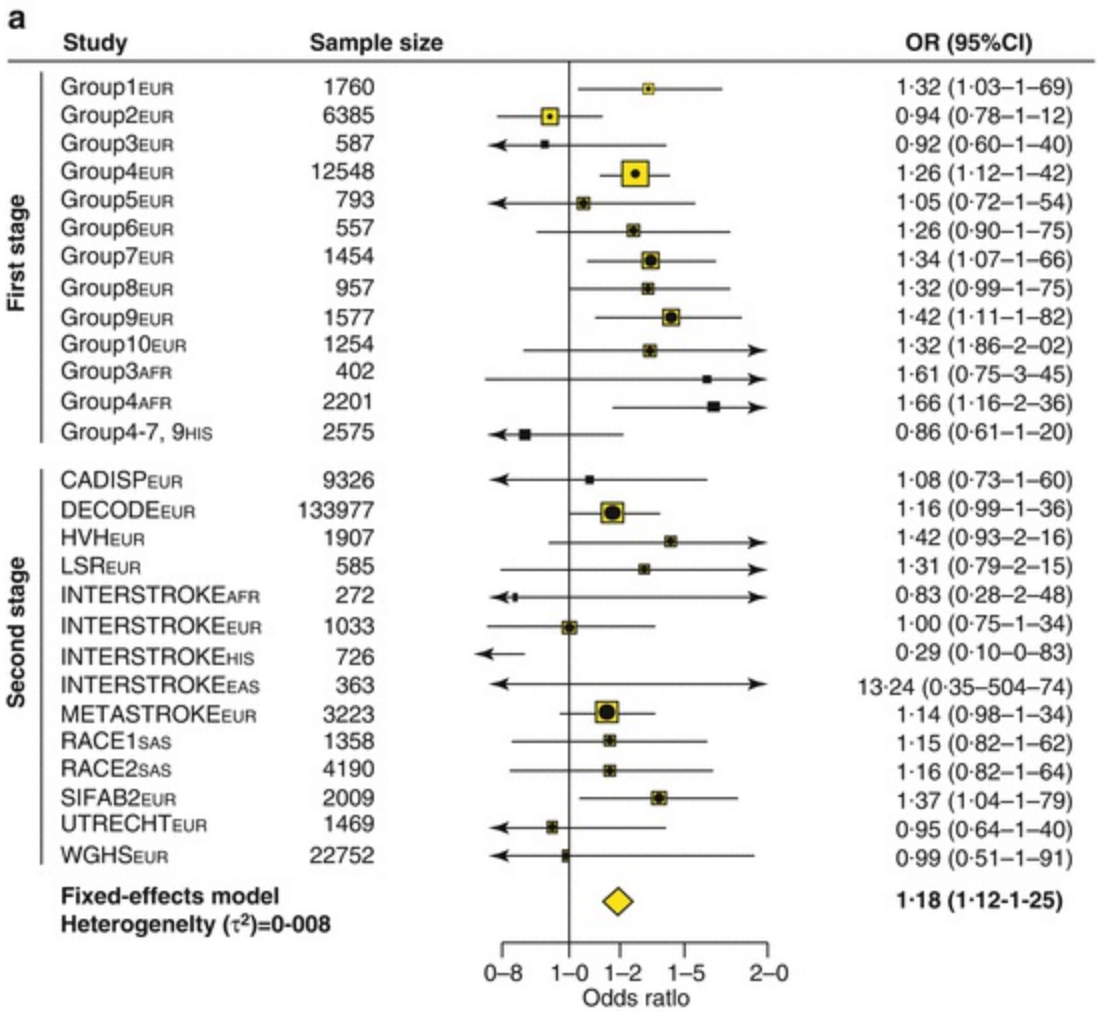
DNA Methylation and Epigenetics

Epigenetics is a burgeoning field with the potential to inform residual unexplained heritability in complex diseases such as stroke and carotid disease. A recent study profiled DNA methylation for symptomatic and asymptomatic carotid plaques obtained at endarterectomy and found only weak differences discriminating the two groups [116]. These results were interpreted as supportive evidence that atherosclerotic plaques revert to a less inflammatory phenotype after causing symptoms (i.e. stroke), which generates hypotheses regarding the timing of testing for future research.

Gene Expression and Stroke Subtypes

Gene expression profiling using RNA microarrays is a promising approach for both investigation of pathophysiology and diagnostics of cerebrovascular disease [117]. Data suggest that expression profiles from whole blood can discriminate ischemic stroke from healthy and risk factor-matched controls, as well as those with other cardiovascular disease such as acute myocardial infarction [118]. Similarly, expression patterns of 34 genes differentiated high-risk but asymptomatic individuals from those in transient ischemic attacks (TIA) with 100% sensitivity and specificity [119]. Expression profiling can also discriminate TIA from non-ischemic causes of transient neurological symptoms with a high degree of accuracy [120]. Of greater relevance to carotid atherosclerotic disease are recent data differentiating large artery stroke from cardioembolic stroke in the acute setting [121, 122]. One investigation found that a profile of expression for 40 genes differentiated large-vessel stroke from cardioembolic stroke with greater than a 95% sensitivity and specificity [121] (Fig. 12.3). Using this profile to investigate a cohort of cryptogenic stroke, 18% were predicted to be of large-

vessel origin [123]. While these findings require validation in larger and diverse populations, the results imply that RNA expression analysis could 1 day identify symptomatic large artery atherosclerosis in lesions that may not appear severely stenotic on imaging. Conversely, an asymptomatic expression profile might preclude aggressive intervention even when stenosis is present.



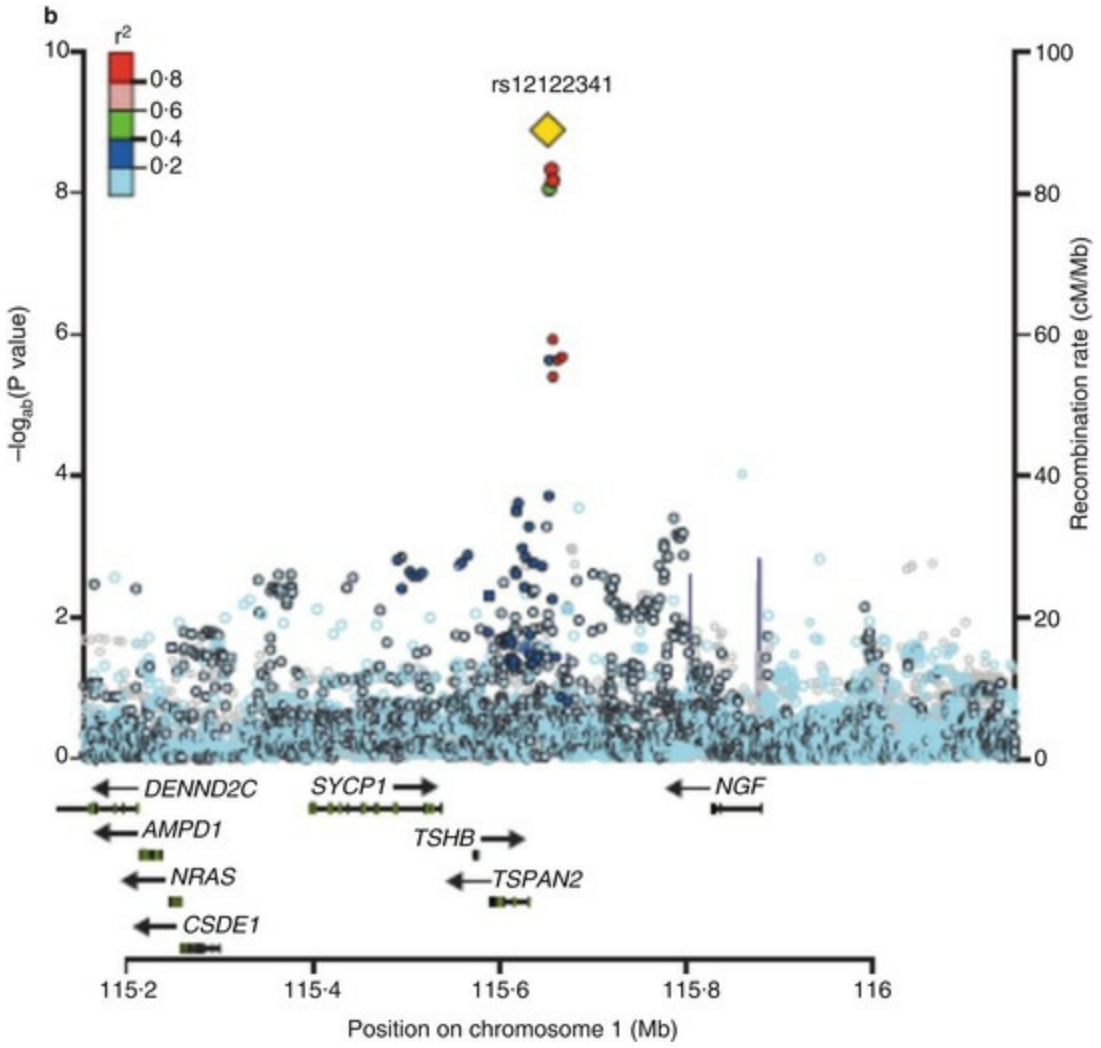


Fig. 12.2 Forest plot (**a**) showing effect sizes for the association of rs12122341 with large artery atherosclerosis-related stroke across the case-control groups included in the first and second stage analyses (**a**). Association of rs12122341 and other SNPs in the region with large artery atherosclerosis-related stroke (**b**). Reproduced from NINDS SiGN and ISCG [106]

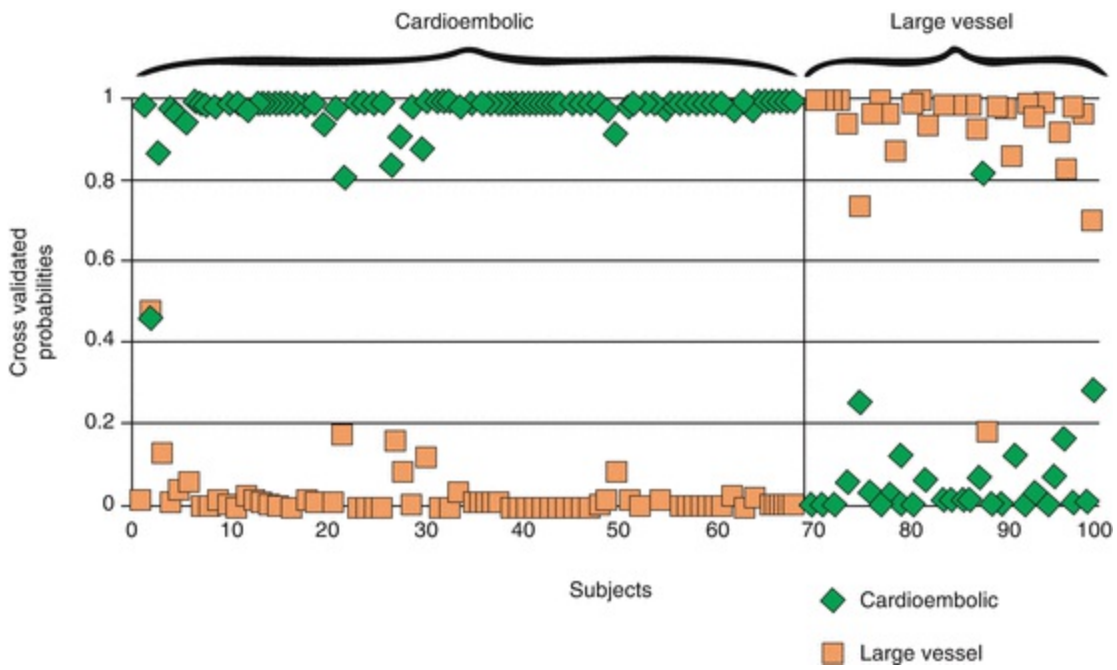


Fig. 12.3 Cross-validation prediction analysis of the 40 genes found to differentiate cardioembolic stroke from large-vessel stroke. The probability of the predicted diagnosis is shown on the *y*-axis. The actual diagnosis is shown on the *x*-axis, where patients with known cardioembolic stroke are shown on the left, and patients with known large-vessel stroke are shown on the right. The probability of a predicted diagnosis of cardioembolic stroke is indicated by the *green diamonds*, and the probability of a predicted diagnosis of large-vessel stroke is indicated by the *orange squares*. Subjects with known cardioembolic stroke were predicted to have cardioembolic stroke for 69 out of 69 samples (100% correct prediction). Subjects with known large-vessel stroke were predicted to have large-vessel stroke for 29 out of 30 samples (96.7% correct prediction). A sample is considered misclassified if the predicted class does not match the known class with a probability greater than 0.5 (Reproduced with permission from Jickling et al. [121])

Gene Expression and Carotid Disease

Gene expression analyses in carotid disease have utilized RNA sampled from whole blood, primarily differentiating patterns of inflammation between large artery stroke and other subtypes. Expression analyses of actual carotid tissue have been limited in part due to the challenges of appropriate control tissue availability. One exciting and unique resource contains paired expression data from human carotid atheroma and adjacent non-atheromatous tissue from 34 individuals. In preliminary analyses using intra-individual comparison, they found that integrin-binding sialoprotein (IBSP) over expressed in atheroma plaque (3.74 fold, p = 1.41E-09) [124] and differential expression of iron metabolism genes (macrophage hemoglobin scavenger receptor (CD163) and heme oxygenase (HO-1)). More recently, using the

same resource, they have found a strong correlation of expression of corticoid and angiotensin receptors in carotid vessels throughout the transition from normal to atheromatous state [124]. A positive feedback loop between angiotensin and cortisol signaling appears to be pro-atherogenic. Another study using biobank specimens with linked clinical data that compared symptomatic and asymptomatic carotid tissue found that high arachidonate lipoxygenase-15B (ALOX15B) expression in carotid lesions was associated with a history of cerebrovascular symptoms [125]. Attempts to correlate expression in plaques with circulating biomarkers have shown early promise [126]. Gene expression from tissue collected from those undergoing symptomatic vs. asymptomatic carotid disease demonstrates striking differences [127]. That study used whole-genome microarrays to investigate differences associated with plaque stability/instability. They found up-regulation of the chemokine (c-c-motif) ligand 19 (CCL19) gene in unstable plaques and verified protein expression differences in a second cohort of patients. They raise the potential of this gene product as a biomarker to identify patients at highest risk of stroke. Another study looking at systemic atherosclerosis (tissue from the aorta, femoral artery, and carotid artery compared to non-atheromatous vascular tissue confirmed up-regulation of inflammatory gene expression on atheromata [128]. Importantly, they found significant differences in the patterns and specific genes expressed between plaques from aortic and carotid arteries suggesting that while atherosclerosis in these arteries are undoubtedly related, important differences exist.

Future directions might shed further light on large artery atherosclerosis by identifying gene expression signatures in vascular tissues, such as harvested carotid plaque following endarterectomy. A recent pilot study of thirty patients undergoing carotid endarterectomy for both symptomatic (greater than 60% stenosis) and asymptomatic stenosis (greater than 70%) isolated microRNAs from the carotid plaques and identified a significant overexpression in symptomatic plaques of selected microRNAs previously implicated in plaque growth and instability (miR-100, 125a, 127, 133a, 145, and 221) [129]. Further work to elucidate diagnostic biomarkers and therapeutic targets for large artery stroke among these gene expression signatures is warranted.

In an analogous study of gene expression profiling, a panel of 30 genes discriminated plaques from symptomatic and asymptomatic individuals with an accuracy of 78% [130]. The genes represented a broad set of networks, but

most of the genes were transcription factors. The implicated gene networks included hypoxia, chemokines, calcification, actin cytoskeleton and extracellular matrix, and several novel genes not previously implicated in carotid disease or atherosclerosis were identified. They validated the microarray data quantitative PCR and immunohistochemistry. Notably, this was not the first attempt to distinguish symptomatic from asymptomatic plaque using gene expression profiling [131, 132], and others have attempted to correlate microRNA expression patterns in human plaques with clinical features [133].

One can envision how expression data might inform our understanding of the biology and pathophysiology of carotid atherosclerosis and symptomatic conversion. As an example, expression studies implicated proprotein convertase subtilisin/kexin type 9 (PCSK9) in plaque destabilization several years ago [134]. We now recognize PCSK9 as binding the LDL receptor and as the target of recently approved drugs for dyslipidemia and vascular disease.

Clearly, this work is very compelling but a long way from clinical applicability. After replication, subsequent work will require testing expression in peripheral blood or other target tissues in real time and following subjects for cerebrovascular events prospectively to help guide appropriate intervention.

Conclusions

The recent successes in genetic research of ischemic stroke and carotid disease will hopefully herald translation into meaningful understanding of the mechanisms underlying these conditions and offer new targets for treatment and prevention. As a complex and compound phenotype, stroke has been especially challenging to study. These data on large artery stroke and carotid disease point to differing genetic architecture underlying various stroke subtypes, although the potential for shared susceptibility should not be dismissed. Similarly, atherosclerosis may not have the same genetic substrates in all vascular beds, or even subsections of the same vascular bed (e.g., distal ICA versus bifurcation ICA). Advanced genetic techniques incorporating next generation sequencing, epigenetics, and novel gene expression analyses may further inform the pathogenetics of carotid disease and identify new targets for stroke prevention. A recent polygenic risk score

analysis of response to statin therapy demonstrated that those with the greatest genetic liability for carotid and other vascular atherosclerotic disease have a greater burden of subclinical atherosclerosis and, importantly, seem to derive the greatest benefits from statin therapy to prevent progression to symptomatic disease [135].

Answering the clinical questions posed at the beginning of this chapter will require collaboration across clinical and research fields and investigation at all phases of carotid disease including initiation, progression, symptomatic conversion, and treatment response. We must not miss the opportunity during large-scale clinical trials of carotid disease, such as the ongoing CREST-2 study of asymptomatic carotid stenosis, to investigate the genetic determinants of outcome or complications of intervention. Going forward, these genomic and pharmacogenomic investigations must be incorporated and embedded into clinical trials research at the *design* phase. We have made important progress to date, but much remains to be done.

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13. Genetics of Cervical Artery Dissection

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Introduction

Cervical artery dissection (CeAD) is one of the most common causes of ischemic stroke (IS) in young and middle aged adults [1], occurring at a mean age of 44–46 years [2, 3], and seldom beyond the age of 65. In the general population the incidence of CeAD is quite low, estimated at 2.6/100,000 per year [3], which makes it a major challenge to study large samples of patients with this disorder. Pathologically, CeAD is associated with a haematoma in the wall of a cervical artery (carotid or vertebral), secondary either to an intimal tear, or to direct bleeding within the arterial wall due to a ruptured vasa vasorum. The most severe complication of CeAD is intra-luminal thrombus formation leading to cerebral, or more seldom retinal, ischemia. CeAD can also present with “local” symptoms or signs only, including headache, cervical pain, Horner’s syndrome, and cranial nerve compression, or can be asymptomatic.

Although mechanisms and risk factors of CeAD are poorly understood,

neck trauma is an important predisposing factor for CeAD. Rarely, CeAD can occur with major penetrating or non-penetrating traumas [4]. Often, CeAD patients report a minor trauma [5, 6], such as chiropractic manipulation, whiplash injury, or other unusual neck movements, in the days or weeks preceding the dissection. Even minor neck positioning such as balancing a telephone or shaving have been described in CeAD. However, a causal relationship is often difficult to establish, and many cases of CeAD occur without any trauma, suggesting that there must be other susceptibility factors. Recently, hypertension was shown to be associated with a moderately increased risk of CeAD [7, 8], while hypercholesterolemia and overweight appear to be inversely associated with the disease [7, 9]. Other putative risk factors and comorbidities include recent infection [10–13], migraine (especially without aura) [14, 15], hyperhomocysteinemia [16–18], although strength of evidence is more limited for the latter. CeAD also shows an intriguing correlation with other non-atherosclerotic vasculopathies [19], mainly fibromuscular dysplasia (FMD) and reversible cerebral vasoconstriction syndrome (RCVS) [20]. In the largest published FMD registry the frequency of CeAD in FMD patients was 12.1% [21–23]. The frequency of FMD in CeAD registries varies widely, ranging between 5.6% [24] and 21% [25, 26].

A number of arguments suggest that genetic factors may play an important role in the pathophysiology of CeAD, in rare cases as part of a monogenic disorder, and more commonly as part of a multifactorial predisposition [27].

Monogenic Disorders and CeAD

Seldom, CeAD can occur as a complication of known, rare inherited connective tissue disorders [28], mostly vascular Ehlers-Danlos syndrome (caused by mutations in the *COL3A1* gene) [29], but also classic or hypermobile Ehlers-Danlos syndrome (caused by mutations in *COL5A1*, *COL5A2*), Marfan syndrome (*FBN1*), Osteogenesis Imperfecta (*COL1A1*, *COL1A2*), Loeys-Dietz syndrome (*TGFBR1*, *TGFBR2*) [30, 31]. These represent less than 1/1000 of CeAD patients hospitalized in departments of neurology [30]. However, a positive diagnosis of an underlying inherited connective tissue disorder, and precise molecular diagnosis thereof, can have important implications in terms of treatment, preventive measures and

follow-up, as well as genetic counselling [32]. Patients with vascular Ehlers-Danlos syndrome for instance are at increased risk of vessel rupture secondary to endovascular investigations or procedures, at high bleeding risk under anticoagulants, and specific treatments may be prescribed to prevent vascular complications [33]. Hence it is important to screen patients for clinical features and a family history suggestive of an inherited connective tissue disease (of note, family history can be negative due to a high frequency of de novo mutations). It should also be noted that currently large published series of CeAD patients have not systematically screened patients for mutations in genes causing known, inherited connective tissue disorders. Pilot data suggests that such mutations may be more common than suspected based on clinical criteria [34–39].

Vascular Ehlers-Danlos Syndrome

Vascular Ehlers-Danlos syndrome (vEDS or EDS type IV) is a rare autosomal dominant disease, due to a mutation in the *Collagen, type III, alpha 1* (*COL3A1*) gene on chromosome 2q31 (OMIM 130050). The prevalence is estimated at 0.2–1/100,000 [29], and the median survival at 48 years [40]. The diagnosis is suggested clinically by the presence of at least two out of four major criteria [41]: easy bruising, thin skin with visible veins, characteristic facial features (including a thin pinched nose, thin lips, hollow cheeks, prominent staring eyes, lobeless ears), and rupture of arteries, uterus or intestines [29]. The demonstration of either an abnormal type III procollagen synthesis or of a mutation in the *COL3A1* gene is required to confirm the diagnosis. Phenotypic features can be subtle and most patients are unaware of the diagnosis at the time of their first major complication, which usually occurs before age 40 [40].

The reported frequency of vEDS cases in large published series of consecutive CeAD patients ranges between 0.5 and 2% [30, 42–45] while several other large series do not report any vEDS patient. Four studies in a total of 53 CeAD patients have screened for mutations in the *COL3A1* gene [35–38]. One missense mutation, typical of vEDS (glycine substitution in the triple helical region of *COL3A1*), was found in two related CeAD patients, who had however no clinical evidence of vEDS [28]. Among vEDS patients, CeAD seems to be a classical but also relatively rare complication, although this was assessed retrospectively only. In the two largest, partly overlapping, series of biologically confirmed vEDS patients, 2% had a history of CeAD

(only carotid and not vertebral artery dissections were reported in the most recent study) [40, 46].

Even though vEDS is a rare cause of CeAD, it seems important to screen CeAD patients for suggestive clinical signs of vEDS and direct the patient to a specialized geneticist if there is a clinical suspicion. Indeed, if the diagnosis is confirmed, genetic counselling and important preventive measures can be proposed. Endovascular investigations are contra-indicated in vEDS patients, given the high risk of iatrogenic arterial dissection and rupture due to the vulnerability of the arterial wall. While in the general population either anticoagulants or antiplatelet agents are prescribed at the acute phase of CeAD to prevent primary or recurrent ischemic events, as their efficacy has not been compared in a randomized trial [45, 47, 48], in vEDS patients, antiplatelet agents might perhaps be the preferred treatment, since fatal bleeding may occur under anticoagulants [29]. Likewise, while long-term prevention of cerebral ischaemia with antiplatelet agents is often proposed in CeAD patients with residual arterial lesions, in vEDS patients the risk-benefit ratio of long-term antiplatelet therapy should be carefully weighed [49].

Marfan Syndrome

Marfan syndrome (MFS) is an autosomal dominant disease due to a mutation in the *fibrillin-1* (*FBN1*) gene on chromosome 15q21.1 (OMIM 154700). The prevalence is estimated at 1/5000 and the mean survival at 45 ± 17 years [50]. Clinical signs of MFS include musculoskeletal, ocular and cardiac complications, with aortic and mitral valve anomalies, aortic aneurysms and dissections conditioning the outcome. The diagnostic criteria have recently been revised [51].

Large series of consecutive CeAD patients report very low frequencies of MFS (0.6–0.9%) [2, 30, 42, 45] without details on how the diagnosis of MFS was confirmed. Many large series do not report any patient with MFS. In patients with a proven diagnosis of MFS, spontaneous CeAD seems to be exceptional and must be differentiated from proximal aortic dissections extending into the brachiocephalic arteries. In a retrospective analysis of neurovascular complications in 513 MFS patients, not a single case of CeAD was found [52]. No CeAD was reported either in a series of 1013 MFS patients, although central nervous system complications were not described in detail [53].

Loeys-Dietz Syndrome

Loeys-Dietz syndrome (LDS) is a recently identified group of autosomal dominant disorders (OMIM 609192, OMIM 610380, OMIM 610168, OMIM 608967) caused by mutations in the *Transforming Growth Factor Beta Receptor 1* (*TGFBR1)* and 2 (*TGFBR2*) genes (chromosome 9q22 and 3p22), of unknown prevalence [54], whose phenotypes overlap with MFS and vEDS [55]. Three quarters of affected individuals have LDS type I with craniofacial manifestations (ocular hypertelorism, bifid uvula and cleft palate, craniosynostosis); the remaining quarter have LDS type II with cutaneous manifestations (velvety and translucent skin; easy bruising; widened, atrophic scars) [55]. The disease is characterized by aggressive arterial aneurysms and high incidence of pregnancy-related complications including uterine rupture and death [55, 56]. Arterial tortuosity involving head and neck vessels is frequent in LDS patients.

Although CeAD has occasionally been described, according to current published data it seems to be usually associated with aortic dissection, and not to occur in the absence of aortic root involvement, as in MFS [55]. Of note however, in a recent study that performed systematic sequencing of *TGFBR1* and *TGFBR2* exons in 56 consecutive CeAD patients, novel *TGFBR2* disease-causing mutations were identified in two patients, who had only very subtle signs of connective tissue involvement [34].

Other Monogenic Disorders

A number of observations have suggested an association of CeAD with other monogenic conditions, including alpha 1 antitrypsin deficiency, osteogenesis imperfecta, autosomal dominant polycystic kidney disease or hereditary haemochromatosis [57–65], as well as with rare chromosomal disorders such as Turner's syndrome [66, 67], or William's syndrome [68]. It is however unclear whether the simultaneous occurrence of these disorders is more common than would be expected by chance and these conditions can probably not be considered as risk factors for CeAD at the community level.

Some studies have also screened for monogenic causes of CeAD through systematic sequencing of candidate genes [35–38, 69–73], in small series enriched in patients with a family history of CeAD or with morphological abnormalities in the dermal connective tissue. Beside *COL3A1* [35–38], other genes involved in connective tissue homeostasis were screened for potential

disease-causing mutations [35, 69–73]. Only one mutation was found in *COL5A2*, which did not correspond to a typical disease-causing mutations [70].

Genetic Predisposition to CeAD

In the majority of CeAD cases, there is no clinical evidence for an underlying monogenic disease. Heritability estimates are not available. Familial cases of CeAD are rare (<2.5% in published series) and usually do not appear to occur in the context of a known inherited connective tissue disorder [30, 74]. On the one hand these scarce reports of family history of CeAD could be an overestimation, due to recruitment bias through tertiary referral centers, and the fact that several CeAD cases from multiple affected families were included. On the other hand, a family history of CeAD is likely to be underreported because CeAD can occur asymptomatically, and even symptomatic CeAD may have been unrecognised as clinical signs can be subtle and the diagnosis tricky, especially before MRI became widely available. Overall, even though familial occurrence of CeAD seems to be rare, it is slightly more common than would be expected by mere chance given the low incidence of the disease [31].

Several other arguments suggest that genetic factors could play a role in the occurrence of “sporadic” CeAD, as part of a multifactorial predisposition [17]. The common occurrence of multiple dissections (15–20%), the concomitant structural and functional vascular abnormalities such as increased carotid stiffness [75], endothelial dysfunction [76], aortic root dilation [77], or pathological changes in the temporal arteries [78], and the frequent co-occurrence of FMD [23, 26] and RCVS [20] suggests that CeAD patients may have a pre-existing vasculopathy, possibly related to a connective tissue fragility, which could be influenced by genetic susceptibility factors [79, 80]. Besides, in some series about half of CeAD patients were shown to have connective tissue aberrations in their reticular dermis, the most common pattern being composite collagen fibrils and fragmentation of elastic fibres [79, 81, 82]. These abnormalities seem to be transmitted according to an autosomal dominant pattern [80, 83], without fulfilling the diagnostic criteria for any known monogenic connective tissue disease.

Genetic factors could theoretically predispose to CeAD at various levels,

e.g.: (1) by contributing to a weakening of the vessel wall (on top of which environmental factors such as trauma or acute infection could act as triggers), (2) by increasing vulnerability to environmental factors that could have an impact on vascular wall integrity, for instance by modulating inflammatory response to infection, (3) by influencing the occurrence of environmental susceptibility factors of CeAD, such as hypertension, low lipid levels and body mass.

Different approaches have been used to identify genetic variants contributing to CeAD risk.

Linkage Studies

Linkage studies are family-based and consist of simultaneously examining the transmission across generations of both a given phenotype (in this case CeAD) and marker alleles, either genome-wide or in a specific genomic region. Linkage studies for CeAD have been limited by the small number of large families with several members affected by the disease. One linkage analysis performed in a family including three individuals affected by CeAD, using markers flanking the *COL3A1* locus, yielded negative results [38]. Other linkage studies have been performed in families with only one member affected by CeAD but several members presenting the typical skin connective tissue abberrations, described as being associated with CeAD [72, 80, 83]. These studies used microsatellite markers for candidates genes involved in the synthesis of extracellular matrix components [72, 83], and one study performed a whole genome linkage analysis [80]. Despite some suggestive findings none of these studies could formally confirm the presence of genetic linkage.

Genetic Association Studies Based on Candidate Genes

Genetic association studies consist of comparing the frequency of genetic variants between patients and controls. Multiple genetic association studies of CeAD have been published to-date [16–18, 38, 84–96], most of which were negative. Significant associations with three candidate genes were described: *Intercellular Adhesion Molecule 1* (*ICAM-1), COL3A1* and *Methylenetetrahydrofolate reductase (MTHFR)* [17, 18, 38, 86, 92]. The associations with the *ICAM-1* and the *COL3A1* variant should be interpreted with caution, as they were observed in relatively small studies, and have not

been replicated yet [38, 86]. Three studies, of which two overlap, found a positive association between the *MTHFR* 677TT genotype and CeAD [17, 18, 92], while four others did not report any association [16, 84, 85, 97]. A meta-analysis of these studies (including 440 CeAD cases in total) suggested an overall significant association of the *MTHFR* 677TT genotype with CeAD [27]. However, given the small sample size of individual studies and the publication bias favoring candidate gene studies with significant results, it seems crucial to replicate this finding in a larger, independent sample. The *MTHFR* 677TT genotype is associated with elevated homocysteine levels [16–18, 85], which may contribute to endothelial damage or influence elastic properties of the arterial wall [17].

Overall, despite important efforts, no consistent genetic association with CeAD has been identified by this candidate gene approach. Studies have been markedly underpowered, mainly due to the low prevalence of CeAD, which made it difficult to reach sufficiently large sample sizes.

Genome-Wide Association Studies

Definitive data can only be obtained from much larger multicentre genetic association studies, with replication of positive associations in independent samples [98]. International efforts recently enabled to analyse much larger datasets using a genome-wide approach (www.cadisp.org) [99]. Indeed, even when statistical power is optimal, candidate gene association studies are unable to identify novel genetic variants involved in unsuspected pathways, since they are based on what is already known or suspected about the pathophysiology of the disease [100–103]. Genome-wide association studies (GWAS) offer an unbiased approach, consisting of genotyping very large numbers of genetic variants (100,000–5,000,000) distributed across the chromosomes, without requiring any a priori hypothesis. In the past few years this approach has been applied to a number of complex diseases with major successes, leading to the identification of hundreds of novel genes conferring increased risk of many complex diseases [104]. This approach may be equally well suited to CeAD if sufficiently large populations can be collected.

A large, multicenter GWAS of CeAD was recently completed by the CADISP consortium, using DNA samples from over 2000 CeAD patients in total (discovery and replication) [105]. This first CeAD GWAS led to the identification of common intronic genetic polymorphisms in the *PHACTR1* gene associated with CeAD risk (the lower frequency allele [G] of the top

variant, rs9349379, was associated with a lower risk of CeAD) [105]. This association was confirmed in an independent sample. Other highly suggestive associations were observed, with common variants in the *LRP1* gene and the *LNX1* gene, but these require confirmation in independent samples. While the *PHACTR1* variants were equally associated with carotid and vertebral artery dissections, the *LRP1* and *LNX1* variants were associated primarily with carotid artery dissection [105]. Intriguingly, independent GWAS have found the G allele of rs9349379 to be associated with a significantly lower risk of migraine (without aura) [106, 107] and fibromuscular dysplasia [108], and significantly increased risk of myocardial infarction and coronary calcifications [109, 110]. Opposite effects of the same genetic variant on different diseases have been described elsewhere, suggesting either that the same region harbors different causal variants or that the same causal variant has biological effects with opposite implications for each disease. This would be consistent with the aforementioned observation from epidemiological studies that CeAD is not associated with an atherosclerotic risk profile. *PHACTR1* being a major susceptibility locus for CeAD, migraine, fibromuscular dysplasia, and coronary artery disease, understanding the mechanisms by which this locus appears to influence key vascular functions could potentially have major applications for the treatment of these conditions [105]. Interestingly, recent analyses of tissue-specific gene expression have shown that the G allele of rs9349379 is associated with significantly reduced *PHACTR1* expression levels in coronary arteries (data in cervical arteries are lacking). *PHACTR1* is located in a highly conserved genomic region, suggesting a crucial involvement in biological processes [39], but its function is poorly understood. Recent data suggest that *PHACTR1* regulates skeletal and cardiac alpha-actin gene expression, with its expression in the heart being modulated by direct mechanical stretching of cardiomyocytes—whether this applies to mechanical stretching of cervical arteries is unknown [111]. Other data suggest that disruption of the phactr-1 pathway may trigger pro-inflammatory and pro-atherogenic factors [112].

It should be noted that, so far, most genetic studies have been performed in populations of European descent. Clinical and demographic characteristics of CeAD partly differ in other ethnic groups [113, 114], and collecting large samples of dissection patients of other ethnicities will be an important challenge in the near future. Finally, as has been experienced with all other complex diseases, GWAS is likely to identify only a minor proportion of

genetic susceptibility factors for CeAD. Indeed, GWAS has focused mostly on one type of genetic variation so far, namely common single nucleotide polymorphisms. Investigating other types of genetic variation, such as copy number variants, rare variants, as well as epigenetic modifications, will be the next step.

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14. Genetics of Small Vessel Disease

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Introduction

Cerebral ‘small vessel disease’ (cSVD) refers to a mixture of clinical, cognitive, neuroimaging, and neuropathologic abnormalities that arise from different pathologic processes in cerebral perforating arteries and arterioles, capillaries and venules. A major risk for stroke, it is now also seen as the major cause for vascular dementia [1]. Nearly every person over age 80 years displays signs of cSVD, making it one of the hallmarks of normal and abnormal ageing. cSVD has been challenging to tackle mechanistically and genetically. Studies have been constrained by technical difficulties, including visualization of small blood vessels, standardized phenotyping, and access to tissue [2]. Several genes discovered in monogenic disorders have been implicated in cSVD, consequentially these findings have also lead to improved options in diagnosing, advising, and managing patients [3]. The following chapter will discuss current studies and findings in the genetics of cSVD along with their therapeutic implications.

Genetic and Epidemiologic Studies on Small Vessel Disease rely on Surrogate Markers

Quantitative surrogate markers of cSVD (Table 14.1) have been included in genetic studies extensively over the last years as they provide insight into progression of cSVD [

Table 14.1 Quantitative surrogate markers of cSVD

Deep intracerebral hemorrhage (ICH)
Cerebral microbleeds (MBs)
Silent brain infarcts
Microinfarcts
White matter hyperintensities
Lacunar infacts

4], they can be measured reliably, and provide more statistical power than traditional binary (case/control) analyses. However, phenotyping by these surrogate markers alone might introduce bias, because other competing etiologies could be missed by concentrating purely on white matter hyperintensities (WMH) and lacunar infarcts. The specificity for cSVD can be increased by combining multiple radiologic and clinical markers [1]. Similarly, more individuals included in the studies can compensate for lower pathologic specificity. Other less specific surrogate markers than the ones in Table 14.1 include: microbleeds (MBs), which are seen in various forms of cSVD and in cerebral amyloid angiopathy [5], and silent brain infarcts, which mostly fall in the size range of lacunar infarcts and are thus associated with cSVD.

Conventional Risk Factors for Small Vessel Disease have a Genetic Basis

In a traditional, epidemiological sense, high blood pressure is the most important risk factor for cSVD apart from age. Others include diabetes and smoking, and each of these risk factors itself harbors an attributable portion of genetic risk. While some of the risk factors like hypertension have been studied successfully and common and rare variations have been found to be associated with multiple blood pressure traits (e.g., systolic blood pressure, diastolic blood pressure, mean arterial pressure, pulse pressure) [6], other phenotypes have been harder to dissect by traditional genetic means. Nevertheless, the number of common and rare genetic variants known to be

associated with traditional risk factors for cSVD is rapidly growing, especially since the advent of next-generation sequencing (NGS) technologies, making discoveries of rare variation much easier.

