

Original Contribution

Clinical Pre-genetic Screening for Stroke Monogenic Diseases Results From Lombardia GENS Registry

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Background and Purpose—Lombardia GENS is a multicentre prospective study aimed at diagnosing 5 single-gene disorders associated with stroke (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy, Fabry disease, MELAS [mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes], hereditary cerebral amyloid angiopathy, and Marfan syndrome) by applying diagnostic algorithms specific for each clinically suspected disease

Methods—We enrolled a consecutive series of patients with ischemic or hemorrhagic stroke or transient ischemic attack admitted in stroke units in the Lombardia region participating in the project. Patients were defined as probable when presenting with stroke or transient ischemic attack of unknown etiopathogenic causes, or in the presence of <3 conventional vascular risk factors or young age at onset, or positive familial history or of specific clinical features. Patients fulfilling diagnostic algorithms specific for each monogenic disease (suspected) were referred for genetic analysis.

Results—In 209 patients (57.4±14.7 years), the application of the disease-specific algorithm identified 227 patients with possible monogenic disease. Genetic testing identified pathogenic mutations in 7% of these cases. Familial history of stroke was the only significant specific feature that distinguished mutated patients from nonmutated ones. The presence of cerebrovascular risk factors did not exclude a genetic disease.

Conclusions—In patients prescreened using a clinical algorithm for monogenic disorders, we identified monogenic causes of events in 7% of patients in comparison to the 1% to 5% prevalence reported in previous series. (*Stroke*. 2016;47:00-00. DOI: 10.1161/STROKEAHA.115.012281.)

Key Words: CADASIL ■ cerebral amyloid angiopathy, familial ■ Fabry disease ■ genetics
■ Marfan syndrome ■ MELAS syndrome ■ stroke

Although monogenic diseases are considered rare causes of stroke ($\approx 1\%$ – 5% of all strokes),^{1,2} they are probably under diagnosed because physicians may not consider them in the differential diagnosis and because the wide phenotypic spectrum makes differentiation from sporadic cases difficult. Deciding which patients to screen for these diseases presents a considerable clinical challenge. First, there are limited data on the yield of screening for these conditions in stroke populations. Second, there is little guidance on which phenotypic characteristics increase the chance that such screening will be positive. This is particularly important because a family history may be absent for later onset familial conditions, such as stroke. Despite this, the identification of a genetic cause of stroke is important both for the individual patient and to allow presymptomatic testing of other family members, including the possibility of prenatal testing.

The Lombardia GENS project³ was established to (1) determine the frequency of a number of the most common single-gene disorders causing stroke in a well-characterized stroke population in whom there was a clinical suspicion of an underlying genetic cause and (2) develop clinical algorithms that might assist the clinician in deciding in which patients testing for these conditions has a useful yield. Testing was performed for 5 single-gene disorders associated with stroke: cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL; #MIM 125310), Fabry disease (#MIM 300644), mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS; #MIM540000), hereditary cerebral amyloid angiopathy (H-CAA; #MIM 605714, 105150, and 176300), and Marfan syndrome (#MIM 174700).

Patients and Methods

Standard Protocol Approval and Patient Contents

The local ethical committees in all participating centers approved the study. All the patients gave informed consent for genetic testing and participation in the study.

Participants and Procedures

Eighteen clinical stroke centres of the Lombardia region, admitting >100 patients with stroke per year, and 8 high-throughput technology genetic laboratories, performing the diagnosis of the 5 monogenic diseases, participated in the present study (online-only Data Supplement). A consecutive series of patients with ischemic or hemorrhagic stroke or transient ischemic attack (TIA) referred to the clinical participating units were recruited. Stroke physicians or trained researchers collected data on demographics, cerebrovascular risk factors, and detailed neurological and systemic clinical features (migraine, seizures, mood disorders, cognitive disorders, deafness, renal failure, high/low height, miscarriages, acroparesthesia, dysmorphism, ligament laxity, myopathy, and skin changes) on a standardized form.⁴ A detailed familial history of stroke and other related neurological and systemic traits was obtained. The family history was considered positive when at least 1 disease typical disturbance was present in at least 1 proband's first-degree relative.