Small Vessel Disease Has a Genetic Basis

The heritability of cSVD can be partly expressed by the heritability of WMHs. Multiple studies have suggested a narrow-sense heritability of >60% for radiologically confirmed WMHs in family studies [7–10]. Here, heritability refers to the proportion of variation in phenotype explained by genetic factors. Consistent with risk factor correlation, genetic overlap between risk factors and WMHs exists. Heritability estimates of WMHs are usually higher in hypertensive rather than non-hypertensive individuals, suggesting a shared genetic basis. Also, the heritability of small vessel-related stroke and deep intracerebral hemorrhage (ICH) attributable to common genetic variants is estimated to be in the range of 15–30% [11, 12]. The discrepancy is explained by higher impact rare variation in familial cases in comparison to sporadic cases. In fact, there is a growing list of Mendelian cSVDs, some of which are now recognized as an important cause of juvenile stroke [13] as well as vascular dementia.

To study cSVD, monogenic cSVDs sharing etiology including a progressive arteriopathy, subcortical infarcts, white matter disease, and clinical manifestations, in particular stroke and dementia can be used efficiently. After their first description, it soon became clear that hereditary SVDs are genetically heterogeneous and represent different disease entities (Table 14.2) [

Table 14.2 Monogenic form of cSVD. Shown are the path of inheritance (dominant, recessive, X-linked), the gene known as responsible for the disease, the stroke phenotype aside from cSVD associated with the disease, the clinical features most prominent and the diagnostic utilities available.

Disease	**Inheritance**	***Gene***	**Stroke phenotype**	**Clinical features**	**Diagnosis**
CADASIL	Autosomal dominant	*NOTCH3*	**Small-vessel disease**	Migraine with aura	Mutational screening, skin biopsy
CARASIL	Autosomal recessive	*HTRA1*	**Small-vessel disease**	Premature baldness; severe low back pain; spondylosis deformans or disk herniation	Mutational analysis

RVCL-S	Autosomal dominant	*TREX1*	**Small-vessel disease**	Retinopathy, neurologic deficits, cognitive impairment, and psychiatric disturbances	Mutational screening
Fabry's disease	X-linked	*GAL*	Large-artery disease and **small-vessel disease**	Angiokeratoma; neuropathic pain; acroparaesthesia; hypohydrosis; corneal opacities; cataract; renal and cardiac failure	α-galactosidase activity, mutational screening
Sickle-cell disease	Autosomal recessive	*HBB*	Large-artery disease, **small-vessel disease**, haemodynamic insufficiency	Pain crises; bacterial infection; vaso-occlusive crises; pulmonary and abdominal crises; anaemia; myelopathy; seizure	Peripheral blood smear, electrophoresis, mutational analysis
Homocystinuria	Autosomal recessive	*CBS* and others	Large-artery disease, cardioembolism, **small-vessel disease**, 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)
Pseudoxanthoma elasticum	Autosomal recessive	*ABCC6*	Large-artery disease and **small-vessel disease**	Skin changes (increased elasticity and yellow-orange papular lesions); ocular changes (angioid streaks); hypertension	Skin biopsy, mutational screening

14]. What follows are brief descriptions for several hereditary cSVDs.

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) (OMIM: 125310) is the most common monogenic form of cSVD (see Chapter 6), with a prevalence of 5/100,000. The condition is caused by cysteine-mutations in NOTCH3, a transmembrane receptor of the Notch family with a large extracellular domain (ECD) mainly consisting of epidermal growth factor (EGF)-like repeats [15, 16]. Disruption of the cysteine residues of NOTCH3 leading to an unequal number of disulfide-bridges and consequent mis-folding of the protein is the hallmark of CADASIL [17]. More than 200 pathogenic NOTCH3 mutations have been

described, with the majority representing missense mutations, although deletions, duplications, and splice site mutations have also been reported [18]. The resulting unpaired cysteine promotes NOTCH3 aggregation, precisely within the ECD domains and also promotes accumulation in the extracellular space, a critical event in CADASIL pathogenesis.

Whether non-cysteine mutations cause disease or not is still debated. Wollenweber et al. showed that these non-cysteine mutants can aggregate *in vitro* [19]. It thus seems that in some instances, CADASIL can originate from atypical mutations that do not affect cysteines within NOTCH3 ECD.

Cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy (CARASIL) (OMIM: 600142) overlaps with CADASIL in terms of phenotypes including WMH in periventricular and deep white matter, multiple SVS, and vasculopathic alterations of penetrating arteries and arterioles [20, 21]. Compared to CADASIL, CARASIL has a much earlier age-of-onset. Its mode of inheritance is recessive compared to dominant, and its phenotypic manifestations are also not always neurologic, as spondylosis and alopecia are common [20]. First discovered in Japan, there have also been reports of CARASIL cases in other ethnicities. The gene causing CARASIL is HTRA1, encoding high temperature requirement protein A1, a highly conserved serine protease [22]. Homozygous mutation carriers suffer from dramatic alterations in protease activity, consistent with a recessive trait. Heterozygous mutation carriers also show reduced HTRA1 activity, with the non-formation of trimers being one of the mechanisms being discussed [23].

The COL4A1/A2-related angiopathies (OMIM: 607595; 614519) are multi-system disorders with very heterogeneous phenotypes, including porencephaly, an infantile neurologic disorder associated with hemorrhages and hemiplegia [24]. COL4A1 mutations have classically been associated with familial cSVD and a wide clinical spectrum including SVS, ICH, WMH, and cerebral microbleeds. COL4A2 mutations mostly disturb trimer-formation with COL4A1, also leading to familial cSVD [25, 26]. The spectrum of COL4A1 and COL4A2 mutations associated with familial cSVD is broad, as is the spectrum of phenotypic abnormalities, which also includes intracranial aneurysms, as well as ocular, cardiac, and renal abnormalities [27]. Ocular defects within the Axenfeld-Rieger spectrum have been found as well in the mutational spectrum of COL4A1/A2 disorders. A combination of Axenfeld–Rieger syndrome and SVD also being described in patients

carrying mutations in FOXC1 and PITX2 [28], which encode two physically interacting transcription factors. Interestingly, common variants at these loci were recently found to be associated with WMH in the general population.

Retinal vasculopathy with cerebral leukodystrophy (RVCL) (OMIM: 192315) refers to a group of cerebroretinal syndromes originally described as separate disorders: cerebroretinal vasculopathy (CRV) [29]; hereditary endotheliopathy with retinopathy, nephropathy and stroke (HERNS) [30]; and hereditary vascular retinopathy (HVR) [31, 32]. Most patients present with retinopathy at a young age (mean age of onset ~45 years), but subsequently develop severe neurologic symptoms including focal neurologic deficits, cognitive impairment, and psychiatric disturbances [33, 34]. CRV, HERNS, and HVR all map to the DNA exonuclease TREX1 [35]. Mutations lead to a frameshift resulting in a protein with truncated or extended C terminus and retained enzymatic activity, but altered subcellular localization. Because of the pathogenetic basis and the emerging clinical picture with systemic manifestations and conspicuous absence of leukodystrophy, the disease was renamed to ‘retinal vasculopathy with cerebral leukoencephalopathy and systemic manifestations’ (RVCL-S) [36].

Common Genetic Variants and Sporadic Small Vessel Disease

Common Variants in NOTCH3 and COL4A1/A2

Monogenic forms of cSVD can often give insight into sporadic forms of the disease. Association studies in both longitudinal and cross-sectional studies suggest that NOTCH3 and COL4A1/A2 mutations are responsible for an attributable part of sporadic cSVD. NOTCH3 variants were found to be associated with both the presence and progression of WMH in 4773 population-based hypertensive subjects from the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) consortium [37]. Common mutations in the COL4A1/A2 gene cluster were found by the International Stroke Genetics Consortium to be associated with cSVD [38]. It could be shown that these variants in the COL4A1/A2 gene cluster are associated with deep intracerebral hemorrhage (ICH) [38]. The same variants showed suggestive associations with lacunar stroke and WMH volume in symptomatic ischemic stroke cases. Common variants in HTRA1 and

TREX1 associated with sporadic cSVD cases have not been identified thus far, but there is agreement in the stroke community that by increasing sample size and power, variants in these genes will eventually show association at a genome-wide level.

Genome-Wide Association Studies

Genome-wide association studies have historically focused on individual markers of cSVD. In this section, I will discuss these studies separately. Very recently, one study tried to combine multiple cSVD markers with very intriguing results paving the way for more complex studies of multiple surrogate markers of cSVD.

White Matter Hyperintensities

WMH burden was studied in more than 10,000 stroke-free individuals from the CHARGE consortium, describing a single risk locus on chromosome 17q25 as being genome-wide significant [39]. Two tripartite motif (ring finger domain followed by a zinc finger domain and a coiled coil domain)-containing genes, TRIM65 and TRIM47 are currently considered the favored candidates for SVD in this region spanning multiple interesting genes, but other genes in this region (WBP2, MRPL38, FBF1, and especially ACOX1) remain alternative candidates.

While being also associated with WMH volume in stroke patients, these genes have not been found in connection with cSVD related stroke phenotypes in this study [40], suggesting similar, but independent mechanisms for WMH and small vessel stroke.

A recent meta-analysis on a much larger set of GWAS data on WMH identified, among known associations, three new loci (2p16, 2p21 and 10q24) that have been implicated in inflammatory and glial proliferative pathways [41].

Small Vessel Stroke

In ischemic stroke (IS), multiple findings have suggested that GWAS strategies can be successful. Most of the genome-wide signals to date are found for TOAST subtypes of IS, with cardioembolic stroke (PITX2, ZFHX3) and large artery stroke (HDAC9, TSPAN2) leading the field [42,

43]. Several associations have been discovered being related to multiple stroke phenotypes (ABO, 12q24) [44, 45], but clear association signals for small vessel stroke (SVS) are lacking. This might, in part, relate to insufficient sample size, sample heterogeneity, and problems in classifying SVS correctly [1]. The PRKCH gene encoding protein kinase C-η were found to be genome-wide associated with SVS in a purely Japanese population [46]. Variants at PRKCH were subsequently shown to also associate with subcortical silent brain infarcts [47] and with cerebral hemorrhage in the Chinese population [48]. Protein kinase C-η is expressed in vascular endothelial and other cell types, is known to mediate a variety of signaling pathways, and regulates multiple cellular functions including proliferation, differentiation, and apoptosis. The lead SNP of the initial association is monomorphic in European populations, and multiple GWAS in non-Asian samples revealed no signals at PRKCH. Hence, it might be that PRKCH represents a risk locus for SVD-related phenotypes (lacunar stroke and ICH) exclusively in the Asian population. However, this requires confirmation in additional samples. While studying a broader population of all stroke, researchers from the CHARGE consortium identified a genomic region close to FOXF2/FOXQ1 showing a high degree of specificity for SVS [49]. Through translational models, it could be shown that mutations in FOXF2 disrupt normal vessel morphology [50], disrupt brain pericyte differentiation and development and maintenance of the blood-brain Barrier, making FOXF2 a viable candidate as a risk gene for SVS. Larger studies will have to confirm the genome-wide association of this region with SVS.

Intracerebral Hemorrhage

A recent GWAS conducted by the International Stroke Genetics Consortium identified a susceptibility locus on chromosome 1q22, overlapping with the PMF1 and SLC25A44 genes [51]. The single-nucleotide polymorphism rs2984613 (minor allele frequency: 32%), the lead SNP at this locus, was associated with a 33% increase in risk of non-lobar ICH. Interestingly, the 1q22 region was also among the loci reaching suggestive association levels of statistical significance in the above-mentioned GWAS on WMH burden from CHARGE consortium [39]. PMF1 codes for polyamine-modulated factor 1, which is required for normal chromosome alignment and segregation and kinetochore formation during mitosis; SLC25A44 encodes a mitochondrial carrier protein. These findings point to a possible mechanism in energy

metabolism, since variation in mitochondrial genes has been implicated in risk of ICH (see below). Further studies have shown that the influence of rare genetic variation in ICH is minimal [52].

Vascular Dementia

Vascular dementia (VaD), the second biggest group of dementias after Alzheimer's disease is often thought to be largely the result of cSVD. The study of VaD is limited due to diagnostic criteria, but there has been a single GWAS on VaD conducted in 5700 initially dementia-free population-based subjects from the Rotterdam study, 67 of whom developed incident VaD over a mean follow-up of 9.3 ± 3.2 years [53]. A single variant on the X chromosome near the androgen receptor gene reached genome-wide significance, with highly variable odds ratios in the discovery and replication cohorts. This locus has so far not been linked to other SVD manifestations and should be considered with caution, as it might be linked to confounding factors.

Apolipoprotein E

Genetic variation in the APOE gene encoding apolipoprotein E is the strongest genetic risk factor for both Alzheimer's disease and cerebral amyloid angiopathy (CAA). The two SNPs responsible are rs7412 and rs429358 where ε2 (rs7412-T, rs429358-T) is the least frequent and the ε4 allele (rs7412-C, rs429358-C) strongly increases the risk of both conditions. The ε2 allele is protective in Alzheimer's disease but associated with an increased risk of cerebral amyloid angiopathy-related lobar ICH [54–56]. The relationship between markers of cSVD and APOE genotype was independently confirmed in a meta-analysis of 42 cross-sectional and longitudinal studies. APOE ε4 was associated with an increased burden of both WMH and microbleeds, and APOE ε2 was associated with an increased WMH burden. Confirming this, a recent GWAS on genetic modifiers in CADASIL found the APOE ε2 allele to be an independent risk factor for higher WMH volumes in NOTCH3 mutation carriers [57]. This indicates that genetic variation in APOE contributes to different manifestations of cSVD independent of the presence of amyloid pathology. The biologic mechanisms underlying this relationship are still poorly understood. An interesting link between APOE and HTRA1 was discovered recently, further showing that

the interactions are complex [58]. Interestingly, in these first in-vitro study, HTRA1 is an allele-selective ApoE-degrading enzyme that degrades Apo ε4 more quickly than Apo ε3.

Oxidative Phosphorylation Genes and Small Vessel Disease

Focusing on aggregate measures of genetic variation rather than individual SNPs, Anderson et al. [59] identified several variants within a larger set of oxidative phosphorylation genes, which collectively were associated with increased risk of both lacunar stroke and deep hemispheric ICH. The oxidative phosphorylation genes are encoded by mitochondrial as well as nuclear DNA. Lacunar stroke showed associations with genetic risk scores in oxidative phosphorylation as a whole, complex I, and complex IV, and the latter also associated with deep hemispheric ICH. These findings are complemented by another study that found a genetic score of mitochondrial variants to be associated with WMH volume in patients with ischemic stroke [60] and a study finding genes implicated in OXPHOS being associated with subtypes of cSVD [61]. Collectively, these findings suggest that genetic variation in oxidative phosphorylation influences small vessel pathobiology although the exact mechanisms remain to be determined.

Common Variants Implicated in Risk Factors for Small Vessel Disease

Recent GWAS have identified a large number of genetic loci associated with established risk factors for SVD. There are more than 30 known risk loci for blood pressure-related traits [62, 63], and more than 100 known loci for type 1 diabetes [64, 65] or type 2 diabetes [66]; and numbers are steadily growing as sample sizes and genetic data are growing. The effect of these loci on SVD risk has not been systematically explored. However, common variants on chromosome 12q24.12 (see above), a known risk locus for hypertension, type 1 diabetes, and hypercholesterolemia, have recently been shown to also associate with SVS [45]. Whether the association between common variants at this locus and SVS is mediated by the associated risk factors or independent mechanisms remains to be explored.

Combining Genetics of SVS and WMH

As mentioned before, genetic studies have primarily focused on single surrogate markers of cSVD. In a recent study by Traylor et al. [67], the authors tried to combine both WMH genetics and SVS genetics to leverage this information and gain insight into how these markers correlate with each other. In a meta-analysis of the genome-wide significant and suggestive loci ($p < 5 \times 10(-6)$) from community populations (15 single nucleotide polymorphisms in total) and from stroke patients, six independent loci were associated with WMH in both populations. Four of these are novel associations at the genome-wide level: NBEAL1, EVL, C1QL1 and COL4A2. This study shows that the combination of multiple cSVD phenotypes can improve the discovery of genetic markers.

Previous mechanistic studies in humans and experimental models have identified multiple factors and mechanisms that may contribute to vascular and parenchymal injury in SVD. The pathology of inherited SVDs shows considerable overlap with sporadic disease, although some pathologic features of sporadic SVD such as breakdown of the blood–brain barrier have so far not been demonstrated in the monogenic forms. A novel aspect that has recently emerged mostly from genetics and from mechanistic studies in monogenic SVDs is the extracellular matrix (ECM). The ECM has long been recognized as a highly dynamic and biologically active compartment that is also implicated in many conditions including diseases of large arteries [68]. However, only recently, the ECM has emerged as a key target relevant to the pathogenesis of SVDs. In fact, mounting evidence suggests that the ECM takes center stage in multiple forms of SVD. I will demonstrate this on examples of COL4A1/A2 phenotypes, CADASIL, and CARASIL. In all three monogenic disorders, the ECM plays an integral part and these findings might pave the way for efficient treatment.

COL4A1/A2-Related Small Vessel Diseases

Collagens type IV α1 (COL4A1) and α2 (COL4A2) represent the most common components of basement membranes, which are extracellular macromolecular structures anchoring epithelial and endothelial cells to underlying connective tissue [27]. Providing structural integrity, basement membranes also participate in cell–matrix and cell–cell communication by interacting with integrins and binding growth factors, e.g., of the TGF-β

superfamily [69]. The core units of COL4A1/A2 networks are heterotrimers (α1α1α2) whose formation is mediated by long stretches of the amino acid triplet Gly-X-Y within the COL4A1 and A2 collagenous domains. Mutations of the glycine residue within this motif are by far the most common cause of COL4A1/A2-related disorders. However, the functional consequences of these mutations are only partly understood. A simple loss-of-function mechanism is unlikely as mice heterozygous for COL4A1 and A2 null alleles exhibit no overt pathology [70], whereas strains carrying both COL4A1 and A2 mutations show multiple defects, including porencephaly, vascular abnormalities, and intracerebral hemorrhage [71, 72]. Several non-mutually exclusive neomorphic pathomechanisms have been proposed, which could explain the remarkable heterogeneity of disease symptoms. A variety of COL4A1/2 mutations result in intracellular accumulation of mutant heterotrimers and in the activation of endoplasmic reticulum stress [73, 74]. Alternatively, intracellular sequestration of heterotrimers may lead to extracellular deficiency of COL4A1/2 and insufficient collagen matrix formation. However, at present, secretion of mutant trimers and their incorporation into the basement membrane network cannot be excluded as an alternative pathomechanism. This could result in the modification of COL4A1/2-dependent interactions with other extracellular components such as bone morphogenic proteins or cell surface molecules [27]. Ultrastructural studies in COL4A1 mutation carriers and COL4A1 mutant mice show profound structural abnormalities, with thickening and herniation of the basement membrane [75]. In accord with this, COL4A1/A2 mutations are assumed to have profound effects on ECM function.

CADASIL

NOTCH3 belongs to the Notch family of cell signaling receptors and is critical for arterial differentiation and maturation of vascular smooth muscle cells in mice. However, murine NOTCH3 experiments did not always translate well to the human organism. A toxic gain-of-function mechanism is supported by the stereotyped nature of CADASIL mutations (see above) and by data showing that CADASIL-mutant NOTCH3ECD aggregates accumulate in the extracellular space of small arteries, arterioles, and capillaries: First, ultrastructural examination of microvessels from CADASIL patients reveals pathognomonic granular osmiophilic material (GOM) that locates to the vicinity of vascular smooth muscle cells and is NOTCH3-

positive on immunogold labeling [76, 77]; Second, NOTCH3 immunostaining of CADASIL microvessels reveals massive immunoreactivity that is specific for this condition [76]; Third, immunoblotting of brain homogenates as well as homogenates from isolated arteries demonstrates accumulation and aggregation of NOTCH3ECD [78]; And fourth, aggregation and accumulation of mutant NOTCH3 can be recapitulated *in vitro* using scanning for intensely fluorescent targets (SIFT), a confocal method allowing the detection of single-protein particles in solution. When the multimerization behavior of NOTCH3-EGF1–5, a fragment encompassing the mutational hot spot for disease-associated mutations, was analyzed, mutant NOTCH3 showed a much stronger tendency to self-aggregate than the wild-type protein [79]. Moreover, mutant NOTCH3 was shown to form mixed multimers with wild-type NOTCH3 and Thrombospondin-2, known to interact with NOTCH3, providing direct evidence for a pathologic co-aggregation mechanism [80]. These findings were recently extended by the demonstration of a co-aggregation between mutant NOTCH3 and latent TGF-β-binding protein (LTBP-1), a key regulator of the TGF-β pathway [81]. Hence, NOTCH3ECD accumulation and deposition, an early process in CADASIL patients [82] as well as in mouse models [83], is now considered a critical step in the pathogenesis of CADASIL [84].

The pathologic processes triggered by NOTCH3ECD aggregation are poorly understood. Monet-Lepretre et al. [85] identified a variety of ECM proteins enriched together with NOTCH3ECD, clearly suggesting a key role of the ECM in CADASIL. From the list of differentially expressed proteins, tissue inhibitor of matrix proteinases 3 (TIMP3) and vitronectin (VTN) were examined in more detail and shown to co-localize with NOTCH3ECD deposits and to interact with NOTCH3 in vitro. Importantly, TIMP3, a major regulator of metalloproteases in the brain, remains biologically active, which might, in part, explain the fibrotic changes within CADASIL arteries. These data support the critical role of ECM proteins in mediating NOTCH3ECD toxicity. Reducing TIMP3 or VTN has proven to be a viable way to weaken disease manifestations in mice [86] suggesting a potential therapeutic approach with these proteins.

CARASIL

A key role of the TGF-β signaling pathway in SVD is suggested by

observations in CARASIL patients as well as cellular and animal models of this condition. A link with TGF-β signaling was already suspected when HTRA1, which is expressed in multiple TGF-β-relevant tissues including the vasculature, skin, and bone, was identified as the affected gene. [87] TGF-β is also abundantly expressed and has multiple biologic functions including a regulatory role in vascular development [68]. Consistent with their role in vascular biology, members of the TGF-β signaling pathway are implicated in Marfan's syndrome and related vascular conditions [88]. HtrA1 has been suggested to inhibit TGF-β in various experimental systems [89]. In accord with this, Hara et al. [87] found signs of increased TGF-β signaling in CARASIL-affected vessels as well as patient skin fibroblasts. This led the authors to propose the lack of TGF-β processing as a critical disease mechanism [90]. Contrasting these findings, a more recent study in HTRA1-deficient mice and CARASIL patient cells revealed a facilitating role of HtrA1 on the TGF-β pathway [91]. The same study showed that LTBP-1 is a substrate for HtrA1. These findings suggest that HtrA1-mediated proteolysis of LTBP-1 promotes release of TGF-β from the ECM. Loss of HtrA1 function either by gene ablation in mice or by CARASIL mutations in humans likely attenuates LTBP-1 processing and TGF-β signaling. Hence, a lack of LTBP-1 cleavage and subsequent reduction in TGF-β release might represent the critical step in CARASIL pathogenesis. Opposing roles of TGF-β have been previously reported in other vascular conditions such as Marfan's syndrome and related conditions [88]. This might relate to differential effects of TGF-β in different cell types including endothelial vs. vascular smooth muscle cells, but could also be a consequence of genetic modifiers of the TGF-β pathway [92].

In summary, genetics should provide a great deal of mechanistic insight into SVD. Current efforts such as whole-exome sequencing and eventually massive parallel sequencing of the entire genome will likely identify multiple additional variants, both common and rare, in yet unknown genes for SVD. However, functional follow-up of such variants in cellular and animal models remains the key for a better mechanistic understanding of SVD and for the development of novel therapeutic approaches to stroke and dementia.

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15. Non-Caucasian Stroke Genetics

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Introduction: Epidemiology of Racial and Ethnic Differences in Stroke

Stroke is the fourth leading cause of death in the United States, but remains the leading cause of death worldwide [1]. Racial and ethnic differences in stroke incidence and subtype may relate to cultural, environmental or genetic variation. Multiple large databases have identified differences in stroke incidence, age of occurrence and outcomes after stroke by race [1]. Here we review the major epidemiologic differences in stroke by race including incidence, presentation age, mortality and differences by subtype are reviewed as a foundation for the subsequent sections.

The Northern Manhattan Stroke Study is a population-based study that compared incidence rates by race/ethnicity and reported that blacks had a 2.4-fold and Hispanics had a twofold increase in stroke incidence compared to whites [2]. Consistent with variation in incidence, stroke mortality rates similarly are higher among minority populations in the United States and worldwide [1, 3, 4]. Disparities in the prevalence of risk factors, particularly hypertension, partially explains these differences. [5, 6] For example, the rates of untreated hypertension in the NHANES study from 2009 to 2010 was greatest among Hispanics (30.4% compared to non-Hispanic whites (24.5%)

and non-Hispanic blacks (20.3%) [7]. Although treatment rates were not different among whites and blacks, controlled hypertension was significantly less frequent among blacks (47.9%) and Hispanics (40.7%) compared to whites (56.3%) [7]. Thus, phenocopies, stroke occurring from non-genetic causes such as untreated hypertension, may lead to false negative searches for genetic risk factors unless proper adjustment is used.

Similar to the NOMAS study, the population-based Auckland Regional Community Stroke Study found that Asians from the same population as Caucasians had higher incidence of strokes with a particularly high rate of intracerebral hemorrhage [8]. Similar to the US study, Caucasians had similar rates of hypertension, but had higher rates of treatment of hypertension.

To summarize, while non-Caucasian variation in the incidence of stroke exists, so too are there differences in risk factor prevalence. While the prior section focused largely on hypertension, numerous other differences such as in diabetes mellitus, hypercholesterolemia or cardiac disease may also exist. While each of these risk factors are likely complex traits with potential genetic components, consideration of the differences in risk factors and treatment should be accounted for when evaluating the variation of findings by race/ethnicity. A ‘stroke gene’ identified among a largely Caucasian population may not be identified among minority populations, not because it is not a genuine risk, but rather because its effect may be overwhelmed by differences in risk factors and treatment.

In endeavoring to summarize non-Caucasian stroke genetics, it should be noted that available genome-wide association studies (GWAS) among minority populations were lacking or faced difficulties in statistical power to detect effects in important stroke subtypes.

Here we report the data based on different stroke subtypes due to the heterogeneity in the pathology of each subtype. The first major division of subtypes of stroke is ischemic and hemorrhagic stroke. Ischemic stroke accounts for 85% of all strokes. Mechanistically, ischemic stroke mostly falls into three groups: small vessel (also known as lacunar), large vessel (both intra and extra-cranial) and cardioembolic subtypes. Hemorrhagic strokes account for the other 15% of all strokes. We have divided hemorrhagic strokes into intracerebral hemorrhage (ICH) and aneurysmal subarachnoid hemorrhage (aSAH). ICH was further divided into deep and lobar hemorrhage. Arteriovenous malformation, traumatic causes, venous occlusions and drug-induced causes of cerebral hemorrhages were excluded.

An exhaustive review of all potential candidate gene and case-control studies was beyond the scope of the current review. Thus, we elected to report on genome-wide association studies of cerebrovascular disease in non-Caucasian populations as well as comparative results between race/ethnic groups. In performing our review, we searched for the terms “gene,” GWAS,” “race,” “ethnicity,” “stroke,” “Ischemic stroke,” “Intracerebral hemorrhage,” “black,” “Asian,” “Chinese,” “Japanese,” “Hispanic,” in PubMed Database. We then classified articles as case-control or meta-analysis studies, heritability studies, twin studies, GWAS studies or population-based studies. Review of abstracts then led to selection of papers that were reviewed and included.

Race, Genetics and Ischemic Stroke

Ischemic stroke is a multifactorial disorder with considerable phenotypic heterogeneity. There are single-gene disorders associated with stroke, although most strokes in the population are polygenic with important environmental contributions. The polygenic contribution to hypertension, type 2 diabetes mellitus, hyperlipidemia and atrial fibrillation have been documented in humans and in animal models. The actual contribution of each of these risk factors may vary between Caucasian and other ethnic and racial groups. For example, blacks have been found to have a lower prevalence of extracranial atherosclerotic disease despite higher rates of hypertension and diabetes mellitus compared to whites, which may be attributable to genetic causes [9]. Furthermore, there is genetic variability in the response to antiplatelet and anticoagulant medication used for stroke prevention. Genes that have been preferentially found to contribute to ischemic stroke risk in non-Caucasians populations are reviewed here.

Genetics of Ischemic Stroke in Black Cohorts

The Consortium of Minority Population Genome-Wide Association Studies of Stroke (COMPASS) was the first GWAS study conducted with the purpose of identifying stroke susceptibility loci in minority populations [10]. In 2015, a meta-analysis of GWAS data including 14,746 African-Americans identified a novel genome-wide significant association with total stroke (rs447161; $p = 3.9E{-}8$) among African-Americans. This novel locus is near

the aquaporin 9 gene, previously implicated in cerebral energy metabolism and brain ischemia, the aldehyde dehydrogenase 1 family, member A1 and hepatic lipase genes. [10] Although this SNP was not significantly associated with ischemic stroke in the largely Caucasian METASTROKE analyses, it represents an important initial finding, which requires independent replication. In addition, four of seven previously reported loci (PITX2, HDAC9, CDKN2A/CDKN2B and ZFHX3) were associated with stroke among African-Americans.

Other genetic variants have been associated with stroke in the black population. Genetic variations in low-density lipoprotein receptor related proteins 1 and 6 (LRP-1 and LRP-6) have been associated with stroke, migraine, abdominal aortic aneurysm and effectiveness of statins [11]. In the Ischemic Stroke Genetics Study (ISGS), a case-control study, LRP1 was associated with ischemic stroke risk in the black cohort (OR 1.89; p 0.006) but not the Caucasian cohort [12].

To summarize, a novel susceptibility locus has been identified utilizing a large meta-analysis of African-Americans along with replication of identified findings among Caucasian populations.

Genetics of Ischemic Strokes in Asian Cohorts

Large cohorts of stroke patients in Asia have provided insight into specific SNPs that predispose this population to ischemic stroke (Table 15.1). Here we have summarized the results of GWAS studies in specific Asian populations (e.g., Chinese Han, Japanese, Korean, Taiwanese). Exome sequencing was used to screen susceptibility loci in a GWAS study of ischemic stroke among 100 cases and 100 matched controls in a Chinese Han population, which identified two SNPs, rs10489177in C1orf156 gene on Ch1q24, and rs17118 in the XYLB gene on Ch3p21. Both SNPs were subsequently validated in a second cohort [13].

Table 15.1 Genetic contributions in ischemic stroke in non-Caucasian populations

Population	Gene	SNP	Location	Reference
Japanese, Chinese	AGT	rs699	1q42–43	Fukamizu et al. [41]
Japanese	ACE (OR 1.74, CI 0.88–3.42)			Wang et al. [32]; Ariyaratham et al. [19]
Chinese	ACE (OR 1.90, CI 1.23–2.93)			Ariyaratham et al. [19]

Jap/Chinese pooled	ACE (OR 1.82, CI 1.28–2.60)			Ariyaratham et al. [19]
Japanese	PDE-4D			Nakayama et al. [37]
Blacks	LRP-1	rs11172113		Harriett et al. [12]
Chinese	APOE-e4 (OR 2.18, CI1.52–3.13)			Ariyaratham et al. [19]
Japanese	APOE-e4 (OR 1.51, CI 0.93–2.45)			Ariyaratham et al. [19]
Chin/Japan pooled	APOE-e4 (OR 1.77, CI 1.30–2.39)			Ariyaratham et al. [19]
Japanese	PRKCH	rs2230500 (V374I)		Kubo M et al. [42]
Japanese	AGTRCI	rs9943582		Kubo M et al. [42]
Chinese	MTHFR (OR 1.18, CI 0.90–1.56)			Ariyaratham et al. [19]
Korean	MTHFR (OR 1.34, CI 0.87–2.06)			Ariyaratham et al. [19]
Chin/Korean pooled	MTHFR (OR 1.22, CI 0.98–1.52)			Ariyaratham et al. [19]

In the Japanese population, a genome-wide case-control study using 1112 cases of cerebral infarction and age- and sex-matched controls, a non-synonymous SNP in *PRKCH* was identified using 52,608 gene-based tagging SNPs [14]. The association was significant with lacunar infarction in two independent Japanese samples ($p = 5E{-}7$) [14]. This finding was then replicated in a separate population of silent lacunar infarction among 792 subjects [15]. These polymorphisms appear to be unique to certain Asian populations. The finding was not replicated in a Chinese Han population, although allele frequencies were markedly different [16].

In a separate genome-wide association study of 6341 individuals, CELSR1 was recognized as a susceptibility gene for ischemic stroke in Japanese patients in a GWAS study [17]. The study was replicated in two other cohorts, where two of the polymorphisms within the CELSR1 gene remained significant (rs6007897 and rs4044210) [17].

Verhaaren et al. recently published a study of cerebral white matter hyperintensities from 29 population-based cohorts, which included 21,079 individuals [18]. While a number of novel loci were identified among Asians, these findings did not replicate in European, Hispanic and Black subgroups and the direction of association was in the opposite direction [18]. Similarly,

several genes that have been implicated in ischemic stroke in Caucasians (i.e., angiotensin I converting enzyme (ACE), 5,10-methylenetetrahydrofolate reductase (MTHFR) and plasminogen activator inhipitor-1 (PAI-1) SERPINE1) were not found to have a significant association with ischemic stroke in populations of Chinese, Japanese or Koreans [19.].

The Risk Assessment of Cerebrovascular Events (RACE) study includes GWAS data from 1322 cases and 1143 controls and was included as a replication cohort in a METASTROKE paper [20]. Reported associations were consistent across studies, although study-specific p-values were not provided.

To summarize, several large GWAS in Asian populations have identified several novel associations.

Genetics of Ischemic Strokes in Hispanic Cohorts

Although Hispanics have been included as replication samples in stroke GWAS studies and were included in large cohort studies, the results of a Hispanic-specific GWAS effort are not currently available. Even less data is available for genetic polymorphisms in the Hispanic stroke population. However a series of studies utilizing a Caribbean Hispanic subset of the Northern Manhattan Study (NOMAS) examined genetic contributions to carotid intima-media thickness (IMT) as a marker for large vessel stroke using linkage analysis [21]. Heritability ranged from 0.41 to 0.65 across various carotid segments. LOD scores >2 were found on chromosomes 7p and 14q. In comparison, there was no genome-wide significant association for carotid IMT in the NHLBI Offspring Cohort of the Framingham Study, a primarily white cohort.

Comparative Results of Ischemic Stroke Genetics by Specific Candidate Gene

APOE E2, E3, E4. The APOE gene encodes a protein called apolipoprotein E, which combines with lipids to form molecules called lipoproteins [22]. These lipoproteins are responsible for packaging cholesterol and other fats and removing them from the bloodstream. There are three major alleles: ε2, ε3 and ε4. The prevalence of specific alleles is associated with ischemic and hemorrhagic stroke and other pathological processes, such as dementia and macular degeneration [23–25]. There are ethnic variations in the distributions

of the alleles [26], which confer important information about genetic stroke risks. For example, the APOE ε4 allele variant is associated with increased risk of developing atherosclerotic disease, acute coronary syndrome, and stroke. A meta-analysis of 54 studies looking at overall genetic effects of APOE polymorphisms and risk of ischemic stroke in the Chinese population found that the ε4 allele, but not the ε2 allele, was associated with increased risk of ischemic stroke compared to the ε3 allele (OR 2.19, 95% CI 1.90–2.51) [27]. This is further supported by a large meta-analysis of Asian ischemic stroke genetics showing a significantly increased odds of ischemic stroke in Chinese and pooled Chinese and Japanese cohorts, which was not seen in Caucasian controls [19].

Hypertension is an important cause of stroke, and therefore genes that influence blood pressure regulation are frequently proposed as candidates for *causing* ischemic stroke. In Asian and black populations, hypertension, more so than atherosclerosis, is implicated as a risk factor for ischemic stroke. The renin-angiotensin-aldosterone system (RAAS) is involved in blood pressure regulation, vascular remodeling, and the atherosclerotic process [28], and has been associated with ischemic stroke risk [29]. There are several polymorphisms in various proteins throughout the RAAS system that have either been associated with hypertension and/or stroke (i.e., CYP11B2 polymorphism of aldosterone and AT1R mutation in the angiotensin II type 1 receptor. A meta-analysis of ACE insertion/deletion (ACE I/D) polymorphism in ischemic stroke confirmed not only that the polymorphism is a marker of susceptibility of ischemic stroke, but that ethnicity contributed to its heterogeneity of effect [30]. Similarly, angiotensinogen (AGT M235T) polymorphism has also been associated with elevated blood pressure and ischemic stroke [31] in Asians. In Han Chinese, a meta-analysis of 58 studies (7168 ischemic stroke cases and 5944 controls) suggested that both the AGT M235T (TT genotype) and ACE I/D polymorphisms contributed to stroke risk [32]. Interestingly, in a Japanese population, it was the M genotype of the AGT gene that was associated with lacunar infarcts, but not hypertension [33] by comparison, in Caucasian populations, no relationship between the AGT polymorphism and ischemic stroke risk was found in two separate meta- analyses [34, 35].

In vascular smooth muscle cells, **phosphodiesterase (PDE)** breaks down cAMP resulting in increased systemic vascular resistance and increased blood pressure. Overexpression or over-activity of phosphodiesterase 4D may cause

increased smooth muscle proliferation and immune functions resulting in increased atherosclerosis. Phosphodiesterase 4D polymorphisms (PDE-4D; various haplotypes and SNPS), initially identified in a genome-wide screen for stroke susceptibility genes in an Icelandic population [36], contributes to the risk of stroke in carriers independent of conventional risk factors such as hypertension and diabetes [29]. The gene itself and regulators of its contribution to ischemic stroke risk varies by population. The PDE-4D haplotype also varies between populations, as reviewed by Munshi and Kaul [29]. For example, in the Japanese population, in addition to the PDE-4D gene, there is another region within the STRK 1 locus that confers susceptibility to ischemic stroke [37].

Single-Gene Disorders Associated with Ischemic Stroke

Of the handful of single-gene mutations associated with stroke, few have been studied specifically in non-Caucasian populations. The most well studied polymorphism is the substitution of valine for glutamic acid in the β-chain of hemoglobin, encoded by the HBB gene, which causes sickle cell anemia. However, there are multiple genetic mutations within the HBB gene that are now known to cause the same phenotype. Blacks who are homozygous for the HgbS allele are at 200 to 400 times the risk for stroke – initially ischemic, but later in life hemorrhagic stroke. Sickle cell trait (SCT), the heterozygous carrier state of sickle cell anemia has a heterozygous allelic frequency of 7 to 9% in blacks and 0.2% in non-Hispanic whites. Within the Atherosclerosis Risk in Communities (ARIC) population, carriers of the SCT were 1.9 times more likely to have an ischemic stroke compared to HgbAA controls [38].

Another notable single-gene disease-causing mutation is the HTRA1 mutation, which causes cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy (CARASIL); an autosomal recessive disease similar, but more severe than, cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL). The mutation was first found in individuals of Asian descent [39], but has since been found in patients of European descent as well [40].

(C85 gene—homocysteinuria, Mitochondria DNA—MELAS, FBN-1—Marfan Syndrome, COL3A1 genes—Ehlers—Danlos Syndrome Type IV,

ABCC6—Pseduoxanthoma elasticum). No strong evidence found for racial/ethnic disparities.

Race, Genetics and Intracerebral hemorrhage (ICH)

Intracerebral hemorrhage (ICH) accounts for about 15% of strokes annually in the US and up to 33% in Asian populations. Thirty-day mortality rates vary from 40 to 50% [43]. Overall, the incidence of ICH is higher in nonwhites compared to whites [43, 44] and the age of presentation is younger for blacks and Chinese (62 vs. 69 years) compared to Caucasian populations. Risk factors vary by location (deep vs. lobar) and have both environmental and genetic risk factors [45]. Heritability studies suggest a substantial contribution to risk and outcome of ICH [46]. APOE polymorphisms ε2 and ε4 are established risk factors for ICH[47] and ICH recurrence [48]. Their involvement in deep and lobar ICH specifically in black, Asian and Hispanic populations, along with other candidate genes conferring risk for ICH will be reviewed here.

Genetics of Intracerebral Hemorrhage Associated with Deep Hemorrhages

The most important risk factor for deep ICH is hypertension [45]; therefore genetic predisposition for HTN may increase the risk for deep ICH. Using a discovery phase of 1545 cases and 1481 controls, the 1q22 locus, SNP rs1052053, was found to be associated at genome wide levels of significance for deep ICH [49]. Replication studies were performed in racially and ethnically diverse populations with replication occurring in black but not Hispanic populations [49]. The region and SNP have been found to be a genome-wide significant association for white matter hyperintensities detected on magnetic resonance imaging [50].

Although the APOE locus has been known to be associated with lobar ICH for some time, the APOE-ε4 allele has also been associated with deep ICH [47]. In the Perindopril Protection Against Recurrent Stroke Study (PROGRESS), a randomized, double-blind, placebo-controlled study including patients of European and Asian descent, both APOE-ε2 and Apo-ε4 alleles were significantly associated with both deep and lobar hemorrhages, independent of blood pressure. The role of the ε2 and ε4 polymorphisms in

conferring risk for ICH was larger in the Asian cohort compared to the European cohort [51].

Genetics of Intracerebral Hemorrhage Associated with Lobar Hemorrhages

Lobar ICH is associated with cerebral amyloid angiopathy (CAA) and has increasing incidence with age and is associated with APOE ε2 and ε4 alleles in whites and blacks [52]. In a meta-analysis of 18 studies containing 2018 cases and 2143 controls in a Chinese population, e4, but not e2 was associated with ICH [27]. An association was not found in elderly Japanese, suggesting either Type I error or possible racial heterogeneity among Asian populations [53].

Race, Genetics and Aneurysmal Subarachnoid Hemorrhage (aSAH)

Aneurysmal SAH (aSAH) affects about 16,000 Americans annually. The mortality is almost 40% within 30 days [54], and this particular stroke subtype affects the youngest patient population. Risk for intracranial aneurysm (IA) formation is complex, with both genetic factors and environmental factors such as smoking and hypertension. Across geographic regions, family history has shown to be one of the strongest known predictors of aSAH [55–57], such that having a first-degree relative with aSAH confers a sevenfold risk of SAH over the general population [58]. Many of these studies were completed primarily in Caucasian populations, or in mixed populations, with race-matched controls.

Incidence of aSAH varies by race [59] with most epidemiological studies suggesting an increased risk in African Americans [43, 60] and Japanese [61] compared to Caucasians [62]. Although sample sizes were small (43 white vs. 64 Mexican Americans), aSAH was shown to affect Mexican Americans disproportionately compared to Caucasian Americans in the Brain Attack Surveillance in Corpus Christi [63]. Studies using Asian subjects have shown racial disparities in ICA genetics in Chinese, Japanese and Korean populations [64]. Candidate genes for aSAH are summarized in Table 15.2. In this section we have summarized these findings in black, Asian and Hispanic cohorts.

Table 15.2 Genetic contributions to intracranial aneurysm formation and rupture in non-Caucasian populations

Population	Gene	Linkage region	Location	Reference
Japanese	Elastin	D7S2472	7q11	Onda et al. [70]
Japanese, Korean	LIMK1	D7S2472	7q11	Akagawa et al. [75]
Japanese, Korean	Endoglin		9	Takenaka et al. [65]; Joo et al. [66]
Japanese, American, Polish	No-endoglin locus			Onda et al. [67]; Peters et al. [68], Pera et al. [69]
Japanese	LOXL2		5q31	Akagawa et al. [75]
Chinese	Kalikreins			Suo et al. [74]
Japanese	ITM2C	Rs3111754		Yoshida et al. [76]
Japanese	MAPKAP1	Rs10986769		Yoshida et al. [76]
Chinese	APOE-e4			Chen et al. [77]

Genetics of aSAH in Asian Cohorts

The endoglin gene, which forms the transforming growth factor β-binding protein on the surface of human vascular endothelial cells, has been associated with SAH in Japanese and Korean populations. Takenaka et al. initially reported an association between aneurysm development and a SNP within intron 7 of the endoglin gene found higher rates in cases than controls within a Japanese population [65]. A polymorphic variant of the endoglin gene was also associated with IA formation in a hospital-based Korean population, but the insertion-deletion in intron 7 of the gene was not the culprit [66]. However, follow up study by Onda et al. did not find this association in Japanese patients [67]. Endoglin gene was not associated with aSAH in other populations of Pennsylvanian Americans [68] or Polish populations [69].

Elastin (*ELN*), a protein found in connective tissue that allows blood vessels and other tissues to resume their shape after stretching or contracting, has been of great interest in the study of cerebral aneurysm genetics. We have found racial and ethnic disparities in genetic mutations of elastin or genes interacting with the protein. In a Japanese study of sibling pairs, 14 distinct SNPs were identified in the region of the elastin gene, with homozygous patients incurring a 4.39 OR of IA formation (P = 0.002) [70]. This was not replicated in a Caucasian population were eight SNPs within the elastin gene had similar allele frequencies and genotypes in Caucasian cases and controls.

In fact, allele frequencies in control populations differ between Caucasians and Japanese [64]. The lysyl oxidase-like (LOXL) family of genes, which catalyzes cross-linking of collagen and elastin by oxidative deamination of lysine residues, has been studied for their involvement with IA as well. Specifically, the LOXL-2 gene was found to be independently associated with familial IA in Japanese patients. In this population, gene-gene interactions with ELN and LIM kinase 1 (LIMK1) were also significant [71]. In a study focused on the Dravidian Malayalam speaking population of South India, the genotype distribution of LOX variants was markedly different compared to Caucasian populations. There was no association between LOX polymorphisms and IAs was found in this population [72].

In a large, well-controlled GWAS and subsequent replication study of 2431 aSAH/IA subjects and 12,696 controls, there was a significant association between SNP rs6842241, located 1.25 kb upstream from the ENDNRA gene encoding endothelin receptor A, and aneurysm. This finding is clinically interesting because of the role of endothelin-1 on its receptors in IA pathophysiology [73].

Kalikreins were initially found to be risk factors for IA in a Finnish cohort. A follow-up case-control study investigating two SNPs (rs1722561 and rs1701946) in Chinese Han found that the C allele of rs1722561 imparted a decreased risk of sporadic IA [74]. APOE ε4 is associated with increased SAH risk in Chinese [27].

Conclusion

Within the predominately Caucasian genome-wide association studies reported to date, sample sizes of tens of thousands of individuals have been required to identify robust associations, which replicate in independent populations. Such sample sizes have not yet been available for non-Caucasian populations, which offer challenges with respect to risk factors, sample size and availability as well as testing completeness and thereby subtype definition.

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16. Cerebral Venous Thrombosis: Genetic Aspects

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Background

Cerebral venous thrombosis (CVT), an unusual form of stroke affecting the venous circulation rather than the more common arterial circulation, is being diagnosed with higher frequency due to increased awareness and ready access to MR. Multiple conditions have been reported in association with CVT. Risk factors and associated conditions for CVT can be categorized as permanent or transient. The most frequent permanent risk factors are genetic prothrombotic disorders. More than half of CVT patients have more than one associated condition or risk factor, in general a combination of a permanent (e.g. genetic prothrombotic disorder) with a transient risk factor (e.g. oral contraceptives, infection). Several publications from different regions of the world, systematic reviews and meta-analysis demonstrated that inherited thrombophilia increases the risk of CVT, especially in children. The most common genetic thrombophilic disorders which are important risk factors for

CVT are Factor V Leiden and prothrombin G20210A mutations and protein C, protein S and antithrombin deficiencies. Routine screening of thrombophilia in CVT patients is not recommended. Thrombophilia screening may be performed in patients with high pre-test probability to carry severe thrombophilia (i.e., a personal and/or family history of venous thrombosis, young age at CVT, CVT without a transient or a permanent risk factor) to prevent recurrent venous thrombotic events.

Introduction

Cerebral venous thrombosis (CVT) is a type of stroke affecting the dural sinus and the encephalic veins. CVT is less frequent than ischemic stroke or intracerebral hemorrhage, but nevertheless its incidence is comparable to that of acute bacterial meningitis in adults [1]. CVT is more frequent in developing countries, with high pregnancy rates. CVT affects predominantly neonates, children, young adults, and females. CVT is now being diagnosed with higher frequency due to increased awareness and ready access to MR in developed countries. CVT have a more diverse clinical presentation than other stroke types, and may present as isolated headache, intracranial hypertension syndrome, seizures, focal lobar syndrome, encephalopathy or coma. The confirmation of the diagnosis of CVT depends on the demonstration of thrombi in the cerebral veins and/or sinuses by MR/MR venography or CT venography [2]. Multiple conditions have been reported in association with CVT [3] . For some of these conditions there is evidence from case-control or cohort studies that they increase the risk of CVT. Risk factors and associated conditions for CVT can be categorized as either permanent or transient. The most frequent permanent risk factors are genetic prothrombotic disorders and diseases associated with a prothrombotic state, such as antiphospholipid syndrome, nephrotic syndrome and cancer. Examples of transient risk factors are oral contraceptives, puerperium and pregnancy, infections, head trauma and drugs with a prothrombotic action. More than half of CVT patients have more than one associated condition or risk factor, in general a combination of a permanent (e.g. genetic prothrombotic disorder) with a transient risk factor [4]. The prognosis of CVT is in general favorable, with about 4% of deaths in the acute phase and a total of 15% of the patients remaining dependent or dying [4]. The antithrombotic treatment in the acute phase consists on anticoagulation with

either low molecular weight or unfractionated heparin. Those patients presenting in severe condition on admission or who deteriorate despite anticoagulation, local thrombolysis or thrombectomy are an option. The use of thrombolysis/thrombectomy are being evaluated in the TO-ACT randomized clinical trial [5]. Decompressive hemicraniectomy is occasionally necessary to prevent death from herniation in patients with large intracranial venous infarcts or hemorrhages [6]. After the acute phase patients should remain anticoagulated with vitamin K antagonists for a variable period of time, depending on their inherent thrombotic risk and other risk factors for recurrence of venous thrombotic events [7].

Predisposing Genes for CVT: General Aspects and Methodological Issues

The genetic underpinnings of CVT have been investigated using a candidate gene approach. These association studies have focused on a very limited set of genes and polymorphisms previously associated with venous thrombosis outside the brain, such as genes belonging to the coagulation and fibrinolytic systems. A meta-analysis of 26 cases-control studies testing the association of six genes in 1183 CVT cases and 5189 controls of European descent [8] found significant overall association for factor V Leiden (G1691A), prothrombin G20210A and methylene tetrahydrofolate reductase (MTHFR) C677T, with odds ratio ranging from 2.3 to 5.5 and no evidence of interstudy heterogeneity. The other three SNPs (4G/5G in plasminogen activator inhibitor-1, V617F in Janus kinase-2, and G79A in protein Z) were investigated in a smaller number of studies and their meta-analyses revealed no association with CVT [8]. Another meta-analysis of 20 case-control studies confirmed the association of the factor V Leiden and prothrombin G20210A mutations with CVT, and showed an association between antithrombin, protein C and protein S deficiencies and CVT [9]. However, a meta-analysis of nine case-control studies for the MTHFR C677T variant in 382 CVT cases and 1217 controls from several racial backgrounds found no pooled association with CVT and significant heterogeneity among studies, most likely due to the inclusion of all ethnic origins [10]. Even though there is a remarkable consistency in the association of these thrombophilic factors with CVT, most of the original association studies have limited power (typically less than 100 cases per original report), diverse diagnostic and

exclusion criteria, use of convenience controls [9], or other limitations (e.g. population stratification not assessed, lack of adjustment for co-variates and multiple testing).

About a decade ago, the high throughput and unbiased genome-wide association study (GWAS) approach revolutionized the genetic epidemiology field and led to the identification of unanticipated susceptibility genes and novel pathogenic mechanisms for numerous diseases. Surprisingly, no single GWAS has been conducted to date for CVT, even though over a dozen GWAS for VTE (deep vein thrombosis and pulmonary embolism) were published. The association with CVT of several well-established genetic risk factors for VTE has been extensively tested (e.g. F2 or prothrombin, factor V), while other susceptibility genes have been neglected (e.g. ABO blood group, factor XI [F11], fibrinogen gamma chain [FGG], endotelial protein C receptor [PROCR]). Furthermore, a recent meta-analysis of 12 GWAS for VTE in which the association of 6,751,884 SNPs was tested in 7507 VTE cases and 52,632 controls, identified two novel risk factors for VTE (rs78707713 in TSPAN15 and rs2288904 in SLC44A2) validated in replication datasets [11]. Even if no GWAS for CVT is undertaken in the future, at least genes associated with thrombosis in well-built GWAS [11, 12] should be tested in sizeable CVT datasets.

Since the genetic contributors identified for complex diseases through association studies typically explain a small proportion of the heritability, rare variants with a higher impact in disease risk recently started to be searched using next-generation sequencing (NGS) technologies [13]. Unlike GWAS, NGS allows the discovery and investigation of low-frequency, rare (minor allele frequency <5% and <1%, respectively) or private changes (e.g. single nucleotide variants, insertions, deletions, duplications). Pilot studies for hemostatic/pro-inflammatory genes in relatively small datasets have identified an excess of rare missense mutations in anticoagulant genes (PROC, SERPINC1, and PROZ) and ADAMTS13 in deep vein thrombosis cases when compared to controls [14, 15]. Similar studies should be pursued in the near future for CVT on a candidate gene basis (e.g. genomic regions highlighted by GWAS), at the coding region (exome) or whole-genome level in well-characterized and extended datasets (by definition, rare variants can only be found with large sample sizes). Given that CVT is a complex disease with several environmental factors, a detailed characterization of the study participants is crucial to perform gene-environment studies in adequately-

powered studies.

Genetic Diseases Associated with a Higher Risk of CVT

By thrombophilia we mean diseases, conditions or states in which the risk of arterial or venous thrombosis is increased. Thrombophilia can be acquired, genetic or both and be permanent or transient. Several publications from different regions of the world, systematic review and meta-analysis [9, 16] demonstrated that inherited thrombophilia increases the risk of CVT, especially in children [17].

Potential mutations which modify the function in (a) any of the coagulation proteins participating in the coagulation pathways, or in (b) its regulation by natural anticoagulants or in (c) in the fibrinolytic system can induce venous thrombosis, if such mutation results in a “gain of function” of coagulation proteins or “loss of function” of natural anticoagulants or fibrinolytics (Fig. 16.1). The most common genetic thrombophilic disorders which are important risk factors for CVT are Factor V Leiden and prothrombin G20210A mutations and protein C, protein S and antithrombin deficiencies.

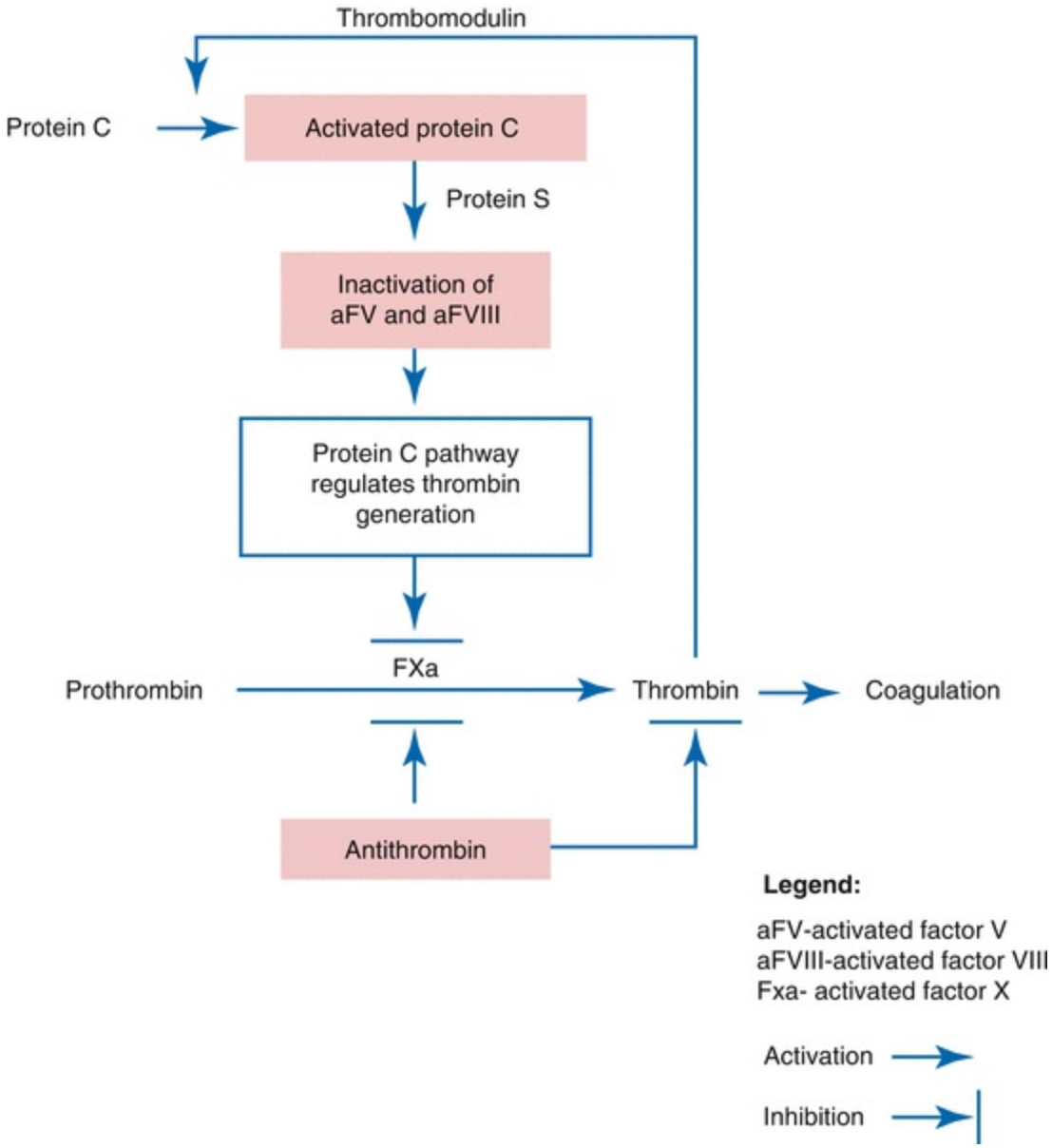

Fig. 16.1 Regulation of the coagulation cascade by circulating anticoagulants

Genetic Thrombophilic Diseases

Genetic thrombophilia is a common risk factor for CVT, being present in 38% of the patients in the International Study of Cerebral Vein and Dural Sinus Thrombosis (ISCVT) [4]. A summary of the main genetic causes of cerebral venous thrombosis (CVT) is provided in Table 16.1. A summary of recommendations for testing for thrombophilia and long-term management of patients with a first episode of CVT is described in Table 16.2.

Table 16.1 Main genetic causes of cerebral venous thrombosis

Disorder	Gene	Inheritance	Prevalence in the general population	Prevalence in patients with CVT (%)	Relative risk of CVT (OR)
Protein C deficiency	PROC	AD	1 in 500 [186]	3 [31] to 8 [44]	11.1 [16]
Protein S deficiency	PROS1	AD	0.026–0.13% (Caucasians) [107] 2% (Japan) [108]	5 [44] to 7.5 [4, 30]	12.5 [16]
Antithrombin deficiency	SERPINC1	AD	1 in 500 to 1 in 5000 [135, 187]	0.9 [4, 30] to 2 [44]	2.7 [16]
Factor V Leiden	F5	AD	5% (carrier, Caucasians) [41]	7.5 [4, 30] to 9 [31]	2.4 [8]
Prothrombin 20210A	F2	AD	2–3% [18, 22, 188]	8 [4, 30] to 20 [31]	5.5 [8]
Hyperhomo-cysteinemia (mild)	MTHFR[a]	AD	6–18% (Caucasians) [152]	10 [4, 30] to 13 [31]	2.3 [8]

[a]677T allele homozygosity

Table 16.2 Summary of recommendations for thrombophilia testing and long term management in patients with a first CVT episode (according to the American Stroke Association guidelines for the Diagnosis and Management of Cerebral Venous Thrombosis [7] and the general recommendations for patients with first venous thrombotic event from the British clinical guidelines for testing for heritable thrombophilia [137]

Disorder	Testing in patients with CVT (Baglin et al. [137])	Long term anticoagulation (AC) (Saposnik et al. [7])
Protein C deficiency	Patients with high pre-test probability	Indefinite AC may be considered
Protein S deficiency	Patients with high pre-test probability	Indefinite AC may be considered
Antithrombin deficiency	Patients with high pre-test probability	Indefinite AC may be considered
Factor V Leiden heterozygote	Patients with high pre-test probability	Follow standard recommendation (3–12 M)
Factor V Leiden homozygote	Patients with high pre-test probability	Indefinite AC may be considered
Prothrombin 20210A heterozygote	Patients with high pre-test probability	Follow standard recommendation (3–12 M)
Prothrombin 20210A homozygote	Patients with high pre-test probability	Indefinite AC may be considered
Hyperhomocysteinemia (mild)	Patients with high pre-test probability	Follow standard recommendation (3–12 M)

Combined defects	–	Indefinite AC may be considered

AC anticoagulation, *M* months

Mutations and Polymorphisms in Coagulation Proteins

Prothrombin Mutations

The prothrombin 20210A mutation was described in 1996 and associated with higher prothrombin levels, leading to an increase in the risk of venous thrombosis [18]. As the G20210A mutation is present outside the coding region for prothrombin (3′ untranslated part of the gene), it does not affect neither the actual structure of the prothrombin molecule nor its function as a strong clotting factor when activated into thrombin. Nonetheless, this gain-of-function mutation was found to lead to a 30% higher plasma prothrombin level due to an increased mRNA and protein expression of prothrombin [19]. Another possible described mechanism for the thrombophilic effect of this mutation was the increase in thrombin-activatable fibrinolysis inhibitor (TAFI) caused by the increased prothrombin levels [20].

Prothrombin G20210A mutation is one of the most common defects associated with thrombophilia in Caucasians. Like in the FV Leiden mutation, a strong linkage disequilibrium was established, suggesting a founder effect [21]. Accordingly, the variation of the prevalence of the prothrombin mutation is dependent on the geographic location and ethnic background [22]. The rough prevalence in Caucasians is estimated to be 2–3% but is found in about 6–8% of patients with venous thrombosis, with a higher prevalence in southern than northern European countries [18, 22]. The mutation is rare in Asian, African or in native populations of America and Australia, with exception to Middle East and North Africa countries, in which the prevalence resembles that of European countries.

Although the plasma prothrombin concentrations may be slightly increased, they often fall within the normal range and cannot be used for screening. Polymerase chain reaction (PCR) methods have been used to detect the G20210A prothrombin gene mutation in genomic DNA.

Prothrombin 20210A is considered a moderately strong risk factor for venous thrombosis. Heterozygous carriers have a threefold increased risk of venous thrombosis [18, 23]. The thrombotic risk in patients homozygous for the G20210A prothrombin gene mutation appears to be substantially higher

than that in heterozygotes. As FVL is also a common mutation, compound heterozygotes of FVL and prothrombin mutation are not exceedingly rare (estimated prevalence one in 1000 individuals in the general population) [24]. These patients have a 20-fold increased risk of thrombosis compared to individuals with neither mutation and a 2.6-fold higher risk of recurrent thrombosis than carriers of FVL alone [25]. An increased risk of thrombosis was also observed in patients with G20210A prothrombin gene mutation and other concomitant prothrombotic defects as antithrombin, protein C or protein S deficiency [26]. As in other types of thrombophilia, association with transient precipitant factors is also commonly associated with the venous thrombotic events. Pregnancy and exposure to oral contraceptives are two common conditions associated with the venous thrombotic events [27].

The G20210A prothrombin gene mutation was identified as a risk factor for CVT soon after its description, with an estimated fivefold age-adjusted odds ratio [28, 29]. A similar risk was reported in a more recent systematic review 15 studies including 646 CVT cases and 3690 controls, in which a significant association of prothrombin G20210A with CVT was found with an OR of 5.5 (95% CI, 3.88–7.74) and an estimated population attributable risk for adult CVT of 14% [8]. Prothrombin mutation was detected in 8.4% of the ISCVT cohort [4, 30], but proportions as high as almost 20% of the included patients were identified in large retrospective CVT cohorts [31, 32]. Several CVT studies found that the risk is particularly higher in women receiving oral contraceptives, due to a synergistic interaction between this risk factors [27, 33].

Prothrombin mutation was also confirmed to be a risk factor for first childhood stroke, including CVT, in a systematic review in which the pooled OR for carriers of the polymorphism was 2 [17].

Factor V Leiden Mutation

Activated protein C (APC) is the key component of the protein C anticoagulant pathway. APC with its cofactor protein S acts by cleaving and thus inactivating factors Va and VIIIa [34]. APC also indirectly promotes fibrinolysis [35]. The relatively long half-life of APC in vivo (15–20 min) is a prerequisite for its function as a circulating anticoagulant. More recently, APC has been shown to exert significant anti-inflammatory and neuroprotective effects in several acute and chronic inflammatory diseases, including in animal models of stroke and CVT [36].

When it was first described, in 1993, this defect was termed “APC resistance” (OMIM #188055) based on the observation that the anticoagulant activity of APC was reduced in a modified activated partial thromboplastin time (aPTT) assay in patients from thrombosis prone families [37]. The molecular explanation underlying this observed phenotype was subsequently provided with the identification of one mutation in the Factor V gene, which was called Factor V Leiden (FVL) [38]. FVL results from a point mutation in the factor V gene which substitutes G (codon CGA) with A (codon CAA) at nucleotide 1691 in exon 10 (G1691A) and that causes an aminoacid substitution (an arginine to glutamine substitution) at position 506 in factor V (Arg506Gln) [38]. Although the mutant molecule expresses normal procoagulant activity when activated by thrombin or factor Xa, this mutation abolishes a cleavage site of APC, making the molecule tenfold less susceptible to inactivation [39]. Therefore, the molecule persists longer time in the circulation, resulting in increased thrombin generation and a mild hypercoagulable state [40].

Other Factor V polymorphisms have been associated with thrombosis, particularly the A4070G variant. However, the associated with venous thrombotic events has been shown to be weak in a large meta-analysis (OR 1.24) [23].

FVL is the most common genetic prothrombotic defect in Caucasians, with an overall prevalence of carriers in the general population around 5% [41]. More than 95% of cases of APC resistance are due to the FVL mutation. In unselected patients with first-episode thrombosis it is found in about 20–25% of the cases and in patients with hereditary thrombophilia the proportion increases to almost 50% [42]. However, the prevalence of the mutation is lower in the Middle East and India [43, 44] and is extremely rare in Asian, African and indigenous Australian populations. Even within Europe there are important geographical variations, with a prevalence that varies from 10 to 15% in southern Sweden and Greece to 2–3% in Italy and Spain [45]. Due to the high prevalence of this mutation, homozygosity for FVL occurs in about 1 per 5000 in the Caucasian population. A possible explanation for this world distribution is that FVL arose from a founder mutation after the evolutionary separation of Caucasian from Asians and Africans, more than 20,000 years ago [46]. The high prevalence of the mutations suggests the heterozygous state may be associated with some survival advantage, probably a reduction in bleeding.

The diagnosis of FVL thrombophilia usually involves a coagulation screening test or a DNA analysis of factor V to identify the Leiden mutation. The APC resistance assay involves performing an APTT on the individual's plasma in the presence and absence of a standardized amount of exogenous APC. The newer second generation assays can be used to test plasma from patients receiving anticoagulation. This test has a sensitivity and specificity for FVL approaching 100% [47]. Molecular genetic testing for FVL is performed by a variety of comparable methods using genomic DNA in peripheral blood mononuclear cells [48]. Molecular genetic tests are reliable in individuals on warfarin or heparin anticoagulation and during acute thrombotic episodes. DNA-based testing is recommended as first evaluation in individuals on anticoagulants that interfere with APC resistance results (e.g., dabigatran, argatroban, bivalirudin, rivaroxaban), with strong lupus inhibitors and a prolonged baseline aPTT and those with very low or borderline APC resistance assay values [49]. Although the modified APC resistance assay is highly sensitive and specific for the FVL mutation, all individuals with a positive screening assay should have the DNA test for confirmation and to distinguish heterozygotes, homozygotes, and "pseudohomozygotes" (heterozygous for both FVL and a second mutation causing Factor V deficiency) [50]. When relatives of individuals known to have FVL are tested, the DNA method is recommended.

Heterozygosity for the FVL allele and the associated risk for venous thrombosis are inherited in an autosomal dominant manner. Homozygosity for the FVL allele has a much greater risk for venous thrombosis.

FVL is a weaker risk factor than deficiencies of other natural anticoagulants but it is also far more common. Although a low absolute risk of thrombosis (0.5–0.6%/year) was found in asymptomatic heterozygous carriers of one FVL G1691A mutation [51], the relative risk of venous thrombosis in these individuals is increased about ninefold [23]. In homozygotes these estimates rise to 80-fold [42]. Therefore, thrombophilia associated with FVL is suspected in individuals with a personal or family history of venous thrombosis particularly when there is recurrence and when it occurs at unusual sites, as the cerebral veins and sinuses [52]. FVL was detected in 7.5% of the overall ISCVT cohort [4, 30–32]. However, this study included mainly Caucasians. An Indian study of 612 patients with CVT detected FVL in only 3% of the patients [44] while a strong association of CVT with FVL was described in a Tunisian cohort in which almost 30% of

patients were carriers of the mutation [53]. As an overall effect, a systematic review of 767 CVT cases and 4020 controls confirmed a significant association of FVL/G1691A with CVT (OR = 2.40; 95% CI, 1.75–3.30; P < 0.00001). The estimated population attributable risk for adult CVT was determined to be 6.8% [8]. Although there are several reports of spontaneous CVT associated with FVL [54], the expression of this thrombophilia is strongly influenced by other genetic and environmental factors. Presence of more than one risk factor was indeed detected in almost half of the patients in ISCVT [4]. Double heterozygoty for prothrombin 20210G>A and FVL was described as occurring in 20–40% of symptomatic 20210G>A heterozygotes [18]. Although there is substantial heterogeneity between studies, a systematic review concluded that patients with double heterozygoty have a sevenfold increase in the relative risk for venous thrombosis. Besides, these individuals seem to have an increased risk of thrombosis on unusual locations, such as the cerebral veins and sinus [25]. Likewise, the association of environmental prothrombotic factors and FVL was shown to have a synergistic effect in the risk of CVT. Particularly, the concomitant presence of FVL and use of oral contraceptives was associated with an OR of 30 [27, 33].

The rate of CVT recurrence in patients with FVL was estimated to be increased about 2.5-fold in a retrospective cohort of 145 patients with long-term follow-up [32]. In a small case-control study of 54 patients with CVT followed for a median of 7 years, one of the three patients with FVL had a recurrent thrombotic event (deep vein thrombosis) [55]. These results are approximate to those from studies in patients with FVL and non-cerebral VTE [24]. The risk for recurrence is higher in FVL homozygotes.

Polymorphisms in Other Coagulation Proteins

A case control study in puerperal Indian women found no association between factor VII R353Q polymorphism and CVT [56]. A case control study in Germany [57] and a linkage analysis in Spain [58] indicated that factor XII gene C46T polymorphism was associated with CVT. This was recently confirmed in a case control-study study in South India [59]. The risk was amplified in women who took contraceptives. Promotor polymorphisms of the plasma glutathione peroxidase (GPx-3), a potent antioxidant enzyme, whose deficiency may cause oxidative modification of fibrinogen, was reported to be more frequent in CVT patients than in controls [60], but this

was not confirmed inanother study conducted in Germany [61].

Mutations in Natural Anticoagulants

Natural anticoagulants limit the location and extent of the thrombus. They include antithrombin, tissue factor plasminogen inhibitor, protein C pathway, which consists of protein C, thrombomodulin, endothelial protein C receptor and protein S and protein Z dependent protease inhibitor (Fig. 16.1).

Protein C Deficiency

Protein C is a vitamin K-dependent serine protease synthesized predominantly in the liver [62]. The conversion of protein C to activated protein C results from cleavage of an Arg-Leu peptide bond, which releases a dodecapeptide. This reaction is facilitated by thrombin, particularly when it is bound to the endothelial thrombomodulin. The activated protein C has an anticoagulant function manifested by its ability to proteolytically inactivate Factor V and Factor VIII. These events are enhanced by cofactor protein S, another vitamin-K dependent protein, and also by the presence of Calcium and phospholipids. Activated protein C also has the ability to down-regulate thrombin and suppress thrombin activatable fibrinolytic inhibitor (TAFI) and plasminogen activator inhibitor-1 (PAI-1), therefore upregulating the fibrinolytic system and contributing to maintain a fluid state of blood. The protein C pathway is, therefore, a very important regulator of the coagulation system. Besides, more recently, the protein C pathway has emerged as crucial mediator of inflammation and endothelial barrier protection [63].

Protein C deficiency can be inherited or acquired. Inherited deficiency of Protein C is due to PROC gene (612283) mutations and usually shows an autosomal dominant pattern of inheritance with variable penetrance. Two main techniques are available to measure protein C levels: immunoassays, which measure the antigen, and functional assays, which measure the activity. A functional assay is preferred for the screening since it detects both types of defects. If the result is low, a protein C antigen assay can be considered to identify the deficiency subtype. Commercially available activity assays are either clotting time based or chromogenic [64]. It is important to note, that lupus anticoagulants, increased factor VIII or direct thrombin inhibitors may affect clotting based assays. The timing of testing is also an important consideration when evaluating patients with suspected

protein C deficiency. Patients should not have received oral anticoagulants for at least 10 days prior to testing. However, if discontinuation of warfarin is not possible due to the severity of the thrombotic diathesis, protein C levels can be studied while the patient is receiving heparin, as it does not alter plasma protein C levels [65]. False positives are uncommon when protein C is measured at the time of acute venous thrombosis [66]. A possible increase of protein C during pregnancy, puerperium and oral contraceptive use has also been reported but these changes are still controversial because they were not observed in all studies [67]. Physiologically decreased levels of protein C also occur in newborns (about 35% of normal adult values). Protein C may remain below normal adult values until 16 years of age [68]. Besides, the relatively wide normal range of protein C measurements in the general population occasionally makes it difficult to identify a given individual as having heterozygous protein C deficiency [69]. Patients with a protein C level less than 55 percent of normal are very likely to have the genetic abnormality if acquired causes of deficiency were excluded. Besides oral anticoagulation, vitamin K deficiency, liver disease, severe infection, septic shock, disseminated intravascular coagulation, acute respiratory distress syndrome, postoperative states or l-asparaginase therapy may also be associated with protein C deficiency. Levels from 55 to 65% are consistent with either a deficiency state or the lower end of the normal distribution [70]. To document the presence of protein C deficiency with confidence, it is often also useful to obtain repeat laboratory determinations and to perform family studies to identify an autosomal dominant inheritance pattern [7]. Some guidelines also still recommend testing at least 6 weeks after a thrombotic event [7]. Patients with factor X concentrations below 50% tend to have also higher activated protein C ratios.