Patients were screened for a clinically probable monogenic disorder using a standard procedure (Figure 1). This considered the presence of <3 conventional vascular risk factors (among hypertension, diabetes mellitus, hypercholesterolemia, atrial fibrillation, and smoking), young age at onset (≤ 55 years), positive familial history, or at least 2 associated neurological or systemic clinical features after the exclusion of other specific causes of stroke according to Trial of ORG 10172 in Acute Stroke Treatment criteria. In patients meeting these conditions, specific clinical and radiological diagnostic algorithms for the 5 monogenic diseases were applied and those fulfilling the criteria for that disease (suspected) were tested for that specific disease. The diagnostic algorithms were implemented based on published clinical phenotypic information and diagnostic criteria for CADASIL,⁴ Fabry Disease,⁵ MELAS,⁶ H-CAA,⁷ and Marfan syndrome⁸ (Figure 2). A full description of methods has been previously detailed elsewhere.³

Clinical Definitions Used

Stroke and TIA were defined according to the standard published clinical criteria.^{9,10} The presence of hypertension was defined as a previous diagnosis or repeated detection of a systolic blood pressure >140 mmHg or a diastolic blood pressure >90 mmHg in patients who were not taking antihypertensive medication.¹¹ Diabetes mellitus was defined according to the American Diabetes Association criteria.¹² Hypercholesterolemia was defined as serum cholesterol >200 mg/dL or was considered in patients on treatment

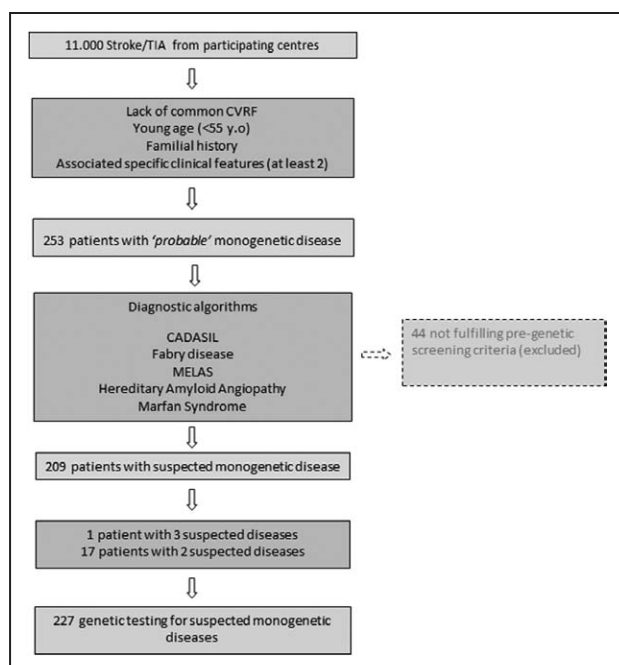


Figure 1. Diagnostic flowchart. CADASIL indicates cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy; CVRF, cardiovascular risk factors; and TIA, transient ischemic attack.

with statins.¹³ Hyperhomocysteinemia was defined as $>13 \mu\text{mol/L}$ for women and $17 \mu\text{mol/L}$ for men. Smoking was considered as present in current smokers or those who had quit smoking in the past 10 years. Psychiatric disorders were recorded as present in any of the following cases: (1) previous diagnosis of a psychiatric disease, (2) previous or current use of antipsychotic or antidepressant drugs, and (3) mood or behavior disorders referred by the patient or his or her family. Cognitive disorders were recorded as present if there was a previous diagnosis of cognitive impairment (mild cognitive impairment or dementia) by a certified specialist or cognitive impairment emerged during the clinical evaluation; the presence of cognitive decline was referred by a next of kin or by the patient and confirmed by neuropsychological testing. Migraine with and without aura was defined according to the Headache Classification Committee of the International Headache Society.¹⁴ Seizures were defined according to the International League Against Epilepsy Commission Report.¹⁵

Genetic Analysis

Blood samples for DNA analysis were collected, and genomic DNA was extracted from peripheral blood leukocytes. Genetic and biochemical analyses were performed using standard procedures as follows:

- CADASIL: direct sequencing of exons 2 to 23 of the *NOTCH3* gene on chromosome 19 (19p13.12).
- Fabry disease: α -galactosidase activity dosage for all suspected men; sequencing of all exons of *GLA* (Xq 22,1) gene in suspected women and in men with decreased α -galactosidase activity or high diagnostic suspicion.
- MELAS: search for m.3243A>G mutation within the mitochondrial *MT-TL1* gene. In cases with a high index of suspicion, including all those in whom a muscle biopsy was consistent with mitochondrial myopathy, other

mtDNA regions were investigated by direct sequencing: *MT-TL1*, *MT-TF*, *MT-TV*, *MT-TQ*, *MT-ND1*, *MT-ND5*, and *MT-ND6*.