Symptomatic heterozygous deficiencies can result in venous thrombosis that occurs spontaneously or during a transient prothrombotic period such as puerperium. Most patients with heterozygotic protein C deficiency remain asymptomatic during childhood. Still, in the Canadian Pediatric Ischemic Stroke Registry, amongst the 123 CVT patients in which tests for prothrombotic disorders were performed, nine cases of protein C deficiency were found [71]. A meta-analysis of observational studies found an association of childhood stroke (arterial or venous) with protein C deficiency with an odds ratio of 8.8 [17]. Patients from thrombophilic families tend to have more severe phenotypes than patients carrying the same defect but who

do not come from such families. The lifetime risk of developing thrombosis in patients with heterozygous deficiency of protein C is estimated as being about sevenfold [72]. The risk is higher in symptomatic families. In a study using unaffected relatives as the reference group, the risk for venous thrombotic events among carriers of protein C deficiency was 24-fold greater than that in non-carriers (1.52% per individual-year). A meta-analysis of 11 case-control and cohort studies including 2554 cases and 9355 controls estimated an overall odds ratio (OR) for venous thrombosis in patients with hereditary protein C deficiency of 7.5 (95% CI 3–18) [73]. There is a gradient of thrombosis risk according to protein C levels. Protein C levels in heterozygous individuals usually range between 35% and 65% of normal [74]. Up to 15% of heterozygotes may have protein C activity levels within the normal range [75]. The annual incidence of recurrent venous thromboembolism in patients with protein C deficiency was estimated to be about 6.0% (3.9–8.7) [76]. The risk appears, therefore, to be increased in comparison to the annual risk of recurrence in the general population with first venous thrombotic event.

Although the deep veins of the legs are the most common sites of disease in patients with protein C deficiency, several studies have shown an association of this type of thrombophilia with CVT. In a large multinational prospective cohort of adult patients with CVT in which about 70% of the patients received screening for genetic prothrombotic conditions, 5% had protein C deficiency. In most of these patients (80%) another condition predisposing for CVT was found, particularly transient risk factors [4, 30]. In a regional Indian study of 612 patients with CVT referred for thrombophilia work-up protein C deficiency was detected in 10% of the males and 7% of the women [44]. In a meta-analysis of two studies analyzing the role of protein C deficiency as a risk factor CVT a significant odds ratio of 11 was found, although with a large confidence interval [16].

Data regarding the specific recurrence rate of venous thrombosis in patients with prior CVT and protein C deficiency are scarce and a large proportion of the patients included in cohorts with long-term follow-up were receiving anticoagulation. Stefano et al. [77] and Vossen et al. [78] described a low annual incidence around 1% for individuals with non-cerebral venous thromboembolism and protein C deficiency receiving long-term anticoagulation, suggesting that prophylaxis is effective in reducing the risk of recurrent thrombosis in patients with familial protein C deficiency.

However, severe thrombophilia (including protein C deficiency) was a risk factor for recurrence in a cohort study evaluating the recurrence of venous thrombosis after CVT after discontinuation of oral anticoagulation [32].

Homozygous protein C deficiencies are exceedingly rare and usually lead to life-threatening prothrombotic diathesis in newborns, namely fatal systemic disseminated intravascular thrombosis along with purpura fulminans [79]. Deficiency of natural coagulation inhibitors, particularly protein C deficiency, also carries an increased risk for warfarin-induced skin necrosis [80]. This skin condition typically occurs during the first days of warfarin therapy in association with large loading doses and is induced by a transient hypercoagulable state but reports of cases occurring beyond 10 days and up to 15 years after initiation of treatment can be found [81]. The dermal manifestations are similar to purpura and occur mostly on areas with increased subcutaneous fat (abdomen, legs, breasts). If treatment with heparin, vitamin K, fresh frozen plasma or protein C concentrate is not rapidly administered, petechiae progress to ecchymoses and hemorrhagic bullae within 24 hours and become necrotic [81, 82].

Protein S Deficiency

Protein S was first discovered in Seattle in 1977 and named after the city of its discover [83]. It is a vitamin K-dependent anticoagulant protein mostly synthesized by the hepatocytes and other sources such as endothelial cells. Human protein S is a single-chain glycoprotein containing 635 amino acid residues. The mature protein S is extensively post-translationally modified and is composed of multiple domains. Protein S plays an important role in the regulation of coagulation, acting as a cofactor to activated Protein C in the proteolytic inactivation of procoagulant factors Va and VIIIa [84]. A direct anticoagulant activity of protein S, independent for activated protein C and mediated by the inhibition of prothrombin formation and proteinase complexes (factor X activating complex), through binding to factor VIII [85] was also described. Approximately 40% of the plasma protein S circulates free in the human plasma, while the remaining 60% circulates bound to C4b-binding protein, a regulator of the classical complement pathway [86]. Although only the free form of the protein has anticoagulant function as cofactor for the activated C protein [87], the complex formed by protein S and C4b-binding protein also have some anticoagulant function, albeit less effective than free protein S [88].

Hereditary protein S deficiency is an autosomal dominant disorder. There are two protein S genes (PROS1 and PROSP) in the human genome but only PROS1 is expressed, whereas PROSP is a pseudogene. Mutations are detected in about 70% the deficient probands [89]. Protein S deficiency is classified based on the levels of total and free antigen and protein S activity [90, 91]. The most common classification, accepted by the International Society on Thrombosis and Haemostasis, differentiates three types of deficits: Type I, in which both free and total protein S levels are decreased, as well as the protein S activity (quantitative deficiency) [92]; type II referring to the decreased protein S activity while total and free antigen levels are within the normal ranges (qualitative deficiency) [93] and type III when only free protein S levels and activity are reduced, but total protein S antigen levels are normal or borderline [94]. More recently, the existence of type III deficiency has been questioned by the finding that both type I and III can be found within the same family, which is considered by some authors as suggestive that the phenotypes are just variants of the same genetic disease [95]. Quantitative (type I/III) deficiencies account for 95% of cases of protein S deficiencies [96]. It has been suggested that the ratio of protein C to total protein S might also be valuable to identify carriers of a PROS1 mutation resulting in quantitative protein S deficiency (type I and type III) [97]. Commercially available immunoassays are used for the determination of total and free protein S and clotting assays to measure the protein S activity (activated protein C cofactor activity). Measurement of free protein S is sensitive to the conditions that affect the concentration of the acute phase reactant C4b-binding protein (pregnancy, oral contraceptive use, acute thrombosis, inflammatory states or cancer), as the increase in the bound form of protein S is associated with a reduction of the unbound free form. Total protein S assays may be more accurate but are only able to detect type I deficiency. Besides, studies investigating members of large kindreds with proven hereditary protein S deficiency have shown that free protein S is more reliable than the total in the discrimination of the carriers and noncarriers [98]. Measurement of protein S activity is subject to multiple interferences, including the presence of activated protein C resistance [99, 100], lupus anticoagulants and high concentrations of prothombin, factor VIIIa or factor VIIa. In addition, measurement of protein S activity is not suitable for the identification of carriers of protein S Tokushima. Acquired causes of protein S deficiency must be excluded in order to diagnose hereditary protein S

deficiency. The most common are hepatic diseases or a vitamin K deficiency but decrease protein S levels have also been associated with pregnancy [101], oral contraception [102] and HIV infection [103]. Therefore, the quantifications should be done after cessation of oral anticoagulation therapy and estrogen contraception or replacement therapy. In patients with indication for prolonged anticoagulant therapy the best strategy to detect protein S deficiency is to give low molecular weight heparin for 2–4 weeks before blood sampling, instead of vitamin K-antagonists. A confirmatory sample should be collected if the first test is positive. In pregnant women, a second evaluation should be done after puerperium. Protein S levels are 30–40% lower in neonates/infants until one year of age. Testing of family members is recommended if inherited deficiency is suspected. The diagnosis of heterozygous protein S deficiency is also hindered by the presence of a pseudogene [104] making the study of this thrombophilia notoriously challenging. So far, almost 300 different PROS1 mutations resulting in loss of function have been identified [90, 105], but the molecular mechanisms leading to reduced plasma protein S levels have only been investigated in a minority of cases, mainly involving missense or splice-site mutations [106].

Although the prevalence of hereditary protein S deficiency in the general population remains largely unknown, it was estimated to be between 0.026% and 0.13% in a large Scottish study among healthy blood donors [107] and there is growing evidence that protein S deficiency may be more prevalent in Asian countries such as Japan, where it was found in approximately 2% of the general population [108]. The point mutation of protein S Lys155Glu, which is named protein S Tokushima, is found at a high frequency of 1 in 55 Japanese people [109]. Given that protein S is one of the major naturally occurring inhibitors of coagulation, acquired or hereditary deficiencies of this protein result in excessive thrombin generation. Familial protein S deficiency has a similar clinical presentation to that observed for protein C deficiency, in which heterozygotes experience are at risk for early and recurrent episodes of venous while homozygotes exhibit a very severe clinical picture with neonatal purpura fulminans [110]. The prevalence of protein S deficiency in unselected patients with a prior episode of venous thrombosis is 2–8%, both in pediatric and adult cases [111, 112] but a prevalence as high as 30% was reported in selected patients with venous thrombosis suspected of having thrombophilia [113, 114]. Type I deficiency is an established risk factor for venous thrombosis [72]. Protein S deficiency was associated with a nearly

tenfold increase in the life-time risk of thrombosis in affected family members, compared with their wild-type relatives [96, 115], although the overall estimated risk in population-based studies is consistently lower [116]. To explain this discrepancy it has been suggested that most subjects defined as having protein S deficiency in population-based studies might have a transitory rather than an inherited protein S deficiency. Besides, data heterogeneity may also be associated with the differences in the risk conferred by different phenotypes and genotypes [117]. Mutations resulting in large protein S derangement can cause more severe secretion defects and functional impairments of protein S, which are likewise reflected in the risk of thrombosis [117]. Likewise, a gradient of risk according with the protein S levels and the need for lower cut-off levels of free protein S (30–40%) for the identification of subjects at risk for venous thrombosis have been proposed in several studies [105]. A recent meta-analysis of 14 case-control and cohort studies including 4955 cases and 9267 controls estimated an overall odds ratio for venous thrombosis in patients with hereditary protein S deficiency of 5.4 (95% CI 3–11). For the previously described reasons, the overall heterogeneity among the studies was statistically significant ($I^2 = 92\%$; $P < 0.00001$) [73].

Depending on the phenotype, approximately half the protein S deficient subjects become symptomatic at 55 years of age. Half of these thromboembolic events will be unprovoked, i.e. not preceded by transient risk factors for venous thrombosis, such as surgery, trauma, immobilization, air travel, pregnancy/puerperium or systemic hormonal contraception or replacement therapy.

Soon after the description of the first cases of thrombosis associated with protein S deficiency in 1984 [118, 119], cerebral venous thrombosis (CVT) was also established as one of the typical manifestations in affected families, both in adults and in pediatric ages [17, 120]. Protein S deficiency was detected in 7.5% of the ISCVT cohort [4, 30]. A regional Indian study of hereditary thrombophilia in more than 600 patients with CVT detected protein S deficiency in 5% of the cases [44]. In a meta-analysis of two studies analyzing the role of protein S deficiency as a risk factor CVT a significant odds ratio of 12 was found, although with a large confidence interval [16]. Combination of protein S deficiency with an acquired precipitating factor like oral contraceptives, pregnancy, puerperium, or malignancy is more common than inherited thrombophilia as a sole trigger for CVT [33, 121]. Although

the results are conflicting across different cohorts, protein S deficiency may also be a risk factor for recurrence of venous thrombotic events in patients with prior CVT [31, 32].

Antithrombin Deficiency

Antithrombin (AT), (called antithrombin III until 1993), is a member of the serine protease inhibitor family (serpin) mainly synthetized in the liver. AT is an inhibitor of thrombin, but it is currently known that AT is also able to inhibit other serine proteases, namely factors Xa, IXa, XIa [122], XIIa, and VIIa when in complex with tissue factor, as well as kallikrein and plasmin. AT is present in plasma in two forms, an active monomer and an inactive "latent" form [123]. AT is believed to be concentrated on the vascular endothelium where it is activated by glycosaminoglycans. Deficiency of AT is the most clinically severe deficiency of a natural anticoagulant.

AT deficiency is a heterogeneous disorder that can be inherited or acquired. Inherited deficiency of AT is due to AT gene mutations and usually shows an autosomal dominant pattern of inheritance with variable penetrance. The gene encoding AT, SERPINC1, is located on the long arm of chromosome 1 (q23–q25.1). Over 250 distinct SERPINC1 mutations causing types I and II AT deficiency have been described. As a vast array of mutations are responsible for hereditary AT deficiencies, and knowing the specific gene mutation does not offer any benefit in the management of affected families, routine molecular characterization is not indicated [124]. The classification of AT deficiency defines heritable type I and type II deficiency states. Type I deficiency is characterized by a parallel reduction in the levels of AT activity and antigen in the plasma and is typically caused by reduced secretion of qualitatively normal AT into the blood. The type II AT defect is where the amount of AT antigen in the plasma exceeds the functional level, which is usually reduced [125]. Type II AT deficiency is currently classified into three broadly defined subtypes: reactive site (type IIRS) in which the defect is at the thrombin binding site at the carboxyterminal end of the molecule, disrupting the enzyme–inhibitor interaction; heparin-binding site defects (type IIHBS), the most common type, where AT binding to heparin is defective; and the pleiotropic effect defects (type IIPE), involving a mutation at the carboxyterminal end of the AT molecule between amino acids 402 and 429 which produces conformational changes in the protein that may be associated with more than

one functional defect. Type IIHBS is associated with very low risk of thrombotic complications compared with other AT defects [126]. Type I deficiency is usually caused by a small deletion or insertion, a larger deletion or a single base substitution, causing to reduced synthesis of the protein. Changes that affect the signal peptide and impair post-translational processing or alter protein stability have also been described. Type II defects are caused by mutations that affect the protein function. Most patients are heterozygous for the defect, although a small number of cases with homozygous type IIHBS defects, which are usually associated with severe thromboembolic events early in life, have been reported [127]. Compound heterozygous inheritance of a severe defect on one allele that results in quantitative deficiency and a missense mutation resulting in a mild qualitative defect on the other has also been reported [128]. Complete quantitative deficiency is thought to be incompatible with life and results in embryonic lethality in mice [129].

Functional and immunological assays are available to detect AT deficiency. Diagnosis can only be reliably made with functional assays (amidolytic assays) because many patients with AT deficiency have quantitatively normal levels of AT in plasma, measured as AT antigen. Nonetheless, when reduced activity levels are identified it is useful to measure the AT antigen levels to distinguish type I from type II hereditary AT deficiency, which have different thrombotic risks [126, 130]. Routine diagnostic AT assays measure AT inhibition of FIIa or FXa in the presence of heparin. Preanalytical variables must be taken into account when testing and when interpreting results. AT activity is reduced in clotted specimens, following thrombosis, after a number of days of heparin therapy, after surgery or trauma, in liver disease, nephrotic syndromes, hemodialysis, asparaginase therapy or with consumption caused by disseminated intravascular coagulation [131]. At least 5 days should have elapsed after stopping heparin therapy before AT measurement can be performed [132]. In patients receiving direct thrombin inhibitors thrombin-based assays should not be used, as these could lead to overestimation of AT levels. Plasma AT level is relatively constant in adults, but may be slightly reduced in subjects taking the oral contraceptive pill or hormone replacement therapy and healthy newborns have approximately 50% of AT activity, reaching adult levels at 6 months of age [133]. Contrary to other natural anticoagulants, AT appears to function as a negative acute-phase reactant protein [134]. Prothrombin time

(PT), activated partial thromboplastin time (aPTT), and thrombin time (TT) are not affected by AT deficiency.

The two major consequences of AT deficiency are increased thrombotic risk and insensitivity to heparin. Patients with AT deficiency, typically with levels of AT below approximately 50 percent of normal (range 40–60%), have increased thrombotic risk. This is thought to be due to excessive thrombin generation and fibrin deposition within the vasculature. This mechanism contrasts with coagulation factor deficiencies, in which heterozygosity for a mutation typically is associated with a benign carrier condition and reduction of plasma activity levels to approximately half of normal rarely confer clinical consequences. The incidence of AT deficiency in healthy populations has been reported to be between 1 in 500 and 1 in 5000 [135], with the variation being due to the different populations studied and detection methods used. The deficiency is equally common in both sexes. The incidence of venous thrombotic events in carriers of AT deficiency is estimated to be 1.0–4.0% per individual-year. A meta-analysis of case-control and cohort studies estimated an overall odds ratiofor venous thrombosis in patients with hereditary AT deficiency of 16 (95% CI 10–27) [73]. A similar high risk had also been calculated in a prior study using unaffected relatives as the reference group, in which the risk for venous thromboembolism among carriers of an AT deficiency was 12-fold greater than that in non-carriers [136]. Antithrombin deficiency was detected in only 0.9% of the ISCVT cohort [4, 30]. A slightly higher prevalence was found in a retrospective cohort of patients with CVT, in which 1.5% of the patients was positive for antithrombin deficiency [31]. A meta-analysis analyzing the role of antithrombin deficiency as a risk factor CVT found a non-significant significant odds ratio of 2.69 was found [16]. Although the estimates of venous thrombotic recurrence vary across studies, prospective and retrospective cohorts found an approximately twofold increased risk of recurrent events in patients with AT deficiency [77, 137]. The incidence of recurrent thrombotic events in a prospective cohort of patients with hereditary AT deficiency was 10.5% per patient-year [77].

Some patients with AT deficiency also may have insensitivity to heparin. This occurs because heparins require that AT inactivate coagulation enzymes. In contrast, direct inhibitors of thrombin or factor Xa do not require AT.

Protein Z Polymorphisms

One case control study reported an association between the G79A polymorphism of the protein Z and CVT [138], but this association was not confirmed in other studies [139] and meta-analysis [8]. A recent study also concluded that PZ G79A polymorphism was not a risk factor for puerperal CVT in Indian women [140].

Polymorphisms in the Fibrinolytic System

The fibrinolytic system is activated in parallel to the coagulation cascade and serves to limit the size of the clot. Fibrinolysis by plasmin (originating from plasminogen) dissolves the fibrin clot into fibrin degradation products. This reaction is catalyzed by tPA (tissue plasminogen activator) or U-PA (urokinase plasminogen activator) released from the endothelium. Fibrinolytic activity is limited by plasminogen activator inhibitor 1 (PAI-1), thrombin activatable fibrinolysis inhibitor (TAFI) and by α2 antiplasmin and α2 macroglobulin, which are plasmin inhibitors.

Tezzon et al. [141] reported a patient with defective t-PA who was also using oral contraceptives who suffered a deep cerebral venous thrombosis.

A few case reports described infants with CVT and the 4G/4G genotype of PAI-1 [142]. No association was found of this polymorphism and CVT in two case control studies with medium sample sizes (<100 cases) [139, 143], except in those patients who also carried factor V Leiden [143]. A subsequent case control study of 136 CVT patients with 1054 population based controls found no association between promotor polymorphisms of PAI-1 and CVT [144]. No association was also detected in the meta-analysis by Marjot et al. [8].

No association has been found between TAFI gene polymorphisms and CVT in acase control study performed in Southern Germany [139] nor in the Tokgoz et al. study [145]. More recently in a Brazilian study, the GTC haplotype for TAFI 505G > A/1040C > T/+1542C > G SNPs was associated with an increased risk of CVT compared to controls [odds ratio2.67, 95% confidence interval: 1.13–6.34] [146].

Genetic Hyperhomocysteinemia

Homocysteine is a sulfur-containing amino acid derived from the metabolism of methionine, an essential amino acid. Impairment in the conversion of methionine to cysteine leads to increased levels of the intermediate product

homocysteine. Cysteine is a precursor of glutathione, which is an important antioxidant.

Hyperhomocysteinemia, which may be inherited or acquired, is defined as a serum homocysteine concentration of >15 mmol/L. Homocystinuria is a rare autosomal recessive disorder characterized by severe elevations in plasma (>100–200 μmol/L) and urine homocysteine concentrations due to a complete deficiency of cystathionine β-synthase. Because of its rarity and early association with other specific manifestations (developmental delay, osteoporosis, ocular abnormalities), besides the increased predisposition to thrombosis, homocystinuria is not further discussed in this chapter.

Less marked elevations in plasma homocysteine (15–40 μmol/L) are much more common, occurring in 5–7% of the population [147]. Although unassociated with the clinical stigmata of homocystinuria, evidence suggests that moderate hyperhomocysteinemia is an independent risk factor for venous thrombosis, including CVT. It has been estimated to increase the risk of venous thrombosis by 1.5- to 2-fold [148].

Elevations in the plasma homocysteine concentration occur mainly due to genetic defects in the enzymes involved in homocysteine metabolism or nutritional deficiencies in vitamin cofactors. Mutations in methylenetetrahydrofolate reductase (MTHFR) (OMIM #236250) or cystathionine β-synthase are thought to be responsible for most of the inherited forms of hyperhomocysteinemia while nutricional deficiencies in folate, vitamin B12, and vitamin B6 have also been postulated as acquired underlying causes [148].

The most common form of genetic hyperhomocysteinemia results from production of a thermolabile variant of MTHFR with reduced enzymatic activity associated with the polymorphism C677T in the MTHFR gene. The different behavior of the variant upon exposure to heat was first shown in 1988, as it lost about 85% of its activity at 46 °C, whereas the wild-type enzyme lost only 63% of its activity [149]. The MTHFR is an important enzyme in the homocysteine metabolism and catalyzes the conversion of 5,10-methylenetetrahydrofolate into 5-methyltetrahydrofolate, the predominant circulating form of folate. Complete absence of MTHFR activity is very rare and leads to severe hyperhomocysteinemia, with levels similar to those seen in homocysteinuria due to cystathionine β-synthase deficiency. The MTHFR 677 T allele causes a 50% reduction in the activity of this enzyme and mildly increased plasma total homocysteine concentrations

[150]. However, its phenotypic penetrance is largely influenced by folate and B12 status, suggesting that patients with the thermolabile version may have a higher folate requirement than individuals with the normal genotype [150, 151].

In Europe, the frequency of C677T homozygosity ranges from 8% (Germany) to 18% (Italy) [152]. Population studies have shown a higher prevalence of the thermolabile variant in Caucasians, Hispanics, and Asians compared to African Americans and Asian Indians [153]. Another functional polymorphism of the MTHFR gene is 1298A > C. MTHFR polymorphism 1298A > C also affects MTHFR activity but without biochemical changes [154].

Homocysteine is readily measured in plasma. Blood samples can be collected in EDTA or citrate anticoagulant and should be centrifuged and plasma separated as soon as possible after collection since homocysteine is spontaneously released from erythrocytes. Without separation, the homocysteine level increases by about 0.5 μmol/h at 22 °C. The sample should be stored on ice until it reaches the laboratory whenever the immediate centrifugation is not possible. Once the plasma is separated, the homocysteine remains stable for up to 96 hours [155]. Since high-protein meals can influence the results, homocysteine should be measured in fasting subjects. Measuring homocysteine immediately after an acute vascular event should be avoided since this may also lead to spuriously high homocysteine [156].

The mechanism by which hyperhomocysteinemia increases the risk of thrombosis is still unclear and appears to be multifactorial. Some of the postulated mechanisms involve nitric oxide, impaired fibrinolysis, endoplasmic reticulum stress and effects in the activated protein C pathway [32, 157].

A 2005 meta-analysis including 8364 cases and 12,468 controls found that the TT variant was associated with a 20% higher risk of venous thrombosis than the wildtype 677CC genotype [158]. Similar results were found in a prospective intervention trial in which the control arm had an estimated risk of venous thrombosis recurrence of 1.13 for each 5-μmol/L higher homocysteine concentration at baseline [159]. However, a more recent meta-analysis only identified a significant increase in the risk of venous thrombotic events in the Chinese/Taiwanese population (odds ratio 1.57), but not in Caucasians [23]. Thus, the overall risk of venous thrombosis in

patients with the C677T MTHFR polymorphism is low.

An association between CVT and this MTHFR C677T polymorphism has also been established. However, although the first estimates pointed to a fourfold increased risk of CVT in patients with hyperhomocysteinemia [10, 160], a systematic review of 15 studies including 646 CVT cases and 3690 controls found a still significant but weaker association for patients with the C677T allele, with an odds ratio of 2.30 [8]. In ISCVT, 10% were carriers of the MTHFR 677 T allele [4, 30]. A large retrospective cohort with 550 CVT patients tested for hyperhomocysteinemia also found that 13% were affected [31]. As described for other types of thrombophilic defects, association of hyperhomocysteinemia and oral contraception have a synergistic effect in the risk of CVT, with an estimated odds ratio of 20 in women with both conditions [160].

Vitamin supplementation, primarily with folic acid, and to a lesser degree with pyridoxine and vitamin B12, is effective in reducing elevated levels of plasma homocysteine in most cases. However, a randomized intervention trial showed that despite lowering of the homocysteine level with such treatment, there is no impact on the risk of recurrence of venous disease [159].

Other Genetic Diseases

Sickle Cell Disease

There are a few reports of CVT in children with sickle cell disease. The first two cases were respectively a 2-year-old boy and a 25-year-old male, presented in coma due to bilateral thalamic infarcts secondary to thrombosis of the cerebral deep venous system [161, 162]. Van Mierlo and colleagues [163] described a 12 year old girl who suffered a CVT after exchange transfusion for a spine pain syndrome. Ciurea and colleagues [164] reported a case of CVT in a patient who developed seizures following exchange transfusion for treatment of acute chest syndrome associated with sickle cell disease. No other hypercoagulable state was identified. In a multicenter European registry of 42 children with CVT, two had sickle cell disease with anemia [165]. A young African-American with sickle cell trait who sustained a carotid dissection after playing football, suffered a CVT 10 year later. Complete workup failed to identify any additional cause for the CVT [166]. An extensive CVT was described in a young man homozygote for sickle cell

disease, who had persistent high hemoglobin levels secondary to hydroxyurea treatment [167]. The most recent reported case (fatal) had a sickle cell trait and was also heterozygote for MTHFR C677T [168].

In sickle cell disease venous thrombosis can occur through several mechanisms. Occlusion of the veins by sickled erythrocytes is the main mechanism. Sickled red blood cell can contribute to the pathogenesis of CVT via abnormal adherence to the vascular endothelium and by hemolysis, which results in endothelial cell activation, a hypercoaguable state, and alterations in vasomotor tone[169]. Other mechanisms include platelet activation, increased adherence of platelets to the endothelium, high expression of adhesion molecules in leucocytes and activation of inflammatory markers [170].

Treatment of CVT includes hydration, exchange transfusion if needed to reduce Hbs levels below 30%, heparin and symptomatic treatment of seizures and increased intracranial hypertension with mechanical ventilation and decompressive hemicraniectomy, without resourcing to mannitol or other diuretics [170].

Genes That Increase the Risk of CVT in Systemic Diseases

The best example of gene mutations which increase the risk of CVT in systemic diseases is the JAK2 V617F mutations in myeloproliferative diseases (MPD) [171]. Of the MPD, both polycythemia vera and essential thrombocythemia can be complicated by CVT [172] and are a rare cause of CVT in prospective series of CVT [4, 172], somewhat more common in middle-aged and elderly patients. Polycythemia vera and essential thrombocythemia are also risk factors for recurrence of CVT and other venous occlusive events [30, 173]. JAK2 V617F mutation is present in almost all patients with polycythemia vera and in 50% of patients with essential thrombocythemia. JAK2 is a tyrosine kinase that has a pivotal role in myelopoiesis in the transduction pathway activated either by erythropoietin, thrombopoietin or granulocyte colony stimulating factor [174].

Besides JAK2 mutation, there are several risk factors for venous thrombosis in MPD. Some are related to the disease, others are unrelated and transient, such as pregnancy/puerperium, oral contraceptives, hormone-replacement therapy and other drugs with prothrombotic actions, infections

and head trauma [174]. Hematological abnormalities associated with thrombosis in MPD can be related to the platelets (increased number of reticulated platelets, of exposure to phosphatidylserine and activation), the red blood cells (plasma membrane phospholipid alterations, aggregates and increased blood viscosity), the white blood cells (increased inflammation and blood viscosity), the clonal cells (JAK 2 V617F mutation) and to the coagulation pathway (reduced protein S, resistance to activated protein C) [174]. A risk score in essential thrombocythemia was recently developed [175] stratifying patients in low, medium and high-risk strata. The high-risk strata carries a 3.56% patients/years risk of thrombosis and is defined by age above 60, cardiovascular risk factors and JAK2 V617F mutation.

The neurologist dealing with a CVT patients can be confronted with MPD in four scenarios: (1) patient with CVT and known MPD, JAK2 V617F being either positive, unknown or negative; (2) patient with CVT where MPD is diagnosed during etiological work up during the acute phase; (3) patient with CVT with high red blood cell or platelet count or other clinical or laboratorial features suggestive of MPD, where MPD is diagnosed during follow up; (4) patient with acute CVT and no clinical or laboratorial features of MPD. In a large study of two databases (CVT and MPD) 27 (3.8%) of CVT patients were diagnosed with MPD: 9 before CVT (1.3%), 4 concomitantly (0.6%) and 14 after CVT (2.0%) [172]. The JAK2 mutation is rare (1–7%) in scenario 4 [176–180], but is more frequent in the other three scenarios, increasing the risk of CVT by 2.26 [181]. A recent study of the prevalence of JAK2 mutation in CVT [179] further illustrates these scenarios. Ten of 152 CVT patients (6.6%) carried the JAK2 V617F mutation. Three had transient risk factors for CVT and five had thrombophilia. Six (3.9% of the total) meet the criteria for MPD during the etiological investigation of CVT (scenario 2) and three (1.9% of the total) additional patients developed the disease during follow up (scenario 3).

Concerning prevention of thrombotic events and treatment of CVT in patients with MPD, primary prevention of arterial events with aspirin or other antiplatelet agent is warranted in the majority of patients. In polycythemia, hematocrit should be kept below 45% by periodic phlebotomies. In high risk patients (older than 65 years of age or previous thrombosis) myelosuppressive therapy with hydroxyurea should be added. Prevention of venous thrombotic events with anticoagulants should be restricted to high risk transient situations, such as prolonged immobilization and some

surgeries. Treatment of CVT follows the guidelines of acute CVT treatment with IV heparin or SC low molecular weight heparin followed by oral anticoagulants (vitamin K antagonists) for a variable period depending on the finding of additional prothrombotic conditions, the activity of the MPD and the bleeding risk [7, 174].

Recommendations for Genetic Studies in CVT

Performing genetic tests in search of a prothrombotic mutation may lead to a final diagnosis of the cause (or of one of the causes) of CVT and may prompt the investigation of additional cases in the family. This potential benefit can be overshadowed by the emotional aspect of the diagnosis of a genetic disease and by over-treatment with prolonged anticoagulation. Diagnostic tests should only be performed if their results lead to a change in patient outcomes. The evidence supporting such beneficial change of outcome is low. No study found that thrombophilia testing decreases death in CVT patients. Three studies reported that patients with thrombophilia had more remote seizures and worse functional outcome compared to patients without thrombophilia [52, 182, 183], while two other studies found no association between thrombophilia and worse functional outcome [4, 184]. Concerning the risk of recurrence of venous thrombotic events in adult CVT patients, four studies gave contrasting results. No effect was found by Miranda et al. [30] and Dentali et al. [31], while an in increased risk effect was identified in two other investigations [32, 185]. Our recommendation is to suggest not performing routine screening of thrombophilia, if the aim is to reduce death, improve functional outcome or to prevent recurrent venous thrombosis in patients with CVT. Thrombophilia screening may be performed in patients with high pre-test probability to carry severe thrombophilia (i.e., a personal and/or family history of venous thrombosis, young age at CVT, CVT without a transient or a permanent risk factor) to prevent recurrent venous thrombotic events.

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17. Stroke Pharmacogenetics

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Introduction

Numerous clinical risk factors predispose to stroke, including atrial fibrillation (AF), hypertension, and hypercholesterolemia (Fig. 17.1). Such risks can be ameliorated with appropriate pharmacotherapy. However, there is variability in response to drugs used for these conditions, which may, at least in part, be genetically determined. For instance, genetic determinants of response to warfarin, statins, antiplatelet agents, and some antihypertensives have been described.

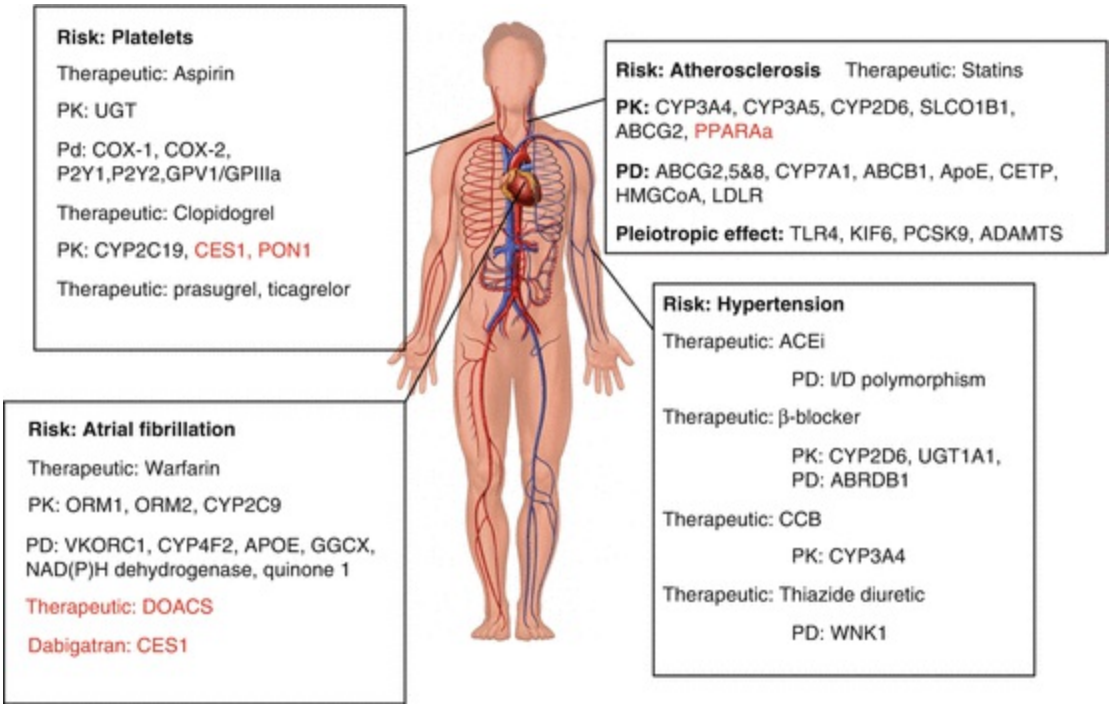

Fig. 17.1 Diagram depicting the pharmaceutical agents targeted at reducing the risk of stroke and the genes implicated in altered clinical efficacy and tolerability

The study of genetic variation in drug response is termed pharmacogenetics. Pharmacogenetics, as a subject and term, came to the fore in the 1950s when landmark discoveries, such as the identification of glucose-6-phosphate dehydrogenase (*G6PD*) variant alleles causing primaquine-induced hemolytic anemia among African-Americans [1], culminated in the coining of the term "pharmacogenetics" in 1959 by the German pharmacologist Friedrich Vogel [2]. In the modern era, following the completion of the human genome project, we now have resources such as the International HapMap Project and the 1000 Genomes Project, which provide an encyclopedia of human genetic variation, not only allowing us to pinpoint the genetic causes of diseases, but also providing us with the tools to characterize factors that determine variability in drug response, both efficacy and toxicity. The ultimate aim, of course, is to ensure that the patient gets the right drug at the right dose at the right time. The ability to evaluate variation at whole genome level has led to the introduction of the term "pharmacogenomics." However, both terms, pharmacogenetics and pharmacogenomics, are used interchangeably.

Pharmacogenetics/pharmacogenomics will make a difference to the use of

some of the drugs in stroke prevention in the near-to-medium term. Furthermore, as more drugs are developed to treat stroke in terms of neuroprotection (rather than prevention), the stratification of treatments through the use of genomic and other technologies will become increasingly important. It is likely that we are at the beginning of a journey: we now have unprecedented access to information about the human genome following its initial sequencing in 2003, resulting in by huge advances in genotyping and sequencing technologies. For example, genome-wide association studies (GWASs) allowing for rapid interrogation of the entire genome relevant to pharmacogenetics while fine mapping with next generation sequencing technologies will allow the identification of causal variants. Indeed, sequencing might become the method of choice to determine genotypes as the cost of sequencing drops, and the informatics tools to analyze the genome become more advanced and pervasive. In the meantime, GWAS is starting to be widely used for pharmacogenomic association studies. Interestingly, even with the small number of GWAS undertaken for drug response to date, it has become apparent that genetic effect sizes are much greater for response to drugs than seen for complex diseases [3]. Despite the challenges in identifying markers for personalizing medicines (covered later in the chapter), novel associations have already been discovered with clinical testing available for several DNA-based pharmacogenetic tests including assays for warfarin sensitivity, CYP2D6, CYP2C19, and UGT1A1. The US Food and Drug Administration (FDA) are now relabeling the product inserts of many drugs to include relevant pharmacogenetic information [4].

In this chapter, we focus on drugs that are currently used in patients with cerebrovascular disease, largely for prevention, and evaluate the pharmacogenetic associations identified to date (Fig. 17.1), and finish off with a section on challenges and how to progress in the future.

Lipid-Lowering Therapy

The lipid-lowering effect of statins is facilitated through inhibition of the rate-limiting enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCoA), preventing the biosynthesis of cholesterol. Their ability to reduce total cholesterol, low-density lipoprotein (LDL) particles and triglycerides and thereby decrease the risk of cardiovascular and cerebrovascular events has been well demonstrated [5]. The Heart Protection

Society showed that simvastatin (40 mg/day) reduced the risk of major vascular events (coronary events, stroke, or revascularization) by 21% in patients with prior stroke or transient ischemic attack (TIA)—absolute risk reduction 1%/year; number need to treat (NNT) = 102 to prevent 1 event per year [6]. While statins are a major advance in the prevention of atherosclerotic arterial disease and are safe in the vast majority of patients, a small percentage of patients can develop adverse effects, the most feared of which is rhabdomyolysis. Indeed, cerivastatin was withdrawn from the market because of an unacceptably high incidence of muscle damage, which in some cases, led to death of patients. Fortunately, such incidences are rare; a British epidemiological cohort study (n = 96/193) revealed a very small but statistically significant increased risk of myopathy in patients taking statins—number needed to harm (NNH) = 10,000/year [7].

There is interindividual variation in the lipid-lowering efficacy of statins. This variability at least partly is due to factors such as gender, age, diet and concomitant drug use. But much of the variability remains unexplained, and genetic factors may play a role. For example, in individuals taking simvastatin 80 mg/day, mean reduction in LDL cholesterol (LDL-c) was 46%; however, in the top 5% of responders, decreases ranged from 63% to 76% as compared to those in the bottom 5% where changes ranged from −23% to +20% following as long as 6 months of treatment [8]. Similarly, there is interindividual predisposition to the occurrence of statin myopathy. Statin-related myotoxicity (SRM) can be divided into seven different categories:

1. SRM0: No muscle symptoms, creatine kinase (CK) elevation <4 times upper limit of normal (ULN)

2. SRM1: Tolerable myalgia, muscle symptoms without CK elevation

3. SRM2: Intolerable myalgia, muscle symptoms, CK <4× ULN, complete resolution on dechallenge

4. SRM3: Myopathy, CK elevation >4× ULN <10× ULN ± muscle symptoms, complete resolution on dechallenge

5. SRM4: Severe myopathy, CK elevation >10× ULN <50× ULN, muscle symptoms, complete resolution on dechallenge

6. SRM5: Rhabdomyolysis, CK elevation >10× ULN with evidence of renal impairment + muscle symptoms or CK >50× ULN

7. SRM6: Autoimmune-mediated necrotizing myositis, HMGCR antibodies, HMGCR expression in muscle biopsy, incomplete resolution on dechallenge [1]

Fortunately, serious muscle toxicity is rare. Findings from a combination of 20 trials in statin-treated patients versus placebo defined the incidence of rhabdomyolysis as 3 per 100,000 person years in those taking simvastatin, lovastatin, atorvastatin, pravastatin, or fluvastatin [9]. This observation could be an underrepresentation; however, as of course, muscle toxicity must inevitably reduce compliance; 25–50% of patients with coronary artery disease (CAD) are noncompliant with a prescribed drug at 12 months with a resultant worsened prognosis [10]. Although the exact cellular mechanism underlying the myopathic effect of statins has not been fully elucidated, there is a clear dose-dependence [11] and increasing risk with increasing blood concentrations [12]. Predisposition to statin-related muscle damage is partly due to environmental factors, for example, drug–drug interactions, but again, genetic factors may be important.

Clinical Efficacy: Pharmacokinetic Factors Determining Variability (Table 17.1)

Table 17.1 Studies investigating the pharmacogenetic factors implicated in the altered pharmacokinetic response to statins

Gene	Allele	Study design	Patient demographics	Statin	Outcomes	Cli im
CYP3A4	A-290G (rs2740574)	RCT	340 subjects 1° hypercholesterolemia	Atorvastatin 10 mg/day	Homozygote carriers of the A-290G promoter variant had significantly higher (+12.4%) posttreatment LDL-c than those	Re eff

					carrying the wild-type allele	
		Prospective cohort	142 Chilean subjects	Atorvastatin 10 mg/day	The A—290G polymorphism was related to greater reduction in Tc and LDL-c variation ($p < 0.001$). Also associated with higher HDL-c variation ($p = 0.017$)	En eff
	M445T (rs4986910)	RCT	340 subjects 1° hypercholesterolemia	Atorvastatin 10 mg/day	Significant and independent association between individuals with the M445T variant and lower (−11.2%) pretreatment LDL-c levels	En eff
	CYP3A4*1G	Prospective cohort	416 Chinese subjects hyperlipidemia	Atorvastatin (217) or simvastatin (199) 20 mg/day	Homozygote carriers of CYP3A4*1G have greatest Tc reduction 6.8 ± 3.3% (*1/*1), 17.8 ± 3.8% (*1/*1G), and 20.9 ± 5.0% (*1G/*1G) following atorvastatin therapy (not seen in the simvastatin-treated subjects)	En eff
PPARA	40 Candidate genes with role in regulating CYP3A4 phenotype	Retrospective cohort	56 volunteers who received single-dose atorvastatin	Atorvastatin	Atorvastatin and its major CYP3A4-dependent metabolite, 2-OH-atorvastatin, showed a reduced 2-OH-atorvastatin/atorvastatin area under the plasma concentration–time curve (AUC0–∞) ratio of 81% (P = 0.044) for PPARA G/A heterozygotes and 50% for the single A/A homozygote	Po rec eff
CYP3A5	CYP3A5*3	Prospective cohort	69 Caucasian subjects hypercholesterolemia	Lovastatin or simvastatin	Mean serum Tc and LDL-c concentration were significantly higher (23% and 24%), respectively, in those possessing the	Re eff

					CYP3A5*1 efficient metabolizer allele	
	CYP3A5*1D CYP3A5*3C CYP3A5*3A (rs5624447)	Prospective cohort	139 subjects (46 African/93 non-African) hypercholesterolemia	Atorvastatin 10 mg/day 4 weeks	Frequencies of the CYP3A5*3C and CYP3A5*1D alleles lower in African's (*3C: 47.8% and *1D: 55.2%) than in non-Africans (*3C: 84.9% and *1D 84.8%, $p < 0.01$). Non-Africans carrying *3A allele (*3C and *1D combined alleles) had lower Tc and LDL-c response to atorvastatin than non-*3A allele carriers ($p < 0.05$)	Re eff
CYP2D6	*3, *4, *5		88 patients hypercholesterolemia	Simvastatin >40 mg/day	Those homozygous for the CYP2D6 mutant alleles were poor metabolizers with greater Tc reduction and increased rate of discontinuation as a result of ADR	En eff

1° primary, *CI* confidence interval, *HR* hazard ratio, *CAD* coronary artery disease, *RCT* randomized control trial, *ApoE* apolipoprotein E, *CETP* cholesteryl ester transfer protein, *LDLR* low-density lipoprotein receptor, *mg* milligrams, *vs*. versus, *LDL-c* low-density lipoprotein cholesterol

Cytochrome P450 Enzymes

With the exception of pravastatin and rosuvastatin, statins are lipophilic molecules that undergo biotransformation via the CYP450 system to hydrophilic metabolites. Therefore, genetic variation in the CYP450 pathway may affect the disposition of statins and account for the variable efficacy, with anywhere between 10% and 50% variation in the magnitude of effect [19]. CYP3A4 is a primary metabolic pathway for simvastatin, atorvastatin, and lovastatin with both CYP2D6 and CYP2C9 additionally involved in fluvastatin [20], pitavastatin, and rosuvastatin metabolism.

CYP3A4

In individuals treated with atorvastatin (10 mg/day), homozygotic carriers of the A-290G promoter variant had significantly higher (+12.4%) posttreatment LDL-c than those carrying the wild-type allele, signifying reduced treatment response [13]. This is possibly as a result of increased, rather than decreased, CYP3A4 transcription compared with the wild-type allele, an effect that has been demonstrated in experiments with a luciferase reporter gene technique in prostate malignancy [21]. However, the opposite finding was identified for this variant in a Chilean population treated with atorvastatin 10 mg/day for 4 weeks in whom individuals with the A-290G variant exhibited a greater reduction in both total cholesterol (Tc) and LDL-c [14]. In addition to the A-290G variant, Kajinami et al. identified a significant and independent association between individuals with the M445T variant and lower (−11.2%) pretreatment LDL-c levels [13]. This difference became exaggerated following treatment, increasing from −11.2% to −17.6%. However, in the case of both A-290G and M445T, the differences in absolute or percent change in LDL-c did not reach statistical significance. In Chinese individuals treated with atorvastatin, the CYP3A4*1G variant was associated with a greater reduction in Tc (20.9 ± 5% vs. 6.8 ± 3.3% wild type) in response to atorvastatin, but not simvastatin [15].

Peroxisome Proliferator-Activated Receptor-α (PPARA)

To date, there are at least 46 known coding variants of the CYP3A4 gene (dbSNP, http://www.ncbi.nlm.nih.gov/projects/SNP, and CYP allele nomenclature homepage, http://www.cypalleles.ki.se/cyp3a4.htm). However, most occur at very low frequency and the phenotypic effects have been debated at length [2–4]. As a result, it is difficult to attribute the high heritability of the CYP3A4 metabolic function purely to the known genetic variants of CYP3A4. Consequently, Klein and colleagues [5] used a candidate-gene approach in a human liver bank to identify predictive genetic factors. They showed that variation in the peroxisome proliferator-activated receptor-α (PPARA) gene was associated with CYP3A4 phenotype, and that atorvastatin-treated carriers of the rs 4253728 variant had lower levels of the atorvastatin-2-hydroxylated metabolite. Short hairpin mediated knock-out of the PPARA gene in human hepatocytes led to decreased expression levels of

the PPAR-α targets COX1 and CYP3A4 by more than 50% [5]. This may potentially lead to reduced efficacy, but the clinical significance has not been examined.

CYP3A5

Although primarily metabolized by CYP3A4, lovastatin, simvastatin, and atorvastatin are also metabolized by the polymorphic CYP3A5 [22]. Located very close to CYP3A4, individuals only express CYP3A5 if they carry at least one copy of the *1 allele [16]; the CYP3A5*3 variant results in low or undetectable CYP3A5 expression [23]. The CYP3A5*1 efficient metabolizer allele has been associated with reduced efficacy in response to both lovastatin and simvastatin [16]. In non-Africans, carriers of the *3A allele (*3C and *1D combined alleles) had a significantly reduced cholesterol-lowering response than noncarriers [17].

CYP2D6

Individuals homozygous for the CYP2D6 mutant allele have greater Tc lowering (0.23 mmol/L/mg of simvastatin) than (0.10 mmol/L) those possessing the wild-type alleles [18]. Discontinuation of treatment as a result of adverse drug reactions (ADRs) was also greater in the mutant allele group, which was postulated to be due to higher drug concentrations. However, the result overall is surprising because metabolism of simvastatin by CYP2D6 is not a major route (if at all) of elimination of simvastatin. Furthermore, this association has not been convincingly replicated.

Adenosine Triphosphate (ATP)-Dependent Efflux Pumps

ATP-binding cassette (ABC) transporters may be involved in the response to statins because they are involved in both the transport of drugs (a pharmacokinetic pathway) and cholesterol (a pharmacodynamic pathway).

A recent pharmacokinetic study of the ABCG2 polymorphism in Finnish subjects demonstrated a marked increase in atorvastatin area under the curve (AUC) by 72% and of rosuvastatin by 144% in those carrying the c.421AA genotype compared to those with the c.421CC genotype [24]. The clinical impact on efficacy has been demonstrated in both Chinese [25] and

Caucasian [26] populations with greater LDL-c reductions in those carrying the c.421AA variant following treatment with rosuvastatin (10 mg/day). The effect of the ABCG2 421C>A polymorphism on adverse reactions including myopathy and of drug–statin interactions requires further evaluation in clinical studies.

The P-glycoprotein (Pgp) efflux pump is involved in the transport of many drugs from the gastrointestinal (GI) tract into the circulation [27]. Pgp is encoded by the ATP-binding cassette subfamily B (ABCB). Lovastatin, simvastatin, and atorvastatin have been implicated in Pgp inhibition [28], which may result in increased bioavailability and could potentially affect efficacy and adverse reactions. Indeed, in a pharmacokinetic study of three ABCB1 SNPs including 1236C>T, 2677G>T/A, and 3435C>T, the AUCs for both simvastatin and atorvastatin were significantly greater (60% and 55%, respectively) in those homozygous for the TTT haplotype compared to those with CGC [29]. In hypercholesterolemic patients treated with simvastatin, the 1236T variant allele [30] enhances efficacy with respect to Tc and LDL-c lowering; the 3435C variant [31] is associated with smaller reductions in LDL-c but larger increases in HDL.

Pharmacodynamic Factors Determining Variability Adenosine Triphosphate (ATP)-Dependent Efflux Pumps

In addition to their pharmacokinetic effects, the ATP-binding cassette (ABC) transporters are involved in intracellular cholesterol transport and homeostasis of cholesterol biosynthesis. Within the ABCG8 allele, the rs11887534 SNP is significantly associated with a greater LDL-c reduction compared to the wild type (39.6% vs. 36.6%; $p = 0.043$) in hypercholesterolemic patients treated with atorvastatin [32].

7 α-Hydroxylase

In addition, CYP7A1 gene encodes the 7 α(alpha)-hydroxylase enzyme, the rate-limiting step in the bile acid synthesis pathway for cholesterol [33]. Individuals with a homozygous deletion mutation of CYP7A1 are resistant to the lipid-lowering effect of statins [34]. Furthermore, the improved efficacy

of atorvastatin in carriers of the ABCG8 allele is enhanced further in noncarriers of the CYP7A1 (A204C) promoter variant (42.7% vs. 38.2%, p = 0.048) [32]. Similar reduced efficacy has been shown for pravastatin [35] with respect to reduced LDL-c lowering capacity for individuals carrying the A204C promoter variant.

Apolipoprotein E

The presence of polymorphisms in apolipoprotein E (ApoE) gene has been associated with a significantly higher risk of myocardial infarction [36]. ApoE is defined by three alleles, E2, E3 (wild type), and E4 with increasing affinity for the LDL receptor [37]. ApoE protein binds lipids and lipid receptors, modulates the clearance rate of lipids and the lipophilic conversion of VLDL and triglyceride function. Lipoproteins containing the E4 isoform are cleared more efficiently from the circulation, resulting in downregulation of HMG-CoA synthesis [38], and thus theoretically, the efficacy of statins in E4 carriers will be reduced by virtue of already depleted HMG-CoA reductase levels. Indeed, the ApoE E4 variant has been associated with decreased statin efficacy in some trials [39, 40] (Table 17.2); however, others have failed to find a significant association when factors such as age, sex, and body mass index are tconsidered [42]. Similarly, where the ARIC cohort (Atherosclerosis Risk in Communities) identified higher LDL-c, higher intima thickness, and lower HDL cholesterol in those with the E4 as compared to the E2 variant, this was not associated with the development of CAD when adjustments were made for lifestyle factors including smoking, weight and comorbid illness such as diabetes mellitus or hypertension [49]. The GO DART study demonstrated that the E4 variant homozygote was associated with a 32% failure to achieve LDL-c lowering target (2 mmol/L) whereas among those expressing the E2 variant, none failed to achieve target [39]. It is important to mention that although the E4 variant is associated with reduced lipid-lowering efficacy, both the Regression Growth Evaluation Statin Study (REGRESS) [44] and a sub-study of the Scandinavian Simvastatin Survival Study [50] showed that statin therapy is still associated with substantial benefit in terms of angiographic parameters [44] and reduced mortality [50].

Table 17.2 Studies investigating the pharmacogenetic factors implicated in the altered pharmacodynamic response to statins

Gene	Allele	Study design	Patient demographics	Statin	Outcomes	Clinical impact
ABCG8	H19 variant (rs11887534)	RCT, double-blind, placebo control	338 subjects hypercholesterolemia	Atorvastatin 10 mg/day 36 weeks	H19 variant in ABCG8 associated with greatest fall in LDL-c level (p = 0.043) compared to wild-type (39.6% vs. 36.6%; p = 0.043)	Enhance efficacy
CYP7A1	A-204C promoter (rs3808607)				Carriers of A-204C variant had reduced efficacy with respect to LDL-c reduction (38.2% compared to noncarriers 42.7%)	Reduced efficacy
		Retrospective cohort	33 subjects hypercholesterolemia	Pravastatin MDR 9.4 mg/day	Carriers of A-204C variant had reduced efficacy with respect to LDL-c lowering ability as compared to noncarriers (24.3% vs. 33.1%, respectively)	Reduced efficacy
ABCG2	c.421AA genotype (rs2231142)		32/660 healthy Finnish volunteers	Atorvastatin 20 mg/day Rosuvastatin 20 mg/day	Marked increase in the AUC of atorvastatin by 72% and of rosuvastatin by 144% in those carrying the c.421AA genotype as compared to	Increase AUC

					those with the c.421CC	
		Prospective cohort	305 Chinese subjects hypercholesterolemia	Rosuvastatin 10 mg/day	Gene-dose-dependent greater reduction in LDL-c in those carrying the c.421AA variant, 6.9% greater reduction in LDL-C level equivalent to double-dose effect	Enhance efficacy
		Prospective, blinded endpoint RCT	601 Caucasian subjects following MI	Rosuvastatin 10 mg/day or simvastatin 40 mg/day	Those with at least one variant (c.4211AA or CYP3A5 allele) treated with rosuvastatin were 2.3 times more likely to achieve LDL-c target than simvastatin	Enhance efficacy
ABCB1	1236T variant C3435T variant	Crossover PK study	24/534 healthy Finnish volunteers	Simvastatin Atorvastatin	AUC for homozygous TTT haplotype significantly greater (60%) for simvastatin than the CGC homozygote. Replicated for atorvastatin >55%. TTT 24% longer half-life	Increase AUC
	C3435T variant (rs1045642)	Placebo-controlled RCT	334 subjects hypercholesterolemia	Atorvastatin 10 mg/day	Female homozygotes for C3435T experienced significantly	Altered efficacy

					reduced decrease in LDL-c concentrations with larger increase in HDL-c, relevant to variant allele	
	1236T variant (rs1128503)	Prospective cohort	116 subjects hypercholesterolemia	Simvastatin 20 mg/day 6 months	1236T significantly associated with enhanced efficacy (p = 0.042), with respect to lowering of Tc (29% vs. 24.2%) and LDL-c (39.6% vs. 33.8%) compared to wild type	Enhance efficacy
ApoE	ApoE 2,3,4	Outpatient prospective cohort	401 Spanish subjects hypercholesterolemia	Pravastatin 20 mg/day 16 weeks	Once adjusted for age, gender, BMI, and baseline lipid levels, ApoE2 did not significantly influence the plasma lipid and lipoprotein response	Unaffec
		RCT, double-blind, placebo control	328 subjects 1° hypercholesterolemia	Atorvastatin 10 mg/day 12 months	Males with E2 allele significantly reduced LDL-c (44%; p = 0.021), Tc (34%; p = 0.033) and triglyceride levels (27%; p = 0.049) compared to	E2 enhance efficacy

					E3 and E4 alleles	
		Longitudinal observational	1383 diabetic adults	Simvastatin (or alternative expressed as equivalent)	32% of E4 homozygotes failed to achieve LDL-c treatment target compared to those expressing E2 variant, none of whom failed to achieve target	E4 reduc efficacy
		RCT, double-blind, placebo control	320 CHD subjects	Fluvastatin 40 mg/day	ApoE3/3 genotype associated with enhanced efficacy with reductions in Tc (20.4% vs. 15.4%; p = 0.01) and LDL-c (28.7% vs. 22.7%; p = 0.03) compared with 3/3 or 4/4	E3 enhance efficacy
CETP	*Taq1B*	RCT, double-blind, placebo control	807 CHD males	Pravastatin 40 mg/day	B1 variant associated with both higher plasma CETP (mean 2.29 ± 0.62 μg/ml for the B1B1 genotype vs. 1.76 ± 0.51 μg/ml for the B2B2 genotype) and lower HDL-c (34 ± 8 vs. 39 ± 10 mg/dl) B2B2 genotype	B2B2 increase mortality risk

					inadequate response to pravastatin	
		Case–control study	2531 subjects	Various	Individuals expressing B1B2 or B2B2 had significant reduction in CV events when treated with statins compared to B1B1.	B2B2 enhance efficacy
HMGCoA	SNP12 (RS17244841) SNP 29 (rs17238540)	Community-based RCT	1536 subjects	Pravastatin 40 mg/day	Overall statin efficacy reduced by 21.8% and 22.3% for carriers of SNP12 and SNP29, respectively, due to ~19% smaller LDL-c reduction after pravastatin ($p < 0.005$)	Reduced efficacy
		Longitudinal Observational	1601 subjects	Various (expressed as equivalent simvastatin dose)	51% of individuals heterozygous for G allele failed to reach target (<4 mmol/l) compared to the 28% homozygous for the more frequent T allele (adjusted OR (95% CI) for failure 2.93 (1.61–5.34) mmol/l, $p = 0.0005$)	Reduced efficacy
		Prospective	596 Caucasian/326	Simvastatin	Black males	H7 redu

		cohort	African-American	40 mg/day 6 weeks	with H7 haplotype significantly reduced efficacy (LDL-c reduction) compared to noncarriers (−36.9 ± 1.0% vs. −40.6 ± 1.3%; *p* = 0.02)	efficacy
LDLR	Several		Familial hypercholesterolemia	Various	Variable reduction in LDL-c	Inconclu

1° primary, *CI* confidence interval, *HR* hazard ratio, *CAD* coronary artery disease, *RCT* randomized control trial, *ApoE* apolipoprotein E, *CETP* cholesteryl ester transfer protein, *LDLR* low-density lipoprotein receptor, *mg* milligrams, *vs*. versus, *LDL-c* low-density lipoprotein cholesterol, *Tc* total cholesterol, *AUC* area under curve, *BMI* body mass index, *CV* cardiovascular, *SNP* single-nucleotide polymorphism, *OR* odds ratio, *MDR* mean dose range

Cholesteryl Ester Transfer Protein (CETP)

CETP is involved in transport of triglycerides from VLDL and LDL in exchange for cholesterol esters in HDL [38]. Variants of the CETP gene have been associated with variable response to statin therapy. REGRESS demonstrated that in men with coronary atherosclerosis, presence of the B1 polymorphism was associated with both higher CETP concentrations and lower HDL and that this genotype was associated with significant progression of atherosclerosis (decrease in mean luminal diameter: 0.14 ± 0.21 mm for the B1B1 genotype vs. 0.05 ± 0.22 mm for the B2B2 genotype) [44], an effect abolished by pravastatin therapy only in the B1B1 and not the B2B2 genotype group. This was also shown in another study where carriers of the B1B1 polymorphism responded better to atorvastatin, in terms of a 7.2% increase in plasma HDL-c as compared to 6.1% in B1B2 and only 0.5% in B2B2 [51]. By contrast, presence of the B2 allele has been shown to significantly reduce cardiovascular events in response to statin therapy [45]. Although 10-year follow-up of the REGRESS trial showed that B2B2

carriers (CETP gene variation absent) had higher HDL cholesterol, they still had higher all-cause mortality (hazard ratio [HR] 1.30; $p = 0.004$) [52]. Large-scale meta-analyses have confirmed the association between the B2B2 homozygous allele and increased HDL-c as compared to B1B1; however, no association between allele type and response to and efficacy of pravastatin therapy was found [53]. Recently, two regulatory variants have been identified in CETP through the use of splice isoform measurements in human liver samples. The authors identified two SNPs in near complete linkage disequilibrium (LD) tightly associated with Δ9 CETP splicing. Analysis of the WhiteHall II study population [6] showed that both rs5883 and rs9930761 were predictive of increased coronary events in at-risk, male subjects [7].

At present, although clear effects of CETP polymorphisms upon both LDL-c and HDL-c are demonstrable, the clinical significance remains unclear.

HMG-CoA Reductase Enzyme

HMG-CoA reductase (HMGCoR) enzyme plays an important role in cholesterol homeostasis. Single nucleotide polymorphism (SNP) 12 (rs17244841) and SNP 29 (rs17238540) are common, and tightly linked intronic SNPs (linkage disequilibrium [LD] $r^2 = 0.90$; heterozygote prevalence = 6.7% for both), that significantly associate with reduced efficacy in response to pravastatin therapy; about 19% reduced LDL-c lowering compared to those with neither haplotype ($p < 0.001$) [46]. Similarly, the GO-DARTS study, including various statins, demonstrated 51% and 28% failure to reach treatment target in patients carrying either the G allele or T allele for SNP 29 [48]. For simvastatin response, two common HMGCoR haplotypes, H2 and H7, have been associated with reduced LDL-c lowering, most evident in African-American men with haplotype H7 [48].

Low-Density Lipoprotein Receptor (LDLR)

Familial hypercholesterolemia (FH) results from mutations in the LDLR gene. Heterozygous mutations affect 1/500 individuals [54] and increase the risk of death due to cardiovascular events by 100-fold by early adulthood [55]. As a result, statin therapy is critical to the management of this condition. To date, multiple studies [56–66], in different ethnicities with FH taking various statins ± adjunctive therapy, have investigated the link

between mutations in the LDLR gene and the lipid-lowering response to statins. There is great discrepancy, with some studies highlighting a potential genotype-dependent effect [57, 58, 61, 64, 65, 67], while equally others failed to show this association [59, 60, 62, 63]. More recently, in non-FH, a common LDLR-3′ untranslated region haplotype (LDLR5) has been associated with reduced simvastatin efficacy in terms of both Tc and LDL-c reduction in Blacks but not Whites. When combined with the presence of HMGCoA haplotypes (H2 and H7), the response was further reduced [68]. The results are equivocal largely due to lack of statistical power and insufficient sample size. Because of the relative rarity of familial hypercholesterolemia, the gene–drug interactions reported in these studies may not prove generalizable to the population as a whole. However, observations in such patients are crucial to our understanding of nonfamilial hypercholesterolemia.

Pleiotropic Effects

Toll-Like Receptor 4 (TLR4)

Recent experimental work in stroke has identified a role for inflammation in atherosclerotic plaque rupture [69, 70]. TLR4, an activator of the innate immune response to both sterile and infectious injury, has been detected in atherosclerotic plaque lesions in both mice and humans [71, 72]. Indeed, in experimental models, cerebral ischemia is associated with significant overexpression of high-mobility group box-1 (HMGB1), TLR4, and the receptor for advanced glycation end products (RAGE), which together trigger transcription of various proinflammatory mediators through activation of NF-κB. In rodents, both simvastatin and atorvastatin have demonstrated neuroprotective effects [73, 74]. Atorvastatin is associated with improved neurological outcome, significant reduction in infarct size, and decreased expression of HMGB1, TLR4, RAGE, and NF-κ(kappa)B [73]. Carriers of the TLR4 Asp299Gly polymorphism have been shown to have decreased risk of carotid atherosclerosis (OR, 0.54; 9% confidence interval (CI), 0.32–0.98; $p = 0.05$) associated with a blunted atherosclerotic inflammatory response [75]. In men with symptomatic CAD treated with pravastatin, the Asp299Gly polymorphism was significantly associated with a massively reduced risk of cardiovascular events from 29.6% down to 2.0% ($p = 0.0002$) [76] as

compared to noncarriers. To date, this is the only study to link TLR4 genotype and response to statin therapy, and further studies are required.

Kinesin-Like Protein 6 (KIF6)

Prospective studies have suggested an increased risk of CAD in individuals carrying the Trp719Arg allele [77–79]. Three large clinical trials have confirmed that carriers of the Trp719Arg (rs20455) allele display significantly greater benefit with pravastatin compared to noncarriers [80–82]. However, a meta-analysis of 19 studies of 17,000 individuals did not find a link between this variant allele and increased risk of CAD [83]. As in the case for polymorphisms in CETP, this calls into question the clinical utility of a genetic test used to identify those at greatest risk of CAD and by extension, subject to greatest benefit from statin therapy. If it proves to be the case that, although associated with impaired biochemical parameters (Tc and LDL-c), genetic variance is not associated with any alteration to disease risk, then testing for efficacy is not useful. Further prospective studies of users and nonusers of statins at risk of CAD are required to clarify the validity of this interaction.

Proprotein Convertase Subtilisin/Kexin Type 9 (PCSK9)

Cholesterol-rich LDL particles are taken into tissues via LDL-receptor (LDL-R)-mediated endocytosis, a process that is tightly regulated. Proprotein convertase subtilisin/kexin type 9 (PCSK9) mediates degradation of the LDL-R protein, resulting in increased plasma LDL-c. Loss-of-function mutations in PCSK9 are associated with reduced cardiovascular risk as a result of lifelong lowering of LDL-c [84, 85]. Conversely, gain-of-function mutations are associated with elevated LDL-c, similar to levels seen in familial hypercholesterolemia [86]. In some patients with FH, carriage of variant PCSK9 polymorphisms including R46L and N157K [87] and leucine repeat polymorphisms [88] has been associated with greater responsiveness to statins. Statins have been shown to increase PCSK9 levels [89] and recently, the Justification for Use of Statins in Prevention (JUPITER) trial [90] demonstrated rosuvastatin increased PCSK9 levels by 35% in women and 28% in men, accompanied by significant reductions in Tc, LDL-c and triglycerides, compared to the placebo arm. Of those treated with

rosuvastatin, greater reductions in LDL-c were significantly associated with increases in PCSK9 ($r = -0.15$, $p < 0.0005$). Similar results have been shown for patients treated with atorvastatin [91]. Carriage of the R46L allele (rs11591147) was associated with 19% lower PCSK9 and lower baseline LDL-c; however, the investigators did not identify any association between this SNP and altered response to rosuvastatin [90]. Given the mechanism of action of PCSK9, it would be expected that increased levels following statin therapy would be associated with a reduced drug response; however, the converse finding is true. This requires further mechanistic clarification. Several monoclonal antibodies targeting PCSK9 have been developed[1]; however, at present, there is insufficient evidence to recommend the use of PCSK9 levels or genotyping in the prediction or monitoring of statin response.

Genome-Wide Association Studies (GWASs)

GWASs have identified several loci associated with plasma lipids and lipid metabolism [92–95]; a limited few have been performed in response to statin treatment [96–98]. In 2009, the Treating to New Targets study genotyped 1984 individuals over almost 300,000 SNPs across the genome for association with response to atorvastatin 10 mg/day taken for 8 weeks. This study did not identify any SNPs that were found to be significant at the whole genome level ($p > 1 \times 10^{-7}$ for all associations) [97]. In 2010, Barber and colleagues [96] combined GWAS analysis across three clinical trials including a total of 3932 subjects taking simvastatin (40 mg/day, 6 weeks), pravastatin (40 mg/day, 24 weeks), or atorvastatin (10 mg/day, 8 weeks) Of particular significance, Bayesian analysis assigned 84% probability that SNP rs8014194, located within CLMN gene on chromosome 14, was strongly associated with statin-mediated change in total cholesterol ($p = 1.8 \times 10^{-8}$). In addition, SNP rs4420638 located in APOC1 near APOE was assigned 51% probability by Bayesian analysis that it is associated with change in LDL-c. Similarly, the Prospective Study of Pravastatin in the Elderly at Risk/Pharmacogenetic Study of Statins in the Elderly at risk (PROSPER/PHASE) study of pravastatin use in 5244 elderly (72–85 years) subjects identified 42 SNPs reaching the GWAS significant threshold of $p = 5.0 \times 10^{-8}$ in five genomic loci (APOE/APOC1, LDLR, FADS2/FEN1, HMGCR, PSRC1/CELSR5). The top SNP (rs445925, chromosome 19) was

identified ($p = 2.8 \times 10^{-30}$) in the APOC1 gene, located near the APOE gene. Three out of the five loci (APOE/APOC1, HMGCR, PSRC1/CELSR5) have been replicated in two independent, albeit small cohorts [98]. Most recently a pharmacogenetic meta-analysis of 18,956 patients who had undergone GWAS, which was validated in 22,318 patients, identified SNPs in four genes (SORT1, LPA, SLCO1B1 and APOE) as determinants of LDL cholesterol lowering in response to statins [8]. However, these four genes only account for 5% of the variance indicating that it would not be clinically and economically effective to use these genes to determine statin response. Other factors, most likely non-genetic, determine outcome from statins and these are likely to include dose, adherence, disease sub-type, predisposition to vascular disease, overall tolerability, co-medications and interactions, diet and exercise. Further studies integrating all of these factors into one model will likely raise the predictability of statin response.

Statin-Related Myopathy

Several genetic markers have been associated with statin muscle toxicity but, in many cases, have either not been replicated or have an effect size which would not be considered to be of clinical relevance.

Pharmacokinetic Factors Determining Myopathy Risk

Cytochrome P450 Enzymes

There is a lack of uniformity among studies investigating the impact of various CYP enzymes on the tolerability of statins. The CYP2D6*4 allele has been associated with an increased risk of atorvastatin [99] and simvastatin-induced muscle effects [18]. However, other studies have failed to find this association [100]. Surprisingly, no association has been found between CYP3A4 [30, 99, 101] and CYP3A5 [30, 99, 100, 102] polymorphisms and myopathy, despite the importance of CYP3A4 as the primary metabolic pathway for most lipophilic statins.

ATP-Binding Cassette G2 Efflux Transporter (ABCG2)

ABCG2 is located within intestinal epithelial cells, hepatocytes, renal tubule cells, and endothelial cells of the blood–brain barrier [103]. Most statins are substrates of ABCG2 [24, 104, 105], and carriers of the reduced function c421 A>A polymorphism have been associated with greater AUC with respect to rosuvastatin (144%), simvastatin (111%), and atorvastatin (72%) [29, 106], resulting from decreased intestinal efflux leading to greater bioavailability. No studies however have yet identified that ABCG2 polymorphisms predispose to myopathy.

SLCO1B1

One of the main carriers involved in statin transport is the solute carrier organic anion transporter family member 1B1 (SLC01B1), which regulates the influx of statins from the portal blood into the hepatocyte [107]. The SLCO1B1 gene encodes the organic anion-transporting polypeptide 1B1 (OATP1B1) influx transporter and can be divided into five haplotypes: *1a wild type, *1b usual transport activity and *5 (c.521T>C alone), *15 (combined c.521T>C and c.388A>G), and *17, all with reduced transport activity. All statins are substrates of OATP1B1 [103], and carriers of at least one copy of the *5 or *15 have higher statin exposure, as represented by higher AUCs, demonstrated in those taking atorvastatin [108], rosuvastatin [108], pravastatin [109], and simvastatin [110]. A study involving 77 patients with statin-induced myopathy showed the *5 SLCO1B1 c.521T>C SNP to be a significant risk factor [111]. The effects of SLCO1B1 polymorphism are largest with simvastatin, the AUC of the active metabolite being 221% greater in individuals with the c.521CC genotype than those with the c.521TT genotype [110]. Variation in the SLCO1B1 gene, in particular, the *5 allele (rs4149056), has been associated with a higher rate of statin intolerance [112] and high risk of developing myopathy in two large cohorts taking high-dose simvastatin therapy [113] (see Table 17.3). The frequency of the noncoding rs4149056 minor allele is greater than 20% in European populations (HapMap data) and in this ethnicity, is in nearly complete LD (LD = 97) with the nonsynonymous rs4149056 polymorphism [113]. In a replication cohort of individuals taking lower-dose simvastatin (mean 30 mg/day), the odds ratio (OR) of myopathy was 3.2 per genotype or 2.3 per “C” allele [114]. Although lower than the findings in the high-dose subset (OR 4.5 per “C” allele) [113], the risk is similar to the findings in the Heart Protection Study (HPS) cohort [113] (2.6 per “C” allele) of individuals taking 40 mg/day of

simvastatin. Importantly, although the effect was seen only in individuals taking simvastatin and not atorvastatin, the sample size was not sufficiently powered in the atorvastatin group to detect an association, and hence, the impact of rs4149056 on myopathy in alternative statins should not be excluded on this basis. Certainly, in individuals treated with pravastatin, no increase in risk of composite adverse events (discontinuation, myalgia, or CK >3 upper limit of normal) was identified in carriers of the *5 variant. In contrast, those treated with simvastatin, and in particular females, presented a twofold relative risk of mild myopathy coexistent with a greater accumulation of simvastatin metabolites, an effect that does not occur with pravastatin, possibly explaining the difference in outcomes [116]. Likewise, Hermann and colleagues did not identify an association between polymorphisms in SLCO1B1 and atorvastatin-induced myopathy in 13 subjects, despite 2.4- and 3.1-fold higher systemic exposures of the metabolites atorvastatin lactone ($p < 0.01$) and *p*-hydroxy atorvastatin ($p < 0.01$) [102]. However, the limited sample size restricts the validity of this finding. Carriage of the *15 polymorphism has also been associated with increased incidence of myopathy in Japanese individuals taking atorvastatin or pravastatin [101, 115]. In addition, Morimoto et al. defined a novel variant in exon 12 of the SLCO1B1 gene (1628T>G) that has been associated with pravastatin-induced muscle toxicity [115].