- H-CAA: sequencing of exon 17 of *amyloid β -A4 precursor protein (APP)* (21q21,3), exon 2 of *cystatin C (CST3)* 20p11,21, and exons 2, 3, and 4 of *transthyretin (TTR)* genes located at 18q12.1.
- Marfan syndrome: sequencing of all 65 exons of *fibrillin-1 gene (FBN1)* located on chromosome 15q21.1 and all 7 exons of *transforming growth factor-beta receptor, type II (TGF β -R2)* gene on chromosome 3p24.1.

The detailed description of genetic analysis methodology is reported in the Methods section in the online-only Data Supplement.

Statistical Analysis

The χ^2 test was applied to assess the significant differences in frequency of clinical features between patients positive and negative at genetic analysis. The positive predictive value was evaluated for neurological or systemic clinical features and family history compared with the result of genetic tests. The independence of single predictive factors was assessed by logistic regression analysis. All analyses were calculated using STATA 8.0 (StataCorp LP, College Station, TX) and S-PLUS (Suite 44, Level 9, 88 Pitt Street Sydney New South Wales, Australia).

Results

During the observation period (January 2009 to December 2012), the participating centres evaluated $\approx 11\,000$ cases of ischemic or hemorrhagic stroke or TIA. Of these, 253 patients met the criteria for stroke of probable genetic origin and were included in the study by the recruiting centres, but on central review, 44 were excluded because they did not fulfil the inclusion criteria. Therefore, 209 patients with stroke/TIA were included (Figure 1).

Full demographic details of the patients are shown in Table 1. The mean age was 57.4 ± 14.7 years; 45% were women. The index event was stroke in 163 (78%) and TIA in 46 (22%). Of the 163 patients with stroke, stroke was ischemic in 112 (69%) and hemorrhagic in 51 (31%; information missing in 1 case). In 45% of cases, stroke was the first event, whereas 51% of patients had experienced ≥ 2 events in the past history (information was not available in 9 cases).

After following the disease-specific clinical screening algorithms, genetic test was performed for CADASIL in 103 cases (41%), Fabry disease in 33 (13%), H-CAA in 70 (28%), MELAS in 16 (6%), and Marfan syndrome in 5 (2%). Because 18 patients met the criteria for >1 genetic disease, a total of 227 tests were performed in the 209 patients.

A monogenic disease was genetically confirmed in 14 cases: 9 were diagnosed as CADASIL, 1 as Fabry disease, 1 as H-CAA, 2 as MELAS, and 1 as Marfan syndrome. One patient negative for genetic screening for Marfan syndrome was subsequently found to have an Ehlers–Danlos type IV disease (*COL3A1* gene) mutation. Table 2 summarizes the detailed results of genetic analysis.^{16–23}

Clinical and demographic characteristics were compared between individuals positive and negative on genetic testing (Table 3). The only significant difference was a family history of stroke (92% versus 47%; $P=0.002$). It is of note that

<p>1) Subcortical lacunar T2 sequence lesions at MRI.</p> <p>2) At least one of the following sign/symptoms</p> <ul style="list-style-type: none"> <input type="checkbox"/> History of recurrent stroke/TIA <input type="checkbox"/> Migraine with aura <input type="checkbox"/> Dementia <input type="checkbox"/> Major mood disorders <input type="checkbox"/> Familial history of stroke/mood disorders and/or migraine and/or dementia 	CADASIL
<p>1) Not atherosclerotic stroke (mostly vertebrobasilar and lacunar)</p> <p>2) At least one of the following sign/symptoms:</p> <ul style="list-style-type: none"> <input type="checkbox"/> Acroparesthesia/ neuropathic pain (recurrent hands and feet pain) <input type="checkbox"/> White matter hyperintensities detected on MRI <input type="checkbox"/> Angiokeratoma <input type="checkbox"/> Corneal lesions: "Corneaverticillata" <input type="checkbox"/> Seizures <input type="checkbox"/> Renal involvement (proteinuria, chronic renal failure) <input type="checkbox"/> Cardiac involvement 	FABRY DISEASE
<p>1) Stroke-like episodes (mostly cortical and not related to a vascular territory) in patients younger than 45 years</p> <p>2) At least one of the following sign/symptoms:</p> <ul style="list-style-type: none"> <input type="checkbox"/> Myopathy with lactic acidosis <input type="checkbox"/> Seizures <input type="checkbox"/> Migraine <input type="checkbox"/> MRI typical lesions (bitemporal and basal ganglia calcification) <input type="checkbox"/> Cardiomyopathy <input type="checkbox"/> Progressive dementia <input type="checkbox"/> Mental retardation <input type="checkbox"/> Short stature 	MELAS
<p>1) At least one of the following signs</p> <ul style="list-style-type: none"> <input type="checkbox