Table 17.3 Studies involving polymorphisms in the SLCO1B1 gene and association with statin-related myopathy

Gene	Allele/SNP	Study	Patient demographics	Statin	Case/control	Outcomes
SLCO1B1	*5 (rs4149056)	SEARCH RCT	12,064 MI Europeans	Simvastatin 80 mg vs. 20 mg	96/96 80 mg 8 20 mg	OR for myopathy 4.5 (95% CI 2.6–7.7)/copy of "C" allele
		HPS (replication)	20,536 diabetes/vascular disease	Simvastatin 40 mg vs. placebo	161664	OR for myopathy 2.6 (95% CI 1.3–5)/copy of "C allele
			Dutch retrospective case/control	Simvastatin mean 30 mg/day	25/83	rs4149056 genotype significantly associated wi myopathy in
				Atorvastatin		

					simvastatin (OR 2.3/“C” allele) but not atorvastatin
*15 (*1b&*5)		Japanese	Atorvastatin Pravastatin	10/26	Increased incidence of myopathy wit both atorvastatin and pravastati
*5&*15	GO-DARTS observational	4196 diabetics			1275 tolerant to statins and 816 intolerant Carriage of rs4149056 associated wi higher rates o intolerance (OR = 2.05, *p* = 0.043)
*5	STRENGTH	509 subjects hypercholesterolemia	Atorvastatin 10 mg vs. 80 mg Simvastatin 20 mg vs. 80 mg Pravastatin 10 mg vs. 40 mg		Carriers of th *5 allele or female sex were at greate risk of developing composite myopathic events, with * carriage presenting twofold relative risk o mild myopath
&1b, *4, *5, *15	24-h PK study	14 subjects atorvastatin-related myopathy	Atorvastatin	13/15	No associatio between polymorphism in SLCO1B1 and myopathy

RCT randomized control trial, *HPS* Heart Protection Society, *SEARCH* study of the effectiveness of additional reductions in cholesterol and homocysteine, *GO-DARTS* genetics of diabetes audit and research in Tayside Scotland, *STRENGTH* statin response examined by genetic haplotype markers, *PK* pharmacokinetic, *MI* myocardial infarction, *mg* milligrams, *OR* odds ratio, *CI* confidence interval

Pharmacodynamic Factors Determining Myopathy Risk

Coenzyme Q10

Through blocking the intermediate farnesyl pyrophosphate [117], statins have been shown to reduce circulating coenzyme Q10 levels in both animal models [118] and human patients [119]. Coenzyme Q10 protects mitochondria from oxidative stress resulting from the production of free radical species [120]. The consequent mitochondrial dysfunction may contribute to statin myopathy. In a trial of more than 1000 patients treated with atorvastatin or lovastatin, reductions in plasma coenzyme Q10 levels of 38% and 27%, respectively, were noted [121]. An ongoing study of individuals with atorvastatin or rosuvastatin-induced myopathy identified a significant association between statin intolerance and the rs4693075 variant within the COQ2 gene for both rosuvastatin (OR 2.6, 95% CI 1.7–4.4 beta-coefficient 1.86 $p < 0.001$) and atorvastatin-treated subjects (OR 3.1, 5% CI 1.9–6.4 beta-coefficient 2.08 $p < 0.001$) [122]. The COQ2 gene is involved in the electron transport chain and coenzyme Q10 synthesis, and mutations in this gene were associated with severe inherited myopathies in one study [123], however, this was not found in another [111]. A large systematic review concluded that at present, due to a dearth in studies investigating the impact of coenzyme Q10 treatment for statin myopathy, there is insufficient evidence to confirm that coenzyme Q10 is implicated in the pathogenesis [124]. Indeed, some studies have not shown any improvement in tolerability as a result of coenzyme Q10 therapy [125, 126]. However, the supplement is not known to cause any health risk, and a large, prospective trial is warranted. What is more, further genetic studies to identify the impact of genetic variants, particularly in high-risk groups requiring high-dose statin therapy including familial hypercholesterolemia, are necessary.

Genome-Wide Association Studies

GWAS provided the first statistically conclusive evidence linking genetic variants and risk of statin-induced myopathy. SLCO1B1 was associated with definitive or incipient myopathy in individuals taking 80 mg/day of simvastatin [113]. The SNP demonstrating the strongest association with

myopathy was the *5 variant. The attributable risk to patients taking 80 mg simvastatin was 60% for the *5 polymorphism with a C allele (rs4149056) in the SLCO1B1 gene [113]. This association was replicated in patients taking 40 mg/day and corroborated by other investigators [112, 116]. Although a candidate gene study has also shown an association between SLCO1B1 and cerivastatin-induced rhabdomyolysis, a genome-wide study of the same patients showed an association with the ryanodine receptor 2 gene (RYR2), although this did not reach genome-wide significance [127].

Clinical Implications

Statins are widely used, with about 38 million people receiving treatment with statins in the US alone [128]. Although numerous studies support an association between various polymorphisms in the pharmacokinetic and pharmacodynamic pathways and reduced efficacy in terms of Tc and LDL-c reduction, very few studies have investigated whether this leads to clear changes in event reduction. Future endeavors to clarify this association will need to involve larger patient numbers and genome-wide approaches that not only focus on cholesterol lowering but also on reduction in CV events. An important issue here is that not all statins are the same, particularly in relation to their pharmacokinetics, and thus, results obtained with one statin may not necessarily be applicable to another statin. The value of undertaking such pharmacogenetic strategies will also need to be shown to be more clinically and cost-effective than clinical management strategies (increasing dose, changing the statin, using combination therapy) before this will be acceptable in clinical practice [9].

Genetic screening for SNPs, particularly in the SCLO1B1 gene, has shown to predict risk of simvastatin-associated myopathy, particularly in those patients taking higher doses. Indeed, Niemi et al. have recommended use of a genotype-dependent “maximum-dosing strategy” [103] and that simvastatin be avoided in carriers of the c.521T>C SNP. However, the positive and negative predictive value of such genetic tests still remains to be established, and importantly it has not been shown definitively whether the SLCO1B1 polymorphism shows the same effect with other statins such as atorvastatin. Indeed, a potential alternative strategy to reduce the impact of the SLCO1B1 polymorphism in susceptible individuals could include consideration of the use of potent statins that are less sensitive to reduced

SLCO1B1 activity, such as rosuvastatin or fluvastatin, as they utilize additional hepatic uptake transporters including SLCO1B3 and SLCO2B1 [104, 129]. Nevertheless, for simvastatin the European summary of product characteristics warns of the increased risk of myopathy in those individuals on 80 mg carrying the variant SLCO1B1 allele.

Antihypertensive Agents

Hypertension represents one of the most important modifiable risk factors for the development of cardiovascular disease, and the lifetime risk of stroke, in particular, increases dramatically with increasing blood pressure (BP). Following first stroke, patients with a normal BP (<120/80 mmHg) have approximately half the lifetime risk of stroke compared with those with high BP (≥140/90 mmHg) [130]. In individuals with diastolic BP (DBP) greater than 115 mmHg, the NNT to prevent one stroke is 29 [131]. In trials conducted in patients with less severe hypertension (DBP 90–110 mmHg), this increases to 118 [132–134]. Many patients exhibit resistance to antihypertensive agents, which can be defined as "blood pressure that remains above goal in spite of the concurrent use of three antihypertensive agents of different classes." [135] The Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial (ALLHAT) study, including ethnically diverse participants, concluded that 34% of patients taking an average of two medications still remained uncontrolled after 5 years of treatment [136]. Genetic factors have been implicated in response to various antihypertensive agents, but none of them have shown clinical utility that has been adequate enough to translate the findings into clinical practice.

Angiotensin-Converting Enzyme (ACE) Inhibitors

ACE inhibitors are recommended as first-line treatment for elevated blood pressure in individuals under the age of 55.[2] ACE inhibitors exert their hypotensive effect by reducing the activity of the renin–angiotensin–aldosterone system, an important regulator of blood pressure.

There is significant interracial variability in response to ACE inhibitors. Blacks are said to exhibit reduced efficacy, and as a result, ACE inhibitors are not currently recommended as first-line therapy in Blacks of any age. Blacks develop hypertension earlier than Whites, with much higher average

blood pressures and worse disease severity [137]. Mortality due to hypertension is fourfold to fivefold more likely in African-Americans than Whites [138]. Blacks have been shown to have lower levels of plasma renin activity than Whites [139], which may, in part, account for the reduced efficacy of ACE inhibitor monotherapy seen in Blacks compared to Whites. In addition, dietary salt intake has been shown to antagonize the hypotensive effect of ACE inhibitors [140], which may contribute as an environmental factor affecting ACE inhibitor efficacy. Captopril has been shown to lead to 2.7 mmHg greater reduction in diastolic blood pressure in Whites than Blacks ($p < 0.001$) [141]. A systematic review of antihypertensive agents in Black patients identified that calcium-channel blockers were the only agent effective for all blood pressure outcomes in all subgroups of Black participants, including diastolic BP >110 mmHg [142]. The reviewers commented that most investigators titrate doses only moderately. Importantly, substantial improvement in BP response to captopril in African-Americans has been demonstrated in BP-lowering trials that have included dose escalation [143, 144], suggesting that ACE inhibitors can still be an effective treatment in Blacks, providing a higher average dose is used.

Pharmacokinetic Factors Determining Response to ACE Inhibitors

No studies have identified any genetic variables associated with the pharmacokinetic processing of ACE inhibitors.

Pharmacodynamic Factors Determining Response to ACE Inhibitors

Substantial interindividual variability in serum ACE level exists as a result of an insertion/deletion (I/D) polymorphism consisting of a 287 base pair *alu* repeat sequence within intron 6 of the ACE gene [145]. Homozygotes for the deletion allele (DD) exhibit serum ACE levels that are on average twice as high as those homozygous for the insertion allele (II). Heterozygotes (ID) are predictably within an intermediate range [145]. Both the ACE I/D polymorphism and the Met235Thr polymorphism in the angiotensinogen gene have also been associated with the development of hypertension [146–149]. Specifically, for the M235T polymorphism, in hypertensive

patients with previous stroke, ACE inhibitor use has been associated with lower stroke risk in Thr homozygotes (OR = 0.37, 95% CI = 0.14–0.99) than among Met carriers (OR = 1.4, 95% CI = 0.88–2.4; *P* for interaction = 0.02) compared to nonusers of ACE inhibitors [150]. However, in a larger cohort of 4097 hypertensive patients, carriage of the M235T polymorphism did not lead to a significantly increased risk of stroke in users of ACE inhibitors [151].

It would follow that variation in baseline enzyme activity, for example, high ACE levels necessitating higher levels of ACE inhibition required for an equivalent effect, might affect clinical response to ACE inhibitor therapy. Limited data are available regarding the pharmacogenetic impact of ACE variations on therapeutic efficacy in hypertension, and indeed, a relationship between altered baseline ACE activities has not been correlated with significant alteration in blood pressure response to ACE inhibitors. Blood pressure and plasma renin activity 1 h after 50 mg captopril administration in 82 patients with untreated essential hypertension were unaffected by the presence of DD, ID, and II genotypes. Likewise, despite the 56% higher ACE activity in individuals with the DD genotype, with the absolute fall in ACE activity following 10 mg of enalapril being significantly greater in the DD subjects, this did not correlate with a significant fall in mean arterial blood pressure [152]. In 5688 with prior stroke or TIA, the presence of the ACE genotype was not found to modify the BP response to perindopril [153]. Similarly, in two large cohorts (8907 with stable CAD and 3571 with cerebrovascular disease), no significant association between genetic determinants of the renin–angiotensin–aldosterone system and BP response to perindopril were identified, despite several SNPs highlighted as being associated with hypertension [154]. Future, long-term large-scale studies focusing on both ACE inhibitor polymorphisms and associated interlinking pathways are required to delineate the relationship between serum ACE levels and ACE inhibitor efficacy. At present, insufficient evidence exists to recommend pharmacogenetic screening of individuals prior to ACE inhibitor therapy.

Two genetic scoring systems have been devised based on SNPs in the angiotensin II receptor type 1 (AGTR1) and bradykinin receptor B1 (BDKRB1) genes, as well as the ACE gene (ACE) and ABO blood group genes (ABO). These scoring systems have, respectively been shown to predict ACEI efficacy and ACE activity in Europeans with IHD and in

Asians under the age of 40 with hypertension [10–13]. However, a retrospective cohort study based on patients participating in the ECHOS severe heart failure trial failed to find an association between either of the analyzed pharmacogenetic scores and fatal outcomes in ACEI-treated patients with CHF [14].

ACE Inhibitor-Induced Angioedema and Cough

ACE inhibitors are known to cause a nonallergic bradykinin-mediated angioedema in 0.1–0.7% of users [155, 156]. The exact pathophysiology remains unclear; however, bradykinin and substance P have been implicated in the effect [157, 158]. Moreover, black Americans appear to be at greater risk [159, 160], suggesting a possible genetic predisposition to the adverse response. Currently, no marker exists to aid identification of individuals at risk of ACE inhibitor-induced angioedema. During ACE inhibition, substance P and bradykinin are inactivated by aminopeptidase P (APP) and dipeptidyl peptidase IV (DPPIV), respectively [161]. The C2399A (rs3788853) SNP, located within XPNPEP2 gene that encodes for APP, is seen at greater frequency in individuals with ACE inhibitor-induced angioedema (ACEiIA) than in controls [162]. This association has been replicated in a larger cohort (n = 169), and the C-2399A genotype was associated with an increased risk of angioedema in men only (OR 2.17, 1.09–4.32, p = 0.03) [163]. Studies that have investigated whether ACE or bradykinin B_2 gene polymorphisms are associated with development of angioedema have, to date, found no causal association [164, 165]. Due to the rarity of this condition, the sample sizes were small (maximum 65), and hence, a larger population would be required to definitively exclude this association and identify new associations.

ACE-inhibitor induced cough is more common (up to 20% of patients), but the pathogenesis is not completely clear. A GWAS of 1595 cases and 5485 controls identified an intronic SNP in KCNIP4 (rs1495509), which was replicated in two other cohorts, giving a combined P value of 1.9×10^{-9} (OR 1.23) [15]. This is unlikely to be clinically useful but nevertheless provides an insight into mechanisms.

Angiotensin-II Receptor Blockers (ARBs)

No clinically relevant pharmacogenetic studies have been performed for ARBs.

Beta(β)-Blockers

Beta(β)-blockers are recommended in the management of hypertension in those with intolerance or contraindication to ACE inhibitor or angiotensin II receptor blocker (AIIRB) therapy, in those of child bearing potential, and also where there is evidence of sympathetic drive. The antihypertensive effect of β(beta)-blockers results from antagonism of β(beta)1-adrenorecptors expressed on juxtaglomerular cells of the kidney, thereby decreasing the release of renin [166].

Pharmacokinetic Factors Determining Response to β-Blockers

CYP2D6 and UGT1A1

Metoprolol is predominantly metabolized by CYP2D6 [167, 168]. As described previously, CYP2D6 is genetically polymorphic, and carriers of the "poor metabolizer" (PM) phenotype would be expected to display significant increases in the bioavailability and half-life of the drug with consequent increases in both peak and steady-state concentrations. Several studies have identified wide interindividual variation in the plasma concentrations of metoprolol (Table 17.4) [169, 176, 177]. Median dose-corrected plasma concentrations of metoprolol have been shown to be 6.2- and 3.9-fold higher in individuals carrying the PM or "intermediate metabolizer" (IM) phenotypes, respectively [171]. A prospective study of metoprolol in Caucasians showed that plasma metoprolol concentrations were 4.9-fold higher in PMs and that this was significantly associated with greater clinical efficacy in terms of heart rate and mean arterial BP reduction [173]. Similarly, in Russian patients treated with metoprolol following myocardial infarction (MI), the PM phenotype was associated with pronounced bradycardia as compared to the "ultrarapid metabolizers" (URM) in whom metoprolol failed to achieve a therapeutic effect. The mean heart rate was lower in the PMs and increased with increasing number of active CYP2D6 genes ($p < 0.05$) [172]. Conversely, no association between CYP2D6

metabolizer status and the occurrence of adverse events was identified in a prospective trial of Caucasians treated with metoprolol for hypertension [170], and similarly no association with response has been identified in heart failure patients [174]. These studies, however, included small numbers with the PM phenotype, 4 [170] and 8 [174], respectively, as compared to the 17 included in the recent Rau et al. study [173] and must therefore be viewed with caution. It is also interesting to note that a recent twin study [16] showed that the heritability in metoprolol area-under-the-curve was 91%, but CYPD6 accounted for only 39% of the variation indicating that other genetic factors, yet unidentified, are also important.

Table 17.4 Studies investigating the pharmacogenetic factors implicated in the altered pharmacokinetic response to β(beta)-blockers

Gene	Allele	Study	Patient demographics	Drug	Outcome	Implication	A
CYP2D6	*1,*3*-*5,*9 *8,*10,*17	PK	91 CV disease	Metoprolol (>6 m)	Wide variation in plasma concentration, not significantly associated with genotype	No association	I [
	*3–*10, *41	Prospective	121 Caucasian hypertensive	Metoprolol (>6 m)	No association between genotype and adverse effects		F [
	*1&*2 *3–*8,*12,*14,*15 *9, *10, *41		91 Caucasian	Metoprolol median 12.6 m	Median adjusted plasma metoprolol concentration 6.2× and 3.9× higher in PMs or IMs, respectively	Clinical impact not evaluated	F [
	*3,*4,*10	Retrospective cohort, PK	187 Russian acute MI	Metoprolol	PMs exhibit most profound bradycardia. Therapeutic effect not achieved in URMs	URM reduced efficacy	C e
	*3–*6	Prospective double-blind	84 Caucasian (95%	Metoprolol	Metoprolol plasma	PM enhanced	F [

		longitudinal	hypertensive)		concentrations 4.9× higher in PMs	efficacy	
	*10,*41				Greater reductions in HR& BP in PMs		
CYP2D6 UGTA1	*1–*10 TA repeat	Retrospective	93 CHF	Metoprolol or carvedilol	CYP2D6 PMs had higher doses of carvedilol ($p < 0.02$). No association between CYP2D6 or UGT1A1 SNPs alone or in haplotype with clinical response to β-blockers	No association	I a
CYP2D6 UGTA1	*4,*5,*10,*14,*36 *37,*47 *6,*28	Retrospective PK	40 Japanese CHF, angina	Carvedilol	The frequency of CYP2D6*10 and UGT1A1*6 was higher in subjects with low-level glucuronidation activity	Clinical impact not evaluated	T a

PK pharmacokinetic, *CV* cardiovascular, *CHF* chronic heart failure, *MI* myocardial infarction, *>6 m* greater than 6 months, *x* fold, *m* months, *PM* poor metabolizer, *URM* ultrarapid metabolizer

Carvedilol is also metabolized by CYP2D6, along with its major metabolic pathway involving urine diphosphate glucuronosyltransferase 1A1 (UGT1A1) [175, 178]. The frequency of CYP2D6*10 and UGT1A1*6 was significantly higher in subjects found to have low-glucuronidation activity in Japanese patients with heart failure and/or angina [175]. In a separate cohort of heart failure patients, both CYP2D6 and UGT1A1 polymorphisms were not associated with response to carvedilol or metoprolol, despite PMs of CYP2D6 having significantly higher doses of carvedilol [174].

On balance, PK data supports the notion that greater plasma

concentrations of metoprolol and carvedilol may result in a greater clinical effect and/or adverse responses. However, larger, long-term prospective studies including greater numbers of PMs are necessary to fully characterize whether this association is clinically significant.

Pharmacodynamic Factors Determining Response to β-Blockers

ADRB1

Adrenoreceptors are highly polymorphic, and there are several well-described nucleotide polymorphisms that alter receptor function [179], including Ser49Gly and Arg389Gly. Both alleles have functional consequences for the receptor: Arg389 induces a hyperfunctional receptor, and Gly49 increases receptor downregulation [180]. Studies examining the effect of common polymorphisms in the β(beta)1-adrenergic receptor (ADRB1) on blood pressure have been conflicting. For those carrying the ADRB1 Ser49Ser homozygote genotype, BP response to bisoprolol was better than in the Ser49Gly heterozygotes (1.6 and 1.4 mmHg differences in systolic and diastolic BP response, $p = 0.04$ and 0.06, respectively) [181]. Similar results were seen in the ADRB1 Gly389Gly homozygotes, in whom BP response was superior than in those with the Arg389Arg homozygote genotype. This is in direct contrast, however, to previous studies wherein the Arg389Arg homozygote was associated with better BP response to β(beta)-blockers [182–184]. INVEST [185], a randomized, controlled clinical trial of atenolol versus verapamil in patients with hypertension plus coronary artery disease, demonstrated a greater than threefold increased risk of death in those carrying the Arg389 haplotype. The risk associated with this outcome was still diminished by treatment with atenolol [185], suggesting adequate treatment effect despite this genetic predisposition. Atenolol, as compared to verapamil, was seen to exert a particular protective effect in carriers of Ser49-Arg 389, consistent with findings in BP response and in heart failure, suggesting a greater benefit of β(beta)-blockers in this group.

Genome-Wide Association Studies

GWAS involving a total of 318 African American patients with hypertension from two monotherapy studies, 150 treated with atenolol and 168 with

metoprolol. A further cohort of 141 African Americans treated with add-on atenolol therapy to their hydrochlorothiazide regime were also evaluated for genome-wide significant variants with P < 5 × 10(−8) and suggestive variants with *P* < 5 × 10(−7). The study identified better diastolic blood pressure response to beta-blockers in heterozygotes for SLC25A31 rs201279313 deletion versus wild-type genotype. Furthermore, 3-group meta-analysis validated LRRC15 rs11313667 for systolic BP response to β-blocker therapy [17].

GWAS involving Euopean-Americans with new-onset diabetes (NOD) examined patients exposed to either beta-blocker therapy or calcium-channel blocker therapy. This was then replicated in Hispanic and African Americans, and a joint meta-analysis was performed involving a total of 334 NOD cases and 806 matched controls [18]. The study identified increased odds for NOD in patients who were A/A homozygotes for rs11124945 who were exposed to the β-blocker strategy as opposed to calcium-channel blockers. In contrast, rs11124945G allele carriers had lower odds for NOD when exposed to the β-blocker strategy compared with the CCB strategy.

Calcium-Channel Blockers (CCBs)

The dihydropyridine calcium-channel blockers exert their hypotensive effect primarily through decreasing peripheral vascular resistance at the small arteriole level [186].

Pharmacokinetic Factors Determining Response to Calcium-Channel Blockers

CYP3A4

All CCBs are metabolized by CYP3A4 [187], and therefore to varying degrees also act as CYP3A4 inhibitors. CYP3A4 is also responsible for activation of the antiplatelet agent clopidogrel. As a result, three recent studies have shown that coadministration of CCBs with clopidogrel leads to reduced platelet inhibition effect and suggested this may increase the risk of atherothrombotic events (Table 17.5) [188–190]. However, this was not shown to increase cardiovascular risk (composite cardiovascular death, nonfatal MI, nonfatal stroke) in 654 patients with established CAD taking

combined CCB and clopidogrel [191]. Future, large-scale prospective studies are needed to evaluate the relationship between combination CCB and clopidogrel therapy causing reduced platelet inhibition and whether this is affected by CYP3A4 polymorphisms and, indeed, whether this affects future risk of cardiovascular events.

Table 17.5 Studies investigating the pharmacokinetic impact of coadministration of calcium-channel blockers and clopidogrel

Gene	Allele	Study	Patient demographics	Platelet function	Outcome	Clinical implication	Author
(Not assessed)		Prospective observational	200 CHD undergoing PCI	VASP	Platelet reactivity 61% higher in individuals taking both CCB and clopidogrel as opposed to clopidogrel alone (41%)	Clinical impact not assessed	Siller-Matula et al. [188]
			162 post-PCI	LTA and VerifyNow®	Platelet reactivity higher in individuals taking both CCB and clopidogrel as opposed to clopidogrel alone ($p < 0.001$ both assays). Multivariate regression analysis confirmed CCB independent predictor of reduced clopidogrel-mediated platelet inhibition ($p < 0.006$, $p < 0.004$)		Gremmel et al. [189]
			623 undergoing elective PCI	LTA	Amlodipine combined with clopidogrel associated with 2.3× increased risk of clopidogrel poor response		Harmsze et al. [190]

CYP2C19	*2		654 CHD	N/A	Concomitant CCB therapy did not increase the risk of CV event. Carriage of *2 loss-of-function allele did not influence clinical antiplatelet effect	No clinical impact	Peng et al. [191]

CAD coronary artery disease, *CV* cardiovascular, *PCI* percutaneous coronary intervention, *VASP* VASP phosphorylation/flow cytometry, *LTA* light transmission aggregometry, *CCB* calcium-channel blocker, *x* fold

Pharmacodynamic Factors Determining Response to Calcium-Channel Blockers

No studies have identified any genetic variables associated with the pharmacodynamic response to CCBs.

Genome-Wide Association Studies

A genome-wide association study involving 21,267 participants of European ancestry with pharmaceutically treated hypertension did not identify any drug-gene interactions involving calcium-channel blockers that influenced the risk of cardiovascular disease [19].

Thiazide Diuretics

Although the exact antihypertensive mechanism of thiazide diuretics is incompletely understood, the initial hypotensive effect is mediated by modest reductions in both plasma volume and cardiac output [192]. Continuing BP decline is then attenuated by activation of the renin–angiotensin–aldosterone system as a result of hypovolemia [193], a hurdle that can be overcome by coadministration of an ACE inhibitor. The hypotensive effect is maintained in the long term by reversal of the initial effect; the systemic vascular resistance falls as the plasma volume returns to near-baseline, producing an overall increase in the vascular capacity [192].

Pharmacokinetic Factors Determining Response to Thiazide Diuretics

No studies have identified any genetic variables associated with the pharmacokinetic processing of thiazide diuretics.

Pharmacodynamic Factors Determining Response to Thiazide Diuretics

WNK1

Polymorphisms in genes regulating renal sodium transport have been associated with varied response to the thiazide diuretic hydrochlorothiazide. Lysine-deficient protein kinases (WNK) regulate thiazide-sensitive sodium chloride cotransport at the distal nephron. Mutations in two members of the WNK family, WNK1 and WNK4, cause Gordon's syndrome of familial hyperkalemic hypertension [194]. Three SNPs within WNK1 (rs2107614, rs1159744, and rs2277869) have shown significant association with BP reduction in response to hydrochlorothiazide, the additional percentage BP variation ranging between 2% and 4% [195]. This association has not been replicated in other studies.

Genome-Wide Association Studies

In a comparison of "good" and "poor" responders to hydrochlorothiazide, GWAS identified variation in one region on chromosome 12q15 including three successive SNPs (rs317689, rs315135, and rs7297610) that were significantly associated with BP response in Black subjects. Follow-up haplotype analysis was then conducted evaluating 35 tag SNPs within the lysozyme (LYZ), YEATS domain containing 4 and fibroblast growth receptor substrate 2 (FRS2) genes. Validation in a statistically independent data set comprising 291 Black subjects and 294 White subjects confirmed variation in LYZ and YEATS 4 influenced BP response to hydrochlorothiazide [196].

Clinical Implications

At present, the data regarding polymorphisms in genes pertinent to the

handling of antihypertensive agents is inconsistent and has not yet shown a clear outcome effect in hypertension and consequently stroke risk reduction. In particular, there is a dearth of studies investigating the clinical endpoints associated with polymorphisms. Importantly, inconsistencies may result from poor study design (observation, retrospective data), use of different drugs with different metabolism and mechanism and/or duration of action, poor disease phenotype strategy (including different etiology and severity), and the use of candidate SNPs as opposed to a panel of SNPs that may be better representative, certainly in relation to receptor structure. There is currently significant variability in patients' response to antihypertensive therapy. It has been shown that random selection of antihypertensive monotherapy can produce an average 50% successful BP reduction, increasing to 73% success with monotherapy by sequential, as opposed to additive, prescribing [197]. In contrast, the ACCELERATE study showed greater mean systolic BP reduction (6.5 mmHg, 95% CI 5.3–7.7) in patients treated with combination therapy (aliskiren plus amlodipine) as opposed to monotherapy, with similar adverse event rates across the groups. As every physician knows, one of the most significant factors determining lack of response to antihypertensives is nonadherence to therapy. Arguably, improving adherence may have much greater impact in the majority of hypertensive. However, personalization of antihypertensive therapy by incorporating pharmacogenetic information may actually have a more significant role to play in resistant hypertension or, indeed, in the case of ACE inhibitor-induced angioedema, in predicting those at greatest risk of ADR.

Antiplatelet Therapy: Aspirin and Clopidogrel

Platelet physiology underpins the link between atherosclerotic plaque, ischemic stroke risk, and antiplatelet therapy.

Platelet Function

Damage to the lumen of blood vessels results in platelet adhesion to the exposed subendothelial matrix, containing adhesive molecules such as collagen and von Willebrand factor. This prevents hemorrhage and promotes healing by means of a platelet plug. At the site of injury, platelets must therefore (1) adhere to the matrix; (2) activate, recruit further platelets, and

aggregate—termed the extension phase; and finally (3) perpetuate hemostasis by stimulating the clot [198].

Following initial adhesion of platelets to sites of vascular injury, platelets are activated by a number of agonists such as adenosine diphosphate (ADP) and collagen present at the exposed site (Fig. 17.2). These agonists bind to receptors on the surface of platelets (including P-selectin, glycoprotein Ib/IX/V, glycoprotein IIB/IIIa, and collagen receptors.) This triggers a downstream cascade of events that ultimately results in increased platelet-free calcium [200]. The effect of this results in numerous structural and functional changes to the platelet, including stimulating membrane phospholipase A2 activity and release of arachidonic acid (AA). AA is then converted to prostaglandin H2 intermediate via cyclooxygenase 1 (COX-1) and then further metabolized to the potent activator of platelets, thromboxane A2 (TXA_2) [201]. Initial activation of platelets triggers release of platelet granules that act in a para- and autocrine manner to amplify the activation process [202], contribute to primary and secondary hemostasis, and stabilize platelet aggregates by release of fibrinolysis inhibitors [203]. Amplification of platelet signaling occurs through release of dense granules containing abundant ADP, which interacts with purinergic G-coupled receptors P2Y1 and P2Y12. This results in platelet shape change [204, 205], increasing the surface area for contact between nearby cells, and decreased cyclic AMP (CAMP) [206] coupled with increased TXA_2 and P-selectin expression [207], respectively. Both ADP and TXA_2 contribute to recruitment of more circulating platelets and promote structural shape changes that sustain platelet activation throughout clot formation. Antiplatelets therefore can be classified on the basis of their site of action, be that they inhibit (1) platelet adhesion, (2) platelet activation, (3) platelet aggregation, and/or (4) platelet-mediated links with inflammation [208].

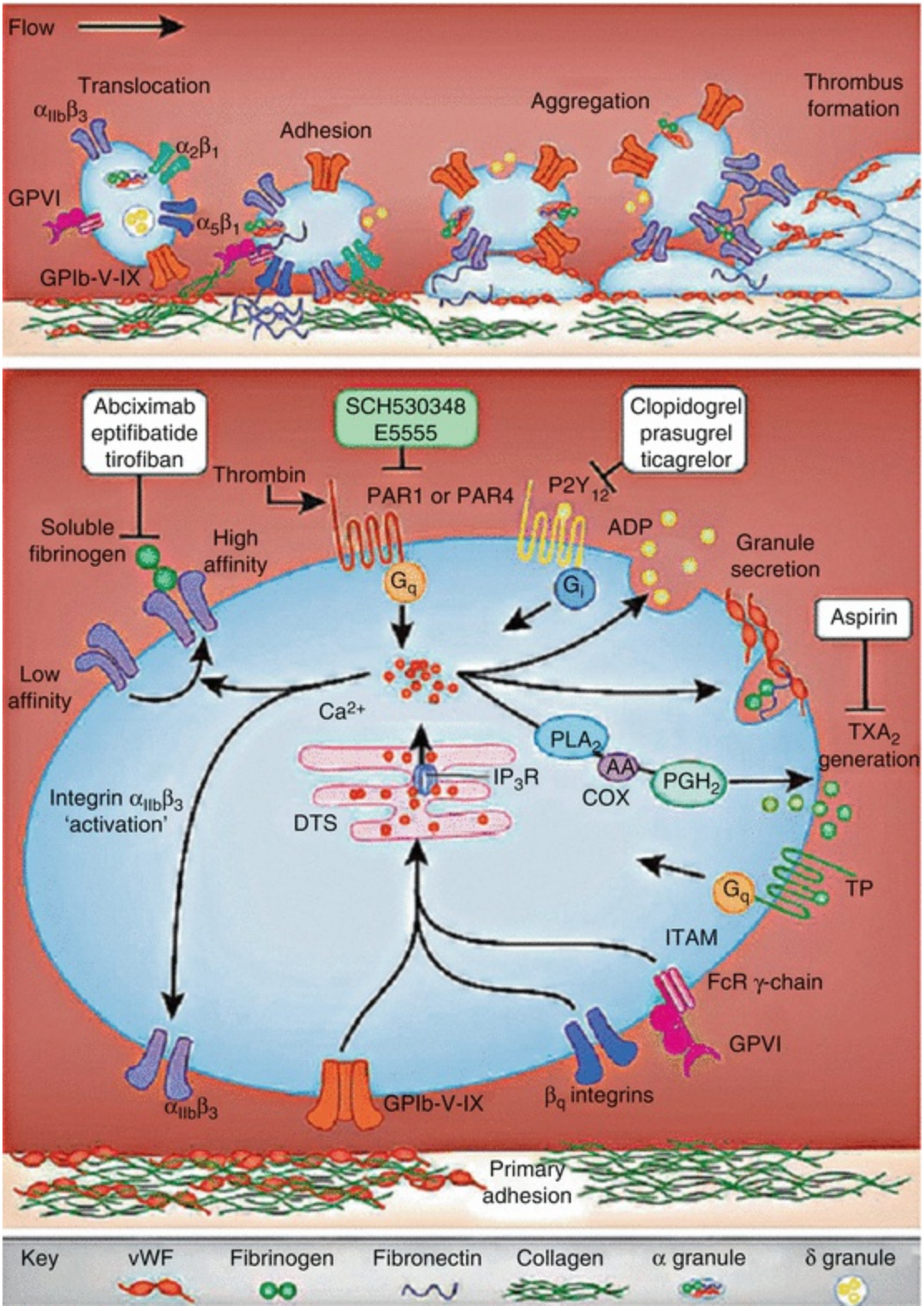

Fig. 17.2 The pivotal role of platelets in thrombosis and the sites of action of currently approved antiplatelet drugs (reprinted by permission from Macmillan Publishers Ltd. [199])

Aspirin

Antiplatelet therapy reduces the risk of stroke for patients at high risk of atherosclerosis and in those with known symptomatic cerebrovascular disease. In patients with transient ischemic attack (TIA) or ischemic stroke of noncardiac origin, antiplatelets are able to decrease the risk of stroke by 11–15% [209]. For secondary stroke prevention, the NNT is 38 to prevent one event per year [210]. However, recurrent event rates remain considerably high with currently available antiplatelet treatment regimes, and there are a number of possible contributing factors.

Low-dose aspirin is the most widely used antiplatelet therapy. Through irreversible inhibition of COX-1 by acetylation of the hydroxyl group of serine 530, aspirin excludes access for AA, thus inhibiting the production of TXA_2 in platelets [211], an effect that persists for the lifetime of the cell (8–11 days) [212]. Daily low-dose aspirin suppresses serum thromboxane B_2 formation (the stable metabolite of TA_2) by at least 95% within 5 days [213] and persists indefinitely with compliance. Aspirin has more than ten times greater selectivity for COX-1 than COX-2 [214].

Unfortunately, the reduced risk of arterial thrombosis cannot be separated from the increased risk of bleeding complications in individuals taking even low-dose aspirin therapy. The mechanisms responsible for aspirin-induced gastrointestinal (GI) ulcers are not yet fully understood, particularly given that most of the antiplatelet effects seen with low-dose aspirin occur in the portal system and the amount of drug actually available in the systemic system is limited [215]. Nevertheless, aspirin-induced prostaglandin depletion results in topical injury to the mucosa and is universal in patients taking nonsteroidal anti-inflammatory drugs (NSAIDS) [216]. Results of a large meta-analysis suggest the relative risk of major GI bleeding with the use of low-dose aspirin as compared with placebo was 2.07 (95% CI: 1.61–2.66), an NNH of 883 for 1 year of treatment [217]. For intracranial bleeding, the NNH is much higher (1333) [217].

Platelets contribute to both homeostasis of the rapidly re-epithelialized lining of the GI tract [218] and also to ulcer healing, by delivery of growth factors including epidermal growth factor and vascular endothelial growth factor [219, 220], necessary to reestablish the vasculature at injury sites.

Aspirin: Licensed Indications

In the UK, low-dose aspirin therapy (75 mg once daily) is licensed for the secondary prevention only of thrombotic cerebrovascular events. In meta-analysis of all randomized clinical trials (RCTs) comparing aspirin and placebo in patients with ischemic stroke or TIA, it was found that aspirin reduces the risk of recurrent stroke and other major vascular events by 13% (95% CI, 6–19%) [221]. Primary prevention, however, is not a licensed indication. The AAA study [222] found no significant difference in primary endpoint (composite of initial coronary event or stroke or revascularization) between patients allocated aspirin or placebo (181 events vs. 176 events; hazard ratio 1.03 [95% CI 0.84–1.27]). Major hemorrhage causing in-hospital admission occurred in 2% of the aspirin-treated patients as compared to 1.2% in the placebo group, resulting in the conclusion that there is no benefit from aspirin use in primary prevention with an increased risk of serious bleeding in asymptomatic individuals. Furthermore, recent meta-analysis of 6 primary prevention and 16 secondary prevention trials of long-term low-dose aspirin for prevention of serious cardiovascular events concluded that aspirin has substantial benefit in secondary prevention only with 22% reduced serious vascular events per year compared with no aspirin (6.7% vs. 8.2%; $p < 0.0001$) [223]. In primary prevention, aspirin reduced serious vascular events by 12%/year compared with no aspirin (0.51% vs. 0.57%; $p < 0.0001$) but significantly increased major bleeds (although they remained uncommon: 0·1% per year with aspirin vs. 0·07% per year without aspirin; $p < 0.0001$) [223].

Aspirin Resistance

Recently, it has been shown that up to 40% of patients may be resistant to the effects of aspirin [213]. Meta-analysis of 20 studies with >2900 patients reported that individuals with aspirin resistance had a significantly increased risk (OR 3.85) of having a cardiovascular or cerebrovascular event [224].

Aspirin resistance can be categorized into three distinct pharmacokinetic and/or pharmacodynamic subtypes [225]:

1. Type 1 aspirin resistance (pharmacokinetic) is the inability of aspirin to suppress TXA2 production following oral administration despite

complete inhibition occurring in vitro.

2. Type 2 aspirin resistance (pharmacodynamic) occurs when aspirin fails to suppress TXA2 both in vivo and in vitro.

3. Type 3 aspirin resistance (pseudoresistance) involves platelet aggregation despite complete inhibition of thromboxane formation.

Aspirin resistance can be divided into clinical resistance, the failure to prevent clinical atherothromboembolic ischemic events [226], and laboratory resistance. Laboratory aspirin resistance is defined as the failure of aspirin to inhibit TXA_2 production or inhibit tests of platelet function (platelet aggregation) that are dependent on platelet thromboxane production [226]. Many in vitro assays are available to detect aspirin resistance (Table 17.6), but the manner in which they assess platelet function varies, and hence reproducibility across the assays is poor. In a study conducted comparing five platelet function tests [227], the prevalence of aspirin resistance varied significantly between the assays used with patients being labeled resistant by one test but not by others. The negative predictive value of each assay was generally high, but positive predictive value was low, implying that each assay is not suitably specific in truly identifying aspirin resistance. Due to this variability, no single test can be recommended as representative of aspirin resistance for use in clinical practice, and hence, the International Society on Thrombosis and Haemostasis working group on aspirin resistance [213] does not currently recommend testing for aspirin resistance in patients using the drug for a cardiovascular indication. Better studies are needed in order to define how and in whom resistance testing should be undertaken.

Table 17.6 Advantages and disadvantages of aspirin resistance/platelet function assays

Assay	Sample	Advantages	Disadvantages
VerifyNow®	Whole blood	Point of care test	Uncertain reproducibility
		Correlated with clinical events	
		Arachidonic acid agonist	
Light transmittance aggregometry (LTA)	Platelet-rich plasma	Correlated with clinical events	Expensive and labor-intensive
		Arachidonic acid agonist	Intraoperator variability
		Most representative of	Poorly reproducible

		pharmacological effect	
			Artificial milieu of PRP may alter platelet response
Platelet function analyzer (PFA)-100	Whole blood	Point of care test	Poorly reproducible
		Correlated with clinical events	Not specific for aspirin resistance
			No defined cut-off closure time for aspirin resistance
			Dependent upon vWF and hematocrit
Urinary/serum thromboxane A2	Urine or serum	Dependent upon COX inhibition	Poorly reproducible
		Correlated with clinical events	Not specific for COX-1 inhibition
			Not specific for aspirin resistance
Plateletworks®	Whole blood	Uses whole blood	Uncertain correlation with other assays
		Point of care test	Limited outcome data
		Arachidonic acid agonist	
Whole blood aggregometry (WBA)	Whole blood	Uses whole blood	Uncertain sensitivity and specificity
		Arachidonic acid agonist	Not correlated with clinical events to date
Multiplate	Whole blood	Uses whole blood	Not correlated with clinical events to date
		Point of care test	
		Correlated to LTA and PFA-100	
		Arachidonic acid agonist	

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PRP platelet-rich plasma, *vWF* von Willebrand factor

There are many potential causes of aspirin treatment failure, including poor adherence and the use of interacting medication including NSAIDs, some of which can be simply remedied. More complex causes, such as the complex interplay between proinflammatory mediators and poor glycemic control in diabetes mellitus affecting protein acetylation of COX-1, or the initial inflammatory state following ACS reportedly related to atherosclerotic

plaque rupture and endothelial dysfunction may require more intensive management strategies. It may be the case that aspirin response is a dynamic phenomenon, responding to particular circumstances. Nevertheless, aspirin resistance is associated with greater cardiovascular risk, and hence, the identification of individuals at greatest risk of resistance is imperative.

Genetic Determinants of Aspirin Response

In a large, population-based study of 2413 participants, heritable factors contributed a third of the variation in laboratory platelet aggregation compared with just 4–7% attributable to measurable covariates [229]. Furthermore, in a study of more than 500 White and African-American families, heritable factors were shown to contribute prominently to variability in residual platelet function after aspirin exposure, with aspirin exposure inhibiting AA-induced aggregation and thromboxane B_2 production by ≥99% ($p < 0.0001$) [230].

Pharmacokinetic Factors Determining Variability

UDP-Glucuronosyltransferase (UGT)

UGT is the microsomal enzyme responsible for glucuronidation reactions and exists as a superfamily of independently regulated enzymes [231]. UGT enzymes convert aspirin to glucuronides and salicyluric acid after glycine conjugation [232]. Various genetic polymorphisms in UGT have been described; however, only UGT1A6 has been associated with variable efficacy of aspirin, specifically with respect to colorectal carcinoma prevention [233–235]. Approximately 50% of Caucasians express UGT1A6 polymorphisms, 40% heterozygosity (UGT1A6*1/UGT1A6*2) and 10% homozygosity (UGT1A6*2/UGT1A6*2) with homozygosity possibly resulting in 50–60% reduction in salicylic acid (SA) glucuronidation [236]. In females receiving aspirin, presence of UTGA6*2 was associated with lower levels of SA reflecting accelerated clearance; thus UTGA6*2 may be implicated in aspirin resistance. However, the functional effects in vivo remain to be elucidated, particularly in high-risk cardiovascular patients. Of note, no association between UGT or CYP2C19 polymorphisms has been associated with prevalence of gastric complaints in cardiovascular patients taking aspirin [237].

Pharmacodynamic Factors Determining Variability

COX-1 and COX-2

Several candidate genes for aspirin resistance have been proposed. SNPs involving COX-1, COX-2, and other platelet genes can modify the antiplatelet effect of aspirin. COX-1, the target enzyme of aspirin, is highly polymorphic, and the C50T polymorphism is the most widely studied, but results have been conflicting. Halushka and colleagues [238] identified two SNPs, A842G and C50T, to be in complete linkage disequilibrium, and those who were heterozygous for the A842G/C50T haplotype showed significantly greater inhibition of prostaglandin H_2 formation by aspirin compared with common allele haplotypes. In 144 patients with stable CAD taking aspirin, Maree et al. [239] found the COX-1 haplotype to be significantly associated with aspirin response with patients being significantly less sensitive to aspirin as determined by AA-induced platelet aggregation ($p = 0.009$) [239]. Similarly, Lepantalo et al. [240] examined polymorphisms in COX-1 on the antiplatelet effect of aspirin in those with CAD. Of the nonresponders detected by AA-induced platelet aggregation, 3 of 5 (60%) carried the rare G allele for the −A842G polymorphism of COX-1 in contrast to 16 of 96 (17%) responders ($p = 0.016$). Conversely, in 125 Tunisian patients with stable CAD, the presence of the C50T mutant allele was not statistically different between aspirin good responders and poor responders, as estimated by urinary thromboxane B_2 excretion [241]. Whereas laboratory studies have shown that the C50T polymorphism is associated with lower response to aspirin, this effect has not been correlated with clinical outcomes (death or further cardiovascular event). In 496 patients admitted to CCU for various indications necessitating low-dose aspirin therapy, 13.3% exhibited the variant genotype; however, this did not correlate with a higher risk of atherothrombotic event [242].

Platelet Receptors P2Y1 and P2Y2 and GPV1/GP IIIa

Similar conflicting results have been shown with the P2Y1 and P2Y12 platelet receptor genes and GPV1/GP IIIa genes [240, 243–247]. A large systematic review by Goodman et al. [248] including 31 studies of 50 polymorphisms in 11 genes demonstrated that the P1A1/A2 polymorphism in the GpIIIa gene was significantly associated with aspirin resistance in healthy

subjects (OR 2.36; 95 CI 1.24 to 4.48; $p = 0.009$). This correlation was only seen with LTA; when PFA-100 was used, the polymorphism appeared to confer aspirin sensitivity, although this was not statistically significant, a limitation acknowledged by the authors.

Genome-Wide Association Studies

GWAS has been performed involving 565 patients before and after starting dual antiplatelet therapy (aspirin and clopidogrel) [20]. Subsequently, significant findings were examined in two independent aspirin-treated groups, 227 patients undergoing primary percutaneous coronay intervention and a further 1000 patients from the International Verapamil SR/Trandolapril Study (INVEST) Genetic Substudy. GWAS identified a strong association between SNPs on chromosome 1q23 and post-dual antiplatelet therapy platelet aggregation. The most strongly associated SNP with dual-antiplatelt response was found to be rs12041331 in the platelet endothelial aggregation receptor-1 (PEAR1) gene.

Clinical Implications

The lack of a standardized laboratory test to reliably confirm aspirin resistance combined with the discordance among studies of heritable resistance, likely resulting from a combination of inadequate phenotype definition, poor sample size, and incomplete genotyping, represents a significant hurdle to the translation of genetic testing of aspirin resistance into clinical practice. Clearly, large-scale trials are necessary to delineate the relationship between laboratory resistance and genetics. It may prove the case that multiple genes are important, genotyping strategies will need to reflect the wider pathway involvement of other genes pertinent to aspirin response, and GWAS will likely be the technique to bridge this gap.

Clopidogrel

Clopidogrel is a widely used second-generation thienopyridine antiplatelet agent. A prodrug, it requires a two-step activation process to its active metabolite R130694 [249] via hepatic CYP450 enzymes. In vivo conversion to the pharmacologically active thiolactone is a process subject to major

competing metabolic pathways, resulting in only 10–15% of clopidogrel dose converting to the active metabolite [250]. The action of clopidogrel depends upon specific and irreversible binding of the thiol group to cysteine residues of the platelet ADP receptor (P2Y12), resulting in reduced ADP-mediated platelet aggregation [251]. The activation cascade is postulated to involve CYP2C19, 1A2, and 2B6 in the first step, and 2C19, 2C9, and 2B6 responsible for the second [249]. Numerous drugs such as erythromycin, ketoconazole, lipophilic statins, and St. John's wort have all been reported to affect clopidogrel activation, through interaction with the CYP3A group of isoenzymes [249, 252]. Importantly, recent evidence of the interaction between clopidogrel and 2C19 substrate proton pump inhibitors (PPIs) supports the notion that CYP2C19 is the primary isoform responsible for clopidogrel activation. Gilard and colleagues demonstrated in 124 patients that coprescription of omeprazole and clopidogrel resulted in a fourfold increase in clopidogrel nonresponse as defined by vasodilator-stimulated phosphoprotein (VASP) phosphorylation [253]. In addition, meta-analysis of 25 studies including 159,138 patients demonstrated that coadministration of PPI and clopidogrel resulted in a 29% increase in the risk of major adverse cardiovascular events versus those taking clopidogrel alone [254].

Clopidogrel response is therefore not uniform, and a subset of patients (up to 25%) may in fact be resistant to its effects. Nonresponse is a complex and multifactorial problem, although it is likely that variability in clopidogrel's activation pathway is a significant contributing factor. Dependence upon CYP enzymes for activation may explain the interindividual variability in response to clopidogrel [249].

Clopidogrel in the Treatment of Cardiovascular and Cerebrovascular Disease

In large, randomized control trials, clopidogrel can be seen to clearly reduce mortality and adverse cardiac and cerebrovascular events. In the Clopidogrel in Unstable Angina to Prevent Recurrent Events (CURE) trial, clopidogrel reduced mortality, nonfatal stroke, and nonfatal myocardial infarction (MI) from 11.4% in the placebo group to 9.3% in the clopidogrel group (relative risk 0.80; 95% CI 0.72 to 0.90; $p < 0.001$) [255]. Specifically, the clopidogrel group had fewer ischemic strokes than placebo (75 vs. 87 RR 0.86; CI, 0.63–11.8), without any increase in hemorrhagic strokes arising as a complication.

Similarly, the Clopidogrel for High Atherothrombotic Risk and Ischemic Stabilization, Management, and Avoidance (CHARISMA) trial provided evidence of benefit for clopidogrel treatment in patients with symptomatic atherothrombosis, with a primary efficacy end point (MI, stroke, or death from cardiovascular cause) of 6.8% compared to 7.3% with aspirin alone (RR 0.93, 95% CI 0.83–1.05; $p = 0.22$) [256]. Again, fewer individuals in the clopidogrel group suffered nonfatal stroke (150 vs. 189 RR 0.79; 95% CI 0.64–0.98; $p = 0.03$) compared to those treated with aspirin alone, without evidence of increased intracranial hemorrhage. Compared to aspirin, the NNT to prevent one more vascular event (ischemia of the cerebral, coronary, or peripheral circulation) for clopidogrel is 92, over a period of about 2 years [257]. A large systematic review and meta-analysis of adverse events associated with aspirin and clopidogrel concluded that compared with clopidogrel, aspirin increases the risk of major GI hemorrhage, but no other form of hemorrhage. Furthermore, the NNH for one major additional bleeding episode with aspirin is 769; however, to prevent one major episode of bleeding, 883 patients would require treatment with clopidogrel instead of aspirin [217].

Clopidogrel Nonresponse

The pharmacodynamic response to clopidogrel displays significant interindividual variability, and recently nonresponse has become an important clinical issue, particularly in the setting of ACS and percutaneous coronary intervention (PCI). In a meta-analysis of 25 studies involving clopidogrel resistance and PCI, Snoep et al. [258] reported a 21% prevalence of clopidogrel nonresponse corresponding to an eightfold increase in the risk of adverse cardiovascular events postprocedure. Like aspirin, clopidogrel activity can be measured using a number of approaches. To date, the gold standard and most reliable method is light transmission aggregometry. Blood samples from individuals in whom platelets readily aggregate (e.g., those unresponsive to clopidogrel) will have the highest degree of light transmission through the sample, as the aggregates fall out of the solution increasing the transparency. This test is not, however, specific for the substrate of clopidogrel, the P2Y12 pathway. In contrast, VerifyNow® (Accumetrics) is P2Y12 specific and has been used to evaluate the presence of both aspirin and clopidogrel resistance. Other methods include ADP-

induced platelet aggregation, VASP phosphorylation/flow cytometry, thromboelastography, and the platelet drop-count method.

Genetic Determinants of Clopidogrel Response Pharmacokinetic Factors Determining Variability

CYP2C19

CYP2C19 appears to be the primary isoform responsible for both activation steps, and importantly, it is highly polymorphic, with around 25 variant alleles described, to date [259]. These can be divided into those that are of the poor metabolizer or loss-of-function phenotypes (*2, *3, *4, 5) and gain-of-function phenotypes (*17). In healthy individuals, the loss-of-function polymorphisms have been shown to significantly reduce the platelet response to clopidogrel [260], as demonstrated by increased platelet aggregation measured by light transmission aggregometry. Platelet aggregation did not change significantly from baseline in *1/*2 heterozygotes (71.8% at day 7; $p = 0.22$ vs. baseline) as compared to the *1/*1 homozygotes wherein platelet aggregation progressively decreased (reaching 48.9% at day 7; $p < 0.07$ vs. baseline) as would be expected. In 227 patients undergoing PCI, carriers of the *2 allele had a 3.4-fold higher rate of cardiovascular event as compared to noncarriers (95% CI, 1.36–8.46, $p = 0.004$).

Numerous studies, predominantly in patients with ACS, have cited a clinical link between the presence of loss-of-function alleles and an increased risk of cardiovascular events [261–263]. In a study of 1477 patients presenting with ACS, presence of a CYP2C19 loss-of-function polymorphism was associated with a higher rate of the primary efficacy outcome (composite of death from cardiovascular causes, non-fatal MI, and nonfatal stroke) than those with wild-type CYP2C19 (12.1% vs. 8.0%; $p = 0.01$); this was consistent with the risk for stroke alone (HR 1.73; 95% CI, 0.68–4.38; $p = 0.25$) [263]. In TRITON-TIMI 38, those carrying CYP2C19 reduced function allele displayed a consistent hazard for nonfatal stroke as compared to noncarriers (0.88% vs. 024%, HR 3.93 95% CI 0.66–23.5) [264]. In 259 patients (<45 years) taking clopidogrel as secondary prevention for ≥1 month following first MI, 5-year event-free survival was much lower in those carrying the loss-of-function CYP2C19*2 polymorphism (HR = 3.69; 95% CI 1.69–8.5; $p = 0.0005$) [262]. Similarly, in 2208 patients treated

with clopidogrel following acute MI, carriers of any two loss-of-function alleles displayed a nearly twofold increase in the risk of adverse cardiovascular events (HR 1.98, 95% CI 1.10–3.58) [261]. A genetic substudy of the PLATO trial examined the effect of ticagrelor versus clopidogrel in treatment of ACS in 10,285 patients. In the clopidogrel group, the event rate at 30 days (cardiovascular death, MI, or stroke) was higher in patients with any loss-of-function *CYP2C19* alleles than those without (5.7% vs. 3.8%, $p = 0{\cdot}028$) [265]. Conversely, combined analysis of 5059 patients with acute coronary syndromes from the CURE and ACTIVE A trials determined that the effect of clopidogrel (reducing the rate of death from cardiovascular causes, nonfatal MI, or stroke) is consistent, regardless of CYP2C19 loss-of-function carrier status [266]. Similarly, no significant difference in the incidence of stroke in 928 patients with acute MI requiring coronary revascularization was found irrespective of genotype status [267]. Furthermore, a large meta-analysis of 32 studies involving 42,016 patients treated with clopidogrel, concluded that there was no clinically significant interaction between the CYP2C19 genotype and cardiovascular events. Analysis of 26 of the “treatment only design” studies (in which all patients were receiving clopidogrel) presence of one or more CYP2C19 alleles was associated with reduced enzyme function (*2, *3, *4,*5, *6, *7, *8), exhibited lower levels of active metabolites, reduced platelet inhibition, and consequently, lower risk of bleeding (relative risk [RR], 0.84; 95% CI, 0.75–0.94; absolute risk reduction of 5–8 events per 1000 individuals) with higher risk of cardiovascular events (RR, 1.18; 95% CI, 1.09–1.28; absolute risk increase of 8–12 events per 1000 individuals). In comparator studies, whereby clopidogrel treatment was nested within an RCT, CYP2C19 genotype was not associated with alteration in cardiovascular end points or bleeding risk ($p > 0.05$). This finding is consistent with previous meta-analysis, wherein no association between CYP2C19 genotype and clinical efficacy of clopidogrel in prevention of thromboembolic events was found [268]. There is still controversy about the utility of CYP2C19 genotyping for later cardiovascular events, while there is more consistency regarding stent thrombosis and CYP2C19 genotype status. There are several RCTs currently in progress to assess the value of pre-prescription CYP2C19 genotyping prior to the use of clopidogrel. More recently, exome sequencing has been used in individuals with extreme phenotypes in terms of platelet reactivity while on clopidogrel; this identified that a rare variant in beta-1,4-galactosyltransferase

2 gene was associated with low platelet reactivity [21]. This finding needs to be replicated in larger cohorts.

PON1

Paraoxonase 1 (PON1) is a major anti-atherosclerotic component of high-density lipoprotein (HDL) and is a crucial enzyme for clopidogrel bioactivation [22]. The common Q192R polymorphism has been shown to determine the rate of active metabolite formation, with QQ192R homozygous individuals showing a considerably higher risk than RR192 homozygous individuals for stent thrombosis and reduced pharmacokinetics following stent implantation. Consistent with this, the authors identified a significantly lower risk of major bleeding (HR = 0.4, 95% CI: 0.2–0.8; P = 0.006) resulting from decreased clopidrogrel response [22]. However, a systematic review and meta-analysis found no evidence of an association observed between PON1 Q192R and platelet reactivity, regardless of the laboratory method used (global mean standardized difference = 0.10; 95% CI, −0.06 to 0.25; P = 0.22) [23]. More recently, PON1 Q192R has been shown to influence relative platelet inhibition rather than on-clopidogrel platelet reactivity [24]. A further study showed that the QQ/QR-genotype was an independent predictor of worse outcome following ACS, associated with higher small-dense LDL levels, a mechanism independent of clopidogrel treatment [25]. Taken together, the impact of PON1 polymorphisms on clopidrogrel pharmacogenetics remains controversial.

CES1

Clopidogrel is a recognized substrate of hepatic carboxylesterase 1 (CES1), being ~85% hydrolyzed to the inactive carboxylate metabolite. Both genetic and environmental factors contribute to the significant interindividual variability in the expression and activity of CES1. Co-incubation studies have shown that CES1 inhibition can lead to significant increases in the metabolic production of both active and intermediate metabolites of clopidogrel. An *in vitro* incubation study using transfected cell lines determined that the catalytic activity of CES1 and its variants G143E and D260fs exhibited null activity for the hydrolysis of both clopidogrel and 2-oxo-clopidogrel, whereas the variants G18V, S82L and A269S were unchanged from wild-type [26]. A large study of over 900 patients showed that carriers of the G143E functional

variant have significantly greater levels of the clopidogrel active metabolite [27].

Genome-Wide Association Studies

In a GWAS, *CYP2C19*2* was shown to be a major determinant of clopidogrel response as measured by ex vivo platelet reactivity in healthy Caucasians. The rs12777823 polymorphism was in strong LD with the CYP2C19*2 variant and was associated with diminished clopidogrel response, and accounted for 12% of the total variation in platelet aggregation to ADP ($p = 4.3 \times 10^{-11}$) [269].

Clinical Implications

There is significant controversy surrounding the association between CYP2C19 genotype and cardiovascular outcome. Accepting the sometimes significant heterogeneity between studies, seven separate meta-analyses have analyzed stent thrombosis risk after PCI and all found a significant increased risk in *2 carriers, with hazard ratios ranging from 1.75 to 3.82, with a median hazard ratio of 2.58 [28–34]. The risk of stent thrombosis in *2 carriers is therefore considered indisputable. In 2010, the FDA added a "boxed warning" to the clopidogrel label advising prescribers of alternative antiplatelet agents or altered dosing in those identified as CYP2C19 poor metabolizers of the drug [270]. In response, both the American Heart Association (AHA) and American College of Cardiology (ACC) have argued that there is currently insufficient evidence to support this warning [271]. In the clinical setting, antiplatelet treatment failure is characterized by the occurrence of thrombotic events despite antiplatelet therapy. As described, this could be due to numerous competing factors beyond simple noncompliance. Decreased activity due to competition with other drugs involving the CYP450 enzyme system or impaired ability to absorb or generate the active metabolite due to altered functioning of the CYP450 system may play a significant role. There is currently no consensus on how resistance to aspirin and/or clopidogrel should be managed in the treatment and/or prevention of stroke.

Clopidogrel Dose Increase

In patients exhibiting inadequate platelet inhibition (<50%) with standard-dose clopidogrel, a maintenance dose of 150 mg/day for 1 month has been shown to increase platelet inhibition as measured by the VerifyNow® assay (27.1 vs. 40.6%; $p = 0.009$) [272]. However, still only 35% of patients actually reached inhibition of ≥50% in this cohort of patients with type 2 diabetes taking dual antiplatelet therapy following PCI. Similarly, in 153 patients with low clopidogrel response (platelet reactivity index >69%) who were randomized to either standard- or higher-dose (150 mg/day) therapy following PCI, significantly lower platelet reactivity index was seen in those taking 150 mg/day than those taking 75 mg/day (43.9% vs. 58.6%; $p < 0.001$) [273]. Interestingly, 20/31 patients in the standard-dose group became responders following increase to 150 mg/day for 2 weeks. In 2011, Mega et al. [274] examined the effect of CYP2C19*2 allele on response to higher doses of clopidogrel. By tripling the standard dose to 225 mg/day, CYP2C19*2 heterozygotes (77) achieved reductions in platelet reactivity (as measured by VASP-PRI and VerifyNow®) to levels achieved with standard-dose therapy (75 mg) in noncarriers. However, even quadruple-dose therapy was unable to achieve comparable levels of platelet function reduction in CYP2C19*2 homozygotes (5). Indeed, in homozygous carriers, even loading doses as high as 900 mg were incapable of overcoming clopidogrel resistance [275]. It may prove the case that homozygote CYP2C19*2 carriers (about 2% of the UK population) would be better served by alternative antiplatelet agents that are not dependent on CYP2C19.

Next-Generation Anti-platelet Agents

Prasugrel, a third generation thienopyridine, is like clopidogrel, an inactive prodrug that must be converted to an active metabolite (R-138727) in order to irreversibly inhibit the platelet P2Y12 receptor. It displays greater and more efficient generation of the active metabolite than clopidogrel and thus greater platelet inhibition [35, 36]. Indeed, it has been shown to inhibit ADP-induced platelet aggregation more rapidly and extensively than both standard and higher doses of clopidogrel in both healthy volunteers and patients with coronary artery disease, including those undergoing PCI [276]. However, where TRITON showed prasugrel to be superior to standard-dose clopidogrel in the ACS/PCI setting, in patients with stroke or TIA, there was in fact net clinical harm due to major bleeding events and in those over the age of 75 or

weighing <60 kg, no net benefit was achieved [276].

In contrast to the FDA boxed warning on the clopidogrel label informing clinicians of the effect of CYP2C19 genotype on efficacy, the prasugrel label indicates that genetic variation in a variety of CYP enzymes, including CYP2C19, does not affect prasugrel's pharmacokinetics or its efficacy. What is more, this pharmacogenetics information was part of the new drug application, and was therefore present on the original label at the time of approval.

Ticagrelor is a cyclopentyl-triazolo-pyrimidine and a direct and reversible P2Y12 antagonist. Like prasugrel, it acts more rapidly and is a more potent platelet inhibitor than clopidogrel and importantly, did not significantly increase major bleeding compared with clopidogrel in phase II studies. However, ticagrelor is associated with apparently dose-dependent dyspnea and requires twice-daily dosing, which may affect patient compliance. The PLATO trial randomized patients with ACS to either ticagrelor or clopidogrel. Overall, the trial demonstrated a significant reduction with ticagrelor in the primary endpoint (composite of death from MI, stroke, or vascular cause) at 12 months; the rate of discontinuation due to dyspnea was higher. Ticagrelor compared with clopidogrel was associated with similar total major bleeding but increased non-CABG (4.5 vs. 3.8%, $p = 0.02$) and nonprocedure-related (3.1 vs. 2.3%, $p = 0.05$) major bleeding. Ticagrelor pharmacokinetics have been shown to be affected by three loci (SLCO1B1, UGT2B7 and CYP3A4) in a GWAS of participants in the PLATO trial, but this was not associated with any differences in clinical outcomes [37].

Oral Anticoagulants

Warfarin

Warfarin (3 α[alpha]-acetonylbenzyl-4-hydroxycoumarin) remains the most widely used oral anticoagulant in the world [277]. There is overwhelming evidence of its efficacy in the prevention of cardioembolic stroke in patients with atrial fibrillation (AF) [278–280], prosthetic heart valves [281, 282] and in patients with postmyocardial infarction complicated by mural thrombus formation [283]. The presence of atrial fibrillation increases the risk of ischemic stroke by fivefold and is essentially a prothrombic state [284, 285]. A meta-analysis involving 2900 participants with nonvalvular AF

demonstrated that adjusted-dose warfarin reduces the risk of stroke by 64% (95% CI, 49–74%) (NNT 37 in primary prevention, 12 in secondary prevention) and all-cause mortality by 26% (95% CI, 3–43%), compared with placebo [286]. As a result, warfarin remains the most established therapy for both the primary and secondary prevention of stroke in patients with atrial fibrillation. However, warfarin's narrow therapeutic range and somewhat unpredictable interindividual variability have posed a significant challenge to clinicians for many years. Consequences of underdosing (leading to a subtherapeutic INR) and overdosing (leading to a supratherapeutic INR) place individuals at risk of thrombotic events and hemorrhage, respectively. Bleeding is often the most feared complication of therapy. Rates of bleeding in patients with AF vary from 0.75% per year to as high as 10% in some cohorts [287, 288] (NNH 283; reported in a large meta-analysis examining major bleeding outcomes [289]).

With at least 30 genes known to regulate its effects [290], it is therefore not surprising that warfarin remains the most studied cardiovascular drug in terms of pharmacogenetic effects. However, it is also important to remember that environmental factors also affect daily warfarin dose requirement (Fig. 17.3), and any improvement in dosing will need to take into account both genetic and environmental factors.

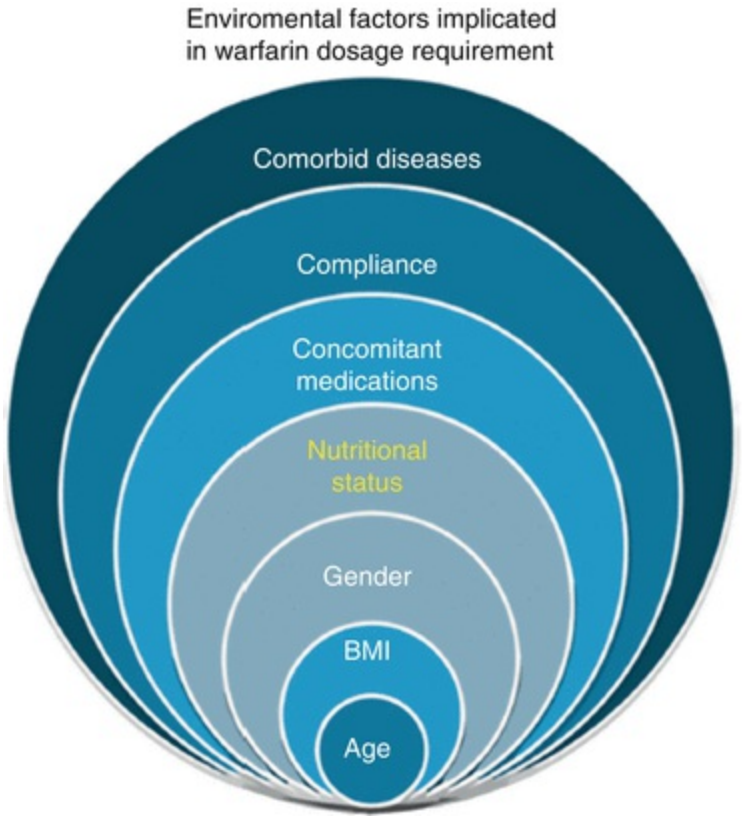

Fig. 17.3 Environmental factors implicated in warfarin dosage requirement

Pharmacology

Warfarin is among a family of 4-hydroxycoumarin anticoagulants with varying molecular structures. It is highly water soluble and achieves more than 95% bioavailability following rapid absorption from the stomach and upper gastrointestinal tract [291]. Warfarin is extensively (~99%) protein bound, the majority to albumin and α(alpha)-1-acid-glycoproteins. Peak plasma concentrations are achieved within 4 h with a relatively small volume of distribution (0.12 L/kg) [292]. Warfarin is a racemic mixture of R and S enantiomers that displays stereoselective metabolism; S-warfarin is approximately three to five times more potent than R-warfarin [293]. The liver is the major site of warfarin biotransformation. Oxidative pathways dominate warfarin's metabolism, with reductive and conjugative pathways of lesser significance [294].

Pharmacokinetic Factors Determining Warfarin

Response

Cytochrome P450 enzymes are well characterized in the metabolism of warfarin and have received the most attention in terms of pharmacogenetics. CYP2C9 predominantly participates in S-warfarin metabolism [295, 296] with CYP3A and CYP2C19 involved to a lesser extent [297]. S-warfarin forms S-7-hydroxywarfarin [295, 296], whereas R-warfarin is metabolized into multiple R-warfarin metabolites by a greater number of P450s, including CYP1A2 and CYP3A4 [295, 296, 298]. Warfarin is eliminated via zero-order kinetics with a mean plasma half-life of 40 h. About 80% of warfarin's metabolites are renally excreted [292].

Orosomucoid 1 Gene [ORM1] and Orosomucoid 2 Gene [ORM2]

Two different genes (orosomucoid 1 gene [ORM1] and orosomucoid 2 gene [ORM2]), which differ in 22 positions, code for the α(alpha)-1-acid-glycoproteins [299, 300]. Warfarin has been shown to preferentially bind to the ORM 2 variant amino acid sequence [300], and in one study, the ORM 1-2 genes were significantly associated with warfarin dosing ($p = 0.0496$) [301]. Significance, however, was not maintained following correction for multiple testing, and other outcomes, such as bleeding and time to achieve maintenance INR, have not been explored.

CYP2C9

CYP2C9 is the main gene responsible for the pharmacokinetics of warfarin. Polymorphisms in CYP2C9 have been extensively described with significant effects on S-warfarin metabolism and hence warfarin efficacy. More than 30 variants in CYP2C9 have been described, although *CYP2C9*2, *3, *5, *6, *8 and *11* represent the main SNP's. The two most common allelic variants in Caucasians, CYP2C9*2 and CYP2C9*3, have reduced enzyme activity (12% and 5%, respectively) compared to the wild-type CYP2C9*1 [302–304]. In Caucasian populations, the frequencies of these genotypes are relatively well described. Around 1% of the population are homozygous for CYP2C9*2 and less commonly (0.4%) for CYP2C9*3. Heterozygous carriers for CYP2C9*2, CYP2C9*3, and CYP2C9*2*3 represent about 22, 15, and 1.4% of the population, respectively. In a landmark paper published in the Lancet, Aithal

et al. demonstrated that these variant alleles were significantly associated with a lower-warfarin-dose requirement (OR 6.21; [95% CI 2.48–15.6]) [305]. A large meta-analysis (involving 7907 Caucasians) compared dose requirements with the wild-type genotype CYP2C9*1*1. It demonstrated that CYP2C9*2*2, CYP2C9*2*3, and CYP2C9*3*3 required significantly lower ($p < 0.05$) warfarin doses (36.0, 56.7, and 78.1%, respectively) [306]. The frequencies of CYP2C9 variants differ significantly in other ethnicities and are less common in Asian and African populations [307]. The variant CYP2C9*1*2 was completely absent in a study in a Han Chinese population [308], in contrast to the one in five Caucasians who are known to possess this allelic variant. The CYP2C9*3 variant, however, is the most commonly reported CYP2C9 polymorphism in Asian populations (1–4%) [309] and has been associated with warfarin sensitivity [310, 311]. In a Japanese study, 5.6% were heterozygous for CYP2C9*3 with the CYP2C9*1*3 genotype being associated with a significantly lower warfarin dose (44.6%; 1.86 ± 0.80 mg/day, $p < 0.001$) than the wild-type CYP2C9*1*1 [312]. In African-American patient groups, CYP2C9*5, *8 and *11 are more commonly alleles and are all associated with a lower weekly warfarin dose [38–40]. The reported allelic frequencies for CYP2C9*2 and CYP2C9*3 polymorphisms vary between 1% and 3% [313, 314], and their pharmacogenetic effects have been less consistently demonstrated [315, 316] in this patient group.

Time to Achieve Stable INR

The CYP2C9 gene has also been associated with an increased length of time to achieve stable dose. In a study involving 185 Caucasian patients, the CYP2C9 variant group required more time to achieve stable dosing (HR, 0.65; 95% CI, 0.45–0.94) with a median difference of 95 days ($p = 0.004$) [317] in comparison to patients possessing the wild-type genotype. Schwarz and his colleagues, however, did not find any significance between CYP2C9 variants and time to achieve therapeutic INR [318]. The evidence for CYP2C9 in relation to achievement of stable warfarin dose and time to achieve therapeutic INR has been less extensively studied in other populations.

Bleeding

In Aithal et al.’s study, patients possessing the CYP2C9 variants were at

increased risk of hemorrhage (rate ratio 3.68 [1.43–9.50]) during induction [305]. Sanderson et al.'s meta-analysis involving 2775 subjects demonstrated that the relative bleeding risk was increased in all variants in comparison to subjects with the wild-type genotype [319]. In patients with the CYP2C9*2 genotype, the relative bleeding risk was 1.91 (95% CI, 1.16–3.17); for CYP2C*3, it was 1.77 (1.07–2.91), and for patients with either of these variant alleles, the relative risk of bleeding was 2.26 (1.36–3.75) [319]. In Hagashi et al.'s cohort study, CYP2C9 was found to be an independent risk factor for bleeding on warfarin initiation (HR 2.39; 95% CI, 1.18–4.86) [317]. As before, the majority of studies in this area have been conducted in predominantly Caucasian populations. One study [320] has, however, reported an increased risk of major hemorrhage among African-American and European-American patients with CYP2C9 variants on warfarin (15.7 vs. 5.8 per 100 patient-years, $p = 0.0015$) [320]. This study is the first to report an association with CYP2C9 and bleeding in African-Americans; however, the CYP2C9 allele frequencies varied significantly across race in this cohort ($p < 0.0001$) leading to potential population stratification bias.

Pharmacodynamic Factors Determining Warfarin Response

Warfarin's 4-hydroxycoumarin ring is the essential component for achievement of its unique anticoagulation properties [321]. This structure binds to vitamin K epoxide reductase (VKOR) and inhibits the reduction of vitamin K epoxide to dihydrovitamin K. This is a cofactor involved in the posttranslational carboxylation of glutamic acid residues in the production of vitamin K-dependent clotting factors [322, 323]. The anticoagulation effect is thereby achieved by inhibition of vitamin K cycling and depletion of clotting factors. The potency of this action depends on its asymmetry at position C9 of the compound.

VKORC1

The target of warfarin's effect is the vitamin K epoxide reductase complex, subunit 1 in the liver. The gene encoding this enzyme (VKORC1) was discovered in 2004 [324] and is located on chromosome 16p11.2. This is the primary gene that has been extensively shown to contribute to warfarin's pharmacodynamic profile. Rieder et al. identified ten common noncoding

VKORC1 single-nucleotide polymorphisms (SNPs) and grouped together five major haplotypes (H1, H2, H7, H8, and H9). These haplotypes had a greater than 5% frequency in European-Americans and were subsequently divided into two groups depending on warfarin dose requirements: group A (comprising H1 and H2) and group B (H7–9). These have been shown to significantly affect warfarin dose requirements, with genotype AA being the most sensitive and requiring the lowest dose and with BB associated with the lowest enzyme activity and hence requiring the highest dose [325]. The dose difference was significant among the three VKORC1 haplotype combinations (ranging from 2.7 ± 0.2 mg in AA to 6.2 ± 0.3 mg in BB [$p < 0.01$]). Schwarz and his colleagues confirmed this relationship [318]. Overall, these haplotypes accounted for 25% of the variation in warfarin dose among patients. Currently, the three most common VKORC1 polymorphisms are 1173C>T, 3730A>G (first described by D'Andrea et al. [326]), and −1639G>A (later described by Yuan et al. and Sconce et al. [308, 327]). The first meta-analysis of VKORC1 polymorphisms included these three common variants to determine their impact on warfarin dose requirement [328]. VKORC1 1173C carriers required 63% (95% CI; 44–82%) higher mean daily warfarin dose than 1733 TT carriers. Additionally, −1639G and 3730A variants required 61% (95% CI; 49–73%) and 32% (95% CI; 4–59%) higher doses than −1639AA and 3730GG, respectively [328]. In the aforementioned polymorphisms, the noncoding variant −1639G>A (guanine to adenine substitution) is the most described VKORC1 SNP. Its association with lower-dose requirements and hence warfarin sensitivity is well documented [311, 325, 329, 330]. It is thought that this promoter SNP has functional effects on protein expression via alteration in the VKORC1 transcription factor binding site [308, 325, 331]. As a result, this polymorphism has been shown to be the best marker of VKORC1 variants associated with warfarin sensitivity [331–333].

The different VKORC1 haplotype frequencies among African, Asian, and European populations in Rieder's study may account for the varied dose requirements in these different ethnic groups. Haplotype A was significantly higher (89%) in Asian-American populations compared with African-Americans (14%) who are known to require a lower and higher maintenance dose of warfarin, respectively ($p < 0.01$) [325]. In a cohort of 226 African-Americans, VKORC1 −1639G>A ($p < 0.002$) and 497T>G ($p < 0.027$) genotypes were significantly associated with warfarin dose requirements

[313].

The VKORC1 haplotype A has been shown to be associated with a shorter time to the INR more than or equal to the lower limit of the therapeutic range (HR 1.64; $p < 0.001$) [334]. Similarly, patients with one or two *VKORC1* haplotype A alleles had a significantly reduced length of time to achieve first INR within the therapeutic range [318]. During warfarin initiation, homozygotes for the VKORC1 −1639 A and G alleles were associated with a longer time to achieve stable dosing ($p < 0.001$) [335]. Studies examining this outcome in other ethnic groups are scarce and have not been validated [336, 337].

A case–control study has demonstrated an association with the VKORC1 1173C>T polymorphism and coumarin-related bleeding. Carriers of at least one T allele had an increased risk of bleeding (crude OR = 1.7; 95% CI, 1.1–2.5) compared to individuals with the wild-type genotype [338]. Other studies, however, have not confirmed any significant associations between VKORC1 variants and risk of bleeding [318, 320, 329].

CYP4F2

The CYP4F2 gene encodes for an essential enzyme in the metabolism of vitamin K. There has been recent interest in the CYP4F2 gene in warfarin pharmacodynamics with varying results. Caldwell et al. reported a clinically important CYP4F2 polymorphism, V433M (rs2108622) that was significantly associated with warfarin dose variation in Caucasian cohorts [339]. This variant accounted for a 1 mg/day difference in stable warfarin dose between TT and CC genotypes [339]. A meta-analysis of 13 studies compared CYP4F2 homozygotes (CC) to carriers of CT and TT genotypes. They required 10.0% (95% CI, 4.0–15.0) and 21.0% (95% CI, 9.0–33.0) higher warfarin doses, respectively ($p < 0.05$) [340]. This increased dose requirement has been confirmed in two further GWA studies [41, 42].

Other Genes Implicated

Finally, several other genes have been implicated in warfarin sensitivity with contradictory evidence, including apolipoprotein E [341, 342], gamma-glutamyl carboxylase (GGCX) [330], calumenin [343], NAD(P)H dehydrogenase, quinone 1 gene [344], and vitamin K-dependent proteins [345, 346]. Their effects on warfarin variability, however, have not been

confirmed in genome-wide association studies (GWAS). To date, four genome-wide association studies have been conducted to determine the genetic variants that have the greatest influence on warfarin's variation in dose requirements [43, 347–349]. Cooper et al. tested for approximately 550,000 polymorphisms in 181 Caucasians and demonstrated that VKORC1 variants had the single most important effect on maintenance dose ($p = 6.2 \times 10^{-13}$). Combining CYP2C9 and VKORC1 genotypes with other environmental factors accounted for approximately 40% of the variance in stable dose [347]. Furthermore, the largest genome-wide association study involving 1053 Swedish patients identified CYP4F2, alongside CYP2C9 and VKORC1 as a third genetic predictor of warfarin dose, contributing to approximately 1.5% of the variance [348]. Cha et al. [349] conducted the first GWAS in an Asian population. VKORC1 was shown to be the greatest predictor of warfarin dose in this cohort (contributing to approximately 25% of variation) and was the only genetic variant to achieve genome-wide significance. CYP2C9 polymorphisms contributed to a lesser extent, and after controlling for both VKORC1 and CYP2C9 variants, CYP4F2 emerged as a further indicator of warfarin's variability in dosage requirements [350]. More recently, a GWAS in a UK cohort conducted a multifactorial analysis, and in addition to VKORC1, CYP2C9 and CYP4F2, identified alcohol consumption, loading dose, cigarette smoking, and interacting medications, as determinants of response to different warfarin phenotypes (including mean weekly dose, stable mean weekly dose and INR > 4 end of first week), with about 58% of variance explained for mean weekly dose [43].

In summary, it has been consistently shown that more than 50% of the variability in warfarin dosage among individuals can be described by CYP2C9 and VKORC1 genotypes in combination with environmental factors such as age and body surface area [308, 325, 351]. Although this still leaves a significant proportion of warfarin's variability yet to be described, it is unlikely any further significant SNPs with clinically relevant effects on warfarin dose will be discovered.

Clinical Implications

Following significant improvement in knowledge of the key genetic components contributing to warfarin's interindividual variability, the FDA amended warfarin's label in 2007 and 2010, to suggest consideration of

genotypic variation in prescribing. As a result, several pharmacogenetic prediction algorithms have been developed [351–353].

The largest sample size used to develop an algorithm is from the International Warfarin Pharmacogenetics Consortium; this algorithm proved significantly better at predicting stable warfarin dose in subjects who required <21 mg weekly (49.4% vs. 33.3%; $p < 0.001$) and required >49 mg weekly (28.4% vs. 7.2%; $p < 0.001$) than the clinical algorithm alone [351]. This would suggest that patients who are at highest risk of adverse events (associated with subtherapeutic and supratherapeutic dosing) benefit most strongly from genotype-based dosing. In recent years, there have been several prospective randomized controlled trials (Table 17.7) comparing clinical (in combination with INR dosing) and pharmacogenetic algorithms. Many have shown that pharmacogenetic dosing algorithms have been associated with more time spent in the therapeutic range than standard dosing [354–357]. The EU-PACT trial [44] showed that pharmacogenomic-guided dosing was superior to fixed dosing regimen but this was not supported by the findings of the COAG trial [45]. Several factors contributed to this variance, including ethnicity (27% Blacks in COAG versus 100% Caucasians in EU-PACT), different control arms, the availability of genotype data prior to warfarin initiation, and the fact that loading doses were not used in the COAG trial [46]. Further research is required to identify relevant genetic variants in other ethnic populations.

Table 17.7 Intervention studies that have evaluated the role of pharmacogenetics in determining warfarin dose requirements

Standard (STD) control Pharmacogenetic (PG) intervention							
Author	***N***	**Ethnicity**	**Study design**	***N***	**Treatment**	***N***	**Algorithm**
Pedro-Botet et al. [44]	427 patients, 211 genotype-guided dosing and 216 control standard-dosing	98.5% Caucasian	Pragmatic, single-blind, randomized, controlled trial	211	Standard dosing: 10 mg, 5 mg, 5 mg	216	Days 1-3 International Warfarin Pharmacogenetic Consortium algorithm, day 4 and 5 dose-revision algorithm that was based on the INR value on da 4

Kuivenhoven et al. [45]	955 patients, 514 genotype-guided dosing and 501 control standard-dosing	Black/hispanic	Multicenter, double-blind, randomized, controlled trial	514	Clinical dose initiation algorithm	501	Genotype-adjusted dosing algorithm
Hillman et al. [354]	117 screened 74 excluded five declined *38 participated*	Not stated	Prospective, randomized, single-blinded pilot trial	20	Standard dosing: 5 mg of warfarin daily on initiation	18	Multivariate dosing model: Age, body size, comorbidities, clinical indication, and genotype
Anderson et al. [355]	206 consented and randomized six withdrew *200 participated*	94.1% Caucasians in PG arm, 94.9% in STD arm	Prospective, randomized trial. The pharmacist who managed dose adjustments was unblinded.	99	Standard dosing,: 10 mg, 10 mg, 5 mg	101	Regression equation: Age, weight, sex, and genotype
Caraco et al.	283	Not stated	Prospective,	93	Standard	92	Warfarin loading

[356]	consented and randomized		randomized, controlled trial. Not blinded. Did not use intention-to-treat analysis		algorithm based on INR response		was guided by si different CYP2C genotype-adjuste algorithms
	98 excluded						
	185 participated						
Huang et al. [357]	156 enrolled	Chinese	Prospective, randomized, single-blinded, controlled trial	70	Standard initiation dose 2.5 mg/day, adjusted with INR	72	Multiple linear regression mode Age, body surfac area, and genotype
	14 excluded						
	142 randomized and participated						
Epstein et al. [358]	896 patients initiating on warfarin matched to 2688 historical controls	Not stated	Prospective, comparative effectiveness study (quasiexperimental design)	2688	Standard warfarin initiation	896	Patients were genotyped for CYP2C9 and VKORC1, and the results (with an interpretation were reported to the physician
							No specified interventions followed deliver of the report

STD standard, *PG* pharmacogenetic, *INR* international normalized ratio, *HR* hazard ratio

Clinically, the use of genotype-guided warfarin has not been universally accepted yet, although it is currently being implemented in some pilot sites in the UK. Certain centers in the US use pharmacogenetics to guide warfarin use. Indeed, a naturalistic study performed by Medco and the Mayo Clinic was able to show that warfarin genotyping reduced hospitalization rates for bleeding and thromboembolism by 43% [357]. However, widespread uptake is likely to be dependent on the results of further ongoing RCTs, for example the genetics-informatics trial (GIFT) of warfarin to prevent deep vein thrombosis, and on the effectiveness and safety of the new oral anticoagulants (dabigatran, rivaroxaban, and apixaban) recently licensed in the US and EU. Whether there will be wholesale replacement of warfarin in all indications or whether a stratified approach will be used is uncertain.

Direct Oral Anticoagulants

Direct oral anticoagulants (DOACs) reversibly inhibit the active sites of circulating and clot-bound thrombin (dabigatran) or clotting factor Xa (rivaroxaban, apixaban and edoxaban). Their onset of action is more rapid than warfarin, with a wider therapeutic window and fewer drug and food interactions. Net absorption of DOACs depends upon the intestinal permeability glycoprotein (P-gp) efflux transporter. Consequently, increased bleeding risks are potentially associated with the concomitant prescription of strong P-gp inhibitors such as amiodarone, verapamil, clarithromycin and antifungal agents, among others. Currently, they do not require therapeutic drug monitoring. However, clinical and genetic factors have been shown to affect DOAC efficacy and safety, and therefore dose adjustments may be required in high risk patients.

The RE-LY trial reported genome-wide SNP associations for both peak and trough dabigatran concentrations [47]. The minor allele of rs8192935, an intronic SNP located in the carboxylesterase 1 gene (*CES1*), was associated with a 12% reduction in peak dabigatran concentrations. Conversely, the minor allele of rs4148738, an intronic SNP located in *ABCB1*, which encodes P-gp, was associated with a 12% increase in peak dabigatran concentrations. Neither rs8192935 nor rs4148738 were associated with clinical outcomes.

Importantly, *CES1* rs2244613 was associated with both decreased dabigatran trough levels and a 33% lower risk of bleeding events per minor allele [47]. These findings require replication.

Rivaroxaban, apixaban and edoxaban are all substrates for P-GP. It may therefore be the case that *ABCB1* gene variants play a role in their availability and disposition. A significant association of allele and genotype frequencies at the ABCB1 SNP rs4148738 and peak, but not trough, apixaban concentrations has been observed in a study of 80 Caucasian patients with atrial fibrillation taking apixaban [48]. Carriers of the AA genotype had significantly lower peak apixaban concentrations than those with the G allele. Conversely, in a phase 1 study involving 459 healthy volunteers, neither allelic variants of ABCB1 C3435T or the SLCO1B1 T521C polymorphism affected edoxaban pharmacokinetics [49]. Other candidate genes include the drug-metabolising CYPs of rivaroxaban (CYP3A4/5, CYP2J2) and apixaban (CYP3A4/5). The sulfotransferases, SULT1A1 and SULT1A2[50], involved in conjugating apixaban into its major metabolite, O-demethyl-apixaban could also exert a genetic influence on apixaban response. Further investigations are certainly warranted.

Conclusions

There has been significant activity in determining the genetic factors determining response to drug used to reduce the risk of stroke. However, none of the genetic factors are routinely tested and incorporate into clinical practice. This is a problem seen in many therapeutics, not just stroke medicine, and is related to many factors including poor study design, inadequate sample sizes, poor phenotyping, lack of replication, and lack of assessment of clinical factors (including co-therapy) that may significantly impact on the response to the index drug. There is no doubt that the path from discovery to clinical implementation is long and arduous. The majority of findings in the area of stroke pharmacogenetics are really stuck in the first translational gap (T1), and very few have progressed to the second translational gap (T2: clinical validity and utility), T3 (implementation), or T4 (effect on public health) [359]. One possible exception to that is warfarin, where the association between two genes (CYP2C9 and VKORC1) and two clinical factors (age and body mass index) is robust. In order to progress the area of stroke pharmacogenetics, it will be important that researchers

undertake a step-wise approach that may not only require comprehensive phenotyping and genotyping, but also multicenter collaboration to allow not only for replication of important findings but also assessment of the effects within different ethnic groups.

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18. Genetic Research and the Law

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Introduction

It has often been said that medicine advances and law restrains.

Legal restraint may sometimes be necessary to protect the interests of society and to protect the welfare of the public. Lawmakers have been more reactive than proactive when it comes to regulating the use of genetic information. At times, the law has even been complicit in the abuse and misuse of genetic information. That is especially true during the eugenics movement. Hideo Kojima expressed the belief that access to genetic information can be beneficial if it is used wisely. Nonetheless, he acknowledged that the possession of genetic information may be abused. Kojima's theory that knowledge comes with benefits and burdens can be illustrated using breast cancer as an example.

Researchers have identified more than 1800 mutations in the BRCA1 gene. Many of those mutations are associated with an increased rise in breast cancer and other types of cancer. Those mutations are present in every cell in the human body and can be passed from one generation to the next. As a result, they are associated with cancers that cluster in families. However, not everyone who inherits a mutation in the BRAC1 gene will develop cancer. That fact does not offer solace to anyone who discovers that they have the

mutations. For example, after Angelina Jolie was diagnosed with the BRAC1 gene, she chose to undergo surgery because her mother died at the age of fifty-six after an eight-year battle with ovarian cancer. Angelina used the knowledge about her genetic predisposition to take steps to remove the possibility that she would get cancer in the future. She benefited by having access to genetic information pertaining to the BRAC1 gene. She treated the surgery as an insurance policy against cancer. However, under some circumstances, someone might have tried to use that genetic information against Angelina. For example, if she was at the beginning of her career, she might have had a difficult time getting acting roles.

When I was a child, I often heard that there were “bad heart genes” on my mother’s side of the family. I was not quite sure what that meant. I just knew that several of my cousins died young after suffering massive heart attacks. One of my aunts lost four of her children to heart disease. When my mother started having shortness of breath and chest pains, her doctor told her that she was just going through “the change.” He kept up that narrative until my sixty-year old mother suffered a massive heart attack and died on a nice fall morning. At that time, I wondered if my mother’s doctor could have done more or if my mother had just been a victim of her family’s “bad heart genes.” My mother died before research revealed the connection between genetics and certain diseases. If testing had shown that she had a genetic predisposition towards heart disease, her doctor might have treated her differently. On the other hand, the existence of that genetic information may have negatively affected my mother in other ways.

After a professional football team drafted my first cousin, the owner asked him to fill out a medical history form. When the team doctors saw the list of his family members who had died of heart disease, they told the team owner that they could not clear him to play. Therefore, although my cousin was in great physical shape, his professional football career ended before it started because he was suspected of having “bad heart genes.” Doctors have a duty to give the patients enough information to enable them to make wise medical decisions. The law is in place to make sure that the information the patients receive is accurate.

An exploration of all of the possible legal issues that may arise because of advances in genetic technology and genetic research is beyond the scope of this chapter. Therefore, I will briefly discuss concerns with regards to genetic privacy, genetic discrimination and genetic commercialization. Legislators

have enacted laws to address each of these issues. Nonetheless, lawmakers must work to ensure that people will be able to avail themselves of beneficial genetic testing without fearing that those test results will be used against them. Lawmakers must also act to prevent genetic information and material from being treated with the same level of respect as a piece of tangible personal property.

Genetic Privacy

Persons may put their genetic privacy at risk by participating in medical and non-medical activities. Every time a person has an encounter with a health care provider he or she faces the possibility that information about his or her genes may be disclosed. Physicians and other health care providers have a common law duty to keep their patients' medical information confidential. However, in some cases, a physician may be legally required to disclose confidential medical information. The doctor may have the obligation or the authority to reveal otherwise confidential medical information under a common law duty to warn. Under certain circumstances, statutes may mandate, prohibit or give the doctor the discretion to disclose.

People are increasingly obtaining genetic information about themselves that, unlike other medical information, may directly concern not only them, but also their genetic relatives. In the United States, at least two courts have recognized a physician's legal duty to warn both a patient and the patient's immediate blood relatives in the event they may be at risk of developing a genetically transmissible condition. This duty exists as an exception to the physician's duty to keep a patient's medical information confidential. It applies even if the relative is not the physician's patient.

A Florida court was faced with a dispute involving the following facts. Doctors treated Marianne New for medullary thyroid carcinoma, a genetically transferable disease. Three years later, New's daughter, Heidi Pate, was diagnosed with an advanced form of the disease. Pate and her husband sued the doctors for failing to warn Pate that she should have been tested for the disease. The Court concluded that the doctors should have informed New about the genetically transferable nature of her disease. In addition, the Court held that when a doctor knows of the existence of third parties who may be impacted by a disease, the doctor's duty to warn extends to them. Nonetheless, the Court determined that to satisfy that duty, the doctor only

had to notify the patient that her disease was genetically transferable [1].

Shortly after the *Pate* case was decided, a New Jersey court heard a similar case. The plaintiff, Pack, suffered from cancerous blockage and multiple polyposis of the colon. Her father had been treated for the same condition when she was a minor. Pack sued the estate of her father's physician claiming that because the disease was hereditary the physician had breached his duty by not informing those who were potentially at risk of developing the condition. Adopting the concept of a "genetic family," the Court ruled in favor of the plaintiff. The Court decided that the physician had a duty to warn of preventable risks from genetic causes.

The Court justified its decision to extend the duty to warn beyond the patient to members of the patient's immediate family by opining that genetic information is not just personal medical information but is simultaneously personal and familial. Unlike the *Pate* Court, the Court in this case noted that a doctor could not always satisfy the duty to warn by just informing the patient of the transferable nature of a disease [2]. After this decision, the New Jersey legislature responded to the complaints of physicians by enacting a genetic information disclosure statute restricting the circumstances under which doctors are required to disclose genetic information. Under the statute, disclosures of the kind mandated by the *Safer* Court are not included among those permitted.

A patient's most sensitive health information is stored in his or her medical records. Traditionally, medical records were paper documents that were relatively secure. A patient could be confident that information contained in those medical records would not be shared without his or her consent. A patient even had to sign a medical release form to get access to his or her own medical records. Times have changed. We live in a world where we do just about everything electronically. We bank, pay our bills, and file our taxes electronically. Thus, it is no surprise that most of our medical records are now stored electronically. In 2009, the Obama administration provided a five-year plan to move hospitals and clinicians away from paper charts by encouraging the use of technology to document the medical records of their patients. Under the plan, health care providers could get federal money to help offset the costs of the electronic medical record systems. There were also penalties for not using an electronic system [3].

Electronic medical records (EMRs) are beneficial to both patients and health care providers. It is easier for patients to access their medical records.

Instead of filling out a release form and waiting to receive their records through the mail, patients can simply use their passwords to gain online access to their records. Thus, patients can instantly keep track of their appointments and medications [4]. Moreover, if a patient sees an error in his or her records, the patient can easily inform the health care provider. Patients also benefit because their medical records follow them, so health care providers always have a good medical history for their patients [5]. This is important in an emergency situation when time may be of the essence [6]. EMRs provide medical professionals with a degree of functionality that paper records cannot. For example, medical errors may be reduced because the health care provider does not have to decipher information in a paper record that might be illegible [7]. Even though start-up costs are expensive, once an EMR system is set up, it is cheaper and more efficient to store data electronically [8].

The compilation of vast amounts of medical and genetic information in a single electronic database to which numerous people in different locations have access undermines personal privacy [9]. Storing medical records electronically increases the possibility that a patient's genetic information may be intentionally or accidentally disclosed [10]. There have been several cases where hospitals have fired employees for looking at the medical records of celebrities [11]. There are probably many more instances where the hospital did not catch the lookers. If those people had to go to the records office and search for the paper files, the likelihood of them taking the time to access those medical records would have probably decreased. The ease of access to the records may have made it hard to resist the temptation of peeking.

Because health information is being stored electronically there is an increased risk of "invisible theft," the stealing of data without taking the physical record [12]. Therefore, the health care provider would never know that the patient's privacy had been compromised. This would be especially troubling in a small town where the disclosure of genetic information could impact the lives of persons other than the patient. Stories about the databases of banks and hospitals being hacked are becoming commonplace. One of the fastest growing crimes is medical identity theft [13]. The law has tried, but has been relatively unsuccessful, to protect the privacy of patient data that is stored electronically [14].

The cornerstone of privacy protection in the medical field is the Health

Insurance Portability and Accountability Act (HIPAA). HIPAA prohibits "covered entities" from disclosing protected health information without first obtaining patient consent [15]. HIPAA protection applies to genetic information [16]. Congress amended HIPAA to establish standards to protect electronic medical records [17]. The mandates of HIPAA are enforced by the Office of Civil Rights (OCR). Recognizing the added burden the existence of EMRs had placed on the OCR, Congress passed the Health Information for Economic and Clinic Health (HITECH) Act. HITECH gave state attorney generals the authority to assist the OCR with its enforcement of HIPAA [18]. A major shortcoming of HIPAA, in this context, is that it only applies to health plans, health care clearinghouses, and health care providers that transmit health information in electronic form in connection with health care transactions. Most EMRs are stored by private third-party companies that do not come under the jurisdiction of HIPAA. Non-covered entities that operate electronic health record databases are not prohibited by HIPAA from disclosing protected health information [19]. Even if these companies seek to protect the information, recent events like the ones that occurred during the 2016 United States presidential election have shown that even the most secure databases can be hacked.

A significant number of private and public-private ventures have responded to the increasing demand for raw material for genetic research by creating biobanks [20]. The banks include large collections of tissue, medical records, disease registries, blood spots, and other information that are collected and maintained by health care providers, academic research organizations, and state and federal governments [21]. These items are made available to researchers. Federal regulations govern the consensual and non-consensual use of medical information and physical specimens in research. Current federal standards require consent for the collection of specimens and personal information but allow nonconsensual use of stored tissue and information [22]. As a result, a great deal of personal genetic information is susceptible to being disclosed without the donor's knowledge or consent. For example, the blood spot cards used for newborn screening for PKU are held by states indefinitely and may be given to researchers. Special privacy and confidentially concerns also arise in genetic research because of the information that specimens and medical records carry about nonparticipating and nonconsenting family members.

In order to discover more information about their genetic make-up,

people are willingly to take the chance that their genetic information may be disclosed. Taking advantage of that quest for knowledge, a growing number of companies are marketing genetic testing kits directly to consumers. Their promotional materials promise to guide customers to healthier lives by predicting their unique risks for developing scores of diseases and telling them how to prevent them. That promise is enticing, so people willingly send samples of their genetic materials through the mail. Direct-to-consumer genetic testing kits are usually marketed to people who are not ill or at high risk for a disease. Those consumers are just curious or concerned about their risk for different disorders. After the company analyzes the genetic samples, the person receives a report identifying his or her risk of developing certain medical conditions. The report usually contains recommended strategies for reducing the indicated risks.

In the scenario described above, the person's genetic privacy may be violated in at least two ways. First, after the person voluntarily turns his or her genetic information over to a member of the postal services, he or she is no longer able to control what happens to it. The mail is not always as secure as the public would like it to be. For example, there have been cases where mail has been discovered abandoned. Therefore, a person who participates in this type of genetic testing runs the risk that someone will get access to their genetic material. In those cases, the person will probably not be harmed by having a stranger gain possession of the sample. The second time at which the person's genetic privacy may be violated is when the company mails the report that contains the analysis of the genetic sample. For instance, the postal worker might accidentally deliver the letter to the wrong address, so someone else might see the test results. There are no laws specifically designed to protect a person's genetic privacy in cases involving organizations that are not subject to HIPAA, the law enacted to insure that health information is protected [23]. The disclosure of a person's genetic information can cause them to be the victim of discrimination.

Genetic Discrimination

The discovery of genetic factors for some diseases led to the presupposition of genetic explanations for other human conditions and opened the door to genetic discrimination. Information gained from genetic research has enabled doctors to determine whether or not a person has a predisposition for certain

diseases. As a result, doctors are able to improve screening and treatment for a number of diseases. Persons who are known to possess genes that are linked to certain conditions may face genetic discrimination. That discrimination has caused persons to be denied health insurance and employment [24]. Currently, lawmakers are taking steps to prevent and remedy that disparate treatment. The regulation of genetic information is influenced by its history. In the case of genetics, for some, that history has not been kind. In fact, the government relied on imperfect science to conclude that some members of the population were genetically defective.

In *Buck v. Bell* [25], one of the first United States Supreme Court opinions dealing with genetics, Justice Oliver Wendell Holmes wrote: “Three generations of imbeciles are enough.” [26] With that proclamation, Justice Holmes unintentionally started a movement that led to the involuntarily sterilization of thousands of people. Carrie Buck gave birth to a baby girl who was a product of her rape [27]. After that, she was placed in a state institution for epileptics and feeble-minded people. Dr. Priddy, the superintendent of the institution, filed a petition to have Carrie sterilized. He claimed that Carrie was feeble-minded. Dr. Priddy also implied that Carrie had inherited her poor mental state from her mother. He believed that Carrie’s mother was feeble-minded because she had given birth to three children, including Carrie, without the benefit of marriage. Dr. Priddy based his request on a state statute authorizing the compulsory sterilization of the intellectually disabled to improve the human race by eliminating persons with defective genes [28]. A guardian for Carrie helped her challenge the proposed sterilization [26].

The Court held that the state’s plan to have Carrie sterilized was justified by a legitimate state interest because Carrie’s mother was believed to be mentally impaired. State legislators thought that sterilization was necessary to prevent Carrie’s defective genes from being passed to another generation. Consequently, the court determined that the statute permitting compulsory sterilization of the unfit did not violate the United States Constitution [29]. After the court ruled against Carrie, she was forced to undergo sterilization. The three generations Holmes referred to were Carrie’s mother, Carrie, and Carrie's daughter. The presumption was that the daughter was also feeble-minded because of her parentage [30].

Approximately 30 states implemented programs that forcibly sterilized low-income, uneducated, or mentally impaired individuals as a selective

population control program [31]. States also enacted legislation requiring that African Americans be screened for the sickle-cell trait. The screening programs were put in place even though individuals who had only one copy of the gene would suffer no physical effects and there was no cure for the disease. Thus, the purpose of the mandatory screening programs was unclear. The programs resulted in widespread confusion about sickle-cell trait and resulted in health insurance and employment discrimination [32]. In response, Congress enacted the National Sickle Cell Anemia Control Act in 1972. That Act provided financial incentives for states to make sickle cell screening voluntary [33]. Presently, the two main areas where genetic information can be misused are health insurance and employment. The two areas are connected because the ability to obtain health insurance for an employee at a reasonable rate often factor into hiring decisions. For example, several companies have refused to hire obese persons and smokers. Lawmakers have taken steps to reduce the likelihood of discrimination in these areas.

Traditionally, state statutes prohibited genetic discrimination by health insurers. They could not use a person's genetic information to justify the denial of health insurance coverage. In addition, health insurers could not rely on genetic information when determining premiums or benefits. The law did allow health insurers to consider genetically linked diseases that had become symptomatic, so they could deny coverage to persons who had pre-existing conditions. For years, the law permitted health insurers to discriminate against people with pre-existing conditions. Therefore, people who needed health insurance the most were not given the opportunity to purchase it. Those persons could get health insurance through their employers. However, the existence of those employees led to the employers paying higher group health insurance premiums. In some cases, the employers' group health insurance coverage was at financial risk. This made employers reluctant to hire persons with pre-existing conditions. As a result, those persons were often left without a job and with no health insurance.

The plight of those persons improved with the passage of the Affordable Care Act (ACA). Under the provisions of that law, individuals and families cannot be denied the opportunity to purchase health insurance because of pre-existing conditions [34]. The Republicans have indicated that, even if the statute is repealed, they will keep that provision. The resolution of the "pre-existing condition" problem has pretty much eliminated discrimination in the health care arena, but employment discrimination continues to be a problem.

The Genetic Information Nondiscrimination Act (GINA) is the first federal law that was enacted to specifically address the issue of genetic discrimination. It focused on the areas where most of the problems exist. The purpose of the statue is to prohibit health insurance providers and employers from using genetic information to disadvantage persons [35]. GINA bars employers from using the genetic information of individuals when making hiring, firing, job placement or promotion decisions [36]. It also prohibits health insurers and health plan administrators from requesting or requiring genetic information from an individual or the individual's family members or using that information for decisions regarding coverage, rates or pre-existing conditions [37].

The enactment of GINA is a step in the right direction. However, the law has some shortcomings. GINA does not prohibit health insurers from raising the premiums for group employer-based insurance once a member of the group manifests a genetic disease [38]. Hence, the employer would have an incentive not to hire a class of people whom the employer believed would necessitate higher health insurance premiums in the future. Health insurers cannot increase premiums on a woman because she has the BRAC1 gene that predisposes her to developing breast or ovarian cancer. However, once the woman is actually diagnosed with breast cancer, she loses GINA protection, so the health insurers are free to increase her insurance rates or the group premiums of her employer. As a result, employers may be hesitant to hire a woman who has a history of breast cancer. It does not make sense that the woman can eventually be discriminated against because of her genetic make-up. To fix this problem, GINA must apply even after the person develops the genetically related disease.

GINA only applies to situations involving genetic information. Genetic information refers to genetic tests or the manifestation of a disease or disorder in family members of the protected person. The statutory definition of genetic information is too narrow. Access to a person's genetic information can be obtained in ways other than genetic test results. For example, a person may have to give a blood or urine sample as a part of a pre-hiring process. That fluid will contain the person's DNA and it does not seem to fit the statutory definition of genetic information. The definition of genetic information should be expanded to include any sample containing a person's DNA. Lawmakers should also strive to make sure that genetic information and genetic materials are not treated like commodities.

Genetic Commercialization

The hope is that genetic research will lead to personalized health care products, reduction of adverse drug reactions, better methods of identifying individuals at risk for diseases, and improvements in preventive care. For that hope to become a reality, there must be cooperation among researchers. Researchers must also be able to gain access to genetic information, tissue samples, and human research subjects. Greed may be the greatest threat to that hope.

This is especially true when avarice influences the manner in which research is conducted. The case of Jesse Gelsinger, a young man with a genetic liver disease, is illustrative of the harm that greed can cause. At the age of eighteen, Jesse became the first known person to die in a gene therapy clinical trial. An investigation revealed that the lead researcher was conducting the research with a private company in which he had a financial interest. Evidence indicated that the financial interests of the lead researcher and the research organization might have influenced the approval and the conduct of the protocol. In a rush to make a profit, the researcher may have been overzealous. He ignored warnings from several independent people who were observing the research. In addition, he failed to note that a previous participant had suffered an adverse reaction similar to the one that caused Jesse's death [39].

Researchers and their financial backers put a great deal of time, energy, and money into clinical genetic research. Conducting genetic research is not cheap. The amount of government funding for genetic research has continuously declined, so researchers have turned to private sources for the money that they need. Because private companies are pouring billions of dollars into genetic research it is reasonable for them to expect a return on their investments. Problems occur when private companies consider genetic research to be just another commercial investment and genetic information and genetic materials to be commodities that can be bought and sold. There is a lot of money to be made from the end-products of genetic research and everyone wants a piece of the pie. Thus, it may be easy for the human component of genetic research to get lost in the process.

The commercialization of genetic research may cause unhealthy competition between persons conducting the research. The focus may shift from searching for cures to genetic diseases to rushing to be the first one to

profit from the research. Myriad exhibited this behavior. After discovering the precise location and DNA sequence of the BRCA1 and BRCA2 genes, Myriad obtained several patents. When Myriad found out that other organizations were isolating the BRCA genes for use in genetic testing, Myriad mailed letters claiming that the genetic testing activities infringed on its patent rights and filed patent infringement claims against researchers who refused to stop the genetic testing. In response, the targeted organizations agreed to quit isolating the BRAC1 and BRAC2 genes. Consequently, Myriad was the sole organization providing genetic testing on the BRCA1 and BRCA2 genes. That monopoly was not meant to benefit the public or to increase our knowledge about those genes. Myriad was just trying to eliminate the competition. Another company joined by medical patients, advocacy groups, and doctors sued to have Myriad's patents declared invalid. The Supreme Court ruled against Myriad after it concluded that isolated DNA could not be the subject of a patent because it involved a segment of DNA that occurred naturally. The Court gave Myriad a small victory by upholding its right to patent DNA that it had synthetically created. In a similar case, a court in Australia held that Myriad could patent the human gene sequence it discovered [40].

Patents are in place to reward persons for their efforts by giving them the right to exclude others from utilizing their inventions for a specific number of years. During that time, anyone who wants to use the product must get permission and pay the patent holder for a license. The system works because it encourages innovation. Myriad spent a great deal of money to finance the research it took to discover the DNA sequence. Therefore, it seems unfair that other researchers get the benefit of that discovery without having to compensate the company. However, Myriad really did not create anything new. The fact that Myriad applied for the patent and sought to prevent other researchers from utilizing the DNA sequence indicates that the company is more concerned about profits than progress when it comes to genetic research. Private companies have to make profits to stay in existence, so it is understandable that Myriad would take steps to protect its investment.

Even though the court ruled against Myriad, there were no real winners and the patients who are hoping to benefit from genetic research are probably the biggest losers. Myriad's attempt to patent the DNA sequence probably hampered future cooperation among genetic researchers. It also damaged the company's image with the public. The decision in the case might have

discouraged other companies from investing in genetic research. Therefore, the quest to find cures for certain genetic diseases and disorders may be slowed a bit. The Court's reluctance to permit Myriad to patent something that is naturally occurring in the human body may send a signal that genetic information and genetic material should not be treated as property. However, that message has not been received.

This case was only a minor setback for private companies like Myriad that are involved in the genetic research business. Those companies are still able to make significant profits as genetic research continues to become more commercialized. Researchers have turned to private companies to get the genetic information and genetic material that they need. As a result, those companies have established databases containing genetic information on millions of people. Most of this information is purchased without the persons' knowledge or permission. Currently, there is no law that prohibits the selling or buying of personal genetic information.

Likewise, private biobanks buy, store, and sell genetic material. Some of the most valuable assets owned by biotech companies are databases containing genetic information. These companies serve a valuable function because they provide genetic researchers with a steady supply of the information that they need. Is it wise to turn over such sensitive data to an entity that is mainly concerned about its own financial gain and the maximization of shareholder profits? There is nothing to prevent these profit-driven organizations from treating genetic databases and biobank materials in the same manner that they would treat any other commercial asset. For example, a company might sell its genetic database or biobank material if it is facing a financial crisis or needs to make a quick profit. When a company files bankruptcy, all of its assets are fair game to its creditors. Is it a good idea to put people's genetic information into the market place?

Companies are not the only ones that have indicated that human tissue should be considered to be property. Researchers and research subjects have asserted property claims to the products of research. Courts have heard several cases in which research subjects claimed that they had a property interest in the results of research produced using their medical tissue and/or information. In those cases, the debate was not over whether or not human tissue should be treated like a commodity. The fights were over who is entitled to the profits. A prime example of that type of case is *Moore v. Regents of the University of California* [41]. The *Moore* case stands for the

proposition that a person only maintains a limited right to control the use of cells that have been excised from his or her body. While Moore was being treated for cancer, his doctors removed his spleen and discovered that it contained unique cells. To obtain more of the cells, the doctors kept Moore coming back to the hospital by leading him to believe that he needed treatment. In fact, the doctors were researching on Moore without his consent. The doctors used Moore's cells to make a cell line. The doctor and the university patented the cell line established with Moore's tissue.

When Moore discovered that they were making millions of dollars, he sued to receive some of the profits. Moore argued that the doctor and the university had converted his property (his tissue) into a cell line without his permission. The Court ruled against Moore because he had voluntarily consented to have his spleen removed. The doctor and the university were found liable for lack of informed consent. They were permitted to profit from Moore's cells, although they made him an unwilling research participant. There have been several reported cases with facts similar to *Moore*. When the public hears that researchers are profiting from their body parts it makes it hard to get test subjects.

Conclusions

In this chapter, I have discussed a few of the legal issues that might arise in the context of genetic research. It is critically important that the research be conducted in a way that protects the privacy of the participants. The main way to achieve that goal is to take steps to ensure that no one discloses confidential genetic information. That task is made more difficult by the fact that researchers are turning to electronic databases to keep up with the massive amount of information they have to decipher. Privacy laws were put in place at a time when most medical information was kept on paper. In fact, numerous health care providers still keep their patients' medical records in paper form. Unfortunately, privacy laws have not kept pace with the ingenuity of persons seeking to steal sensitive health information.

Genetic privacy is crucial because if a person's genetic information ends up in the wrong hands he or she may be the subject of genetic discrimination. Most genetic discrimination occurs in health and employment settings, the two places were persons are the most vulnerable. Congress has enacted statutes to protect people from being discriminated against because of their

genetic predispositions. Nonetheless, the laws contain loopholes and weak enforcement mechanisms. In a profit-driven world, it is not surprising that there is now a market for genetic information and genetic materials. The law makes it clear that a person cannot sell a kidney or a liver; however, that person can sell blood or sperm. It is too early to tell whether genetic material will be treated like the former or the latter. But, we do know that as long as private companies are involved in genetic research commercialization will continue to occur.

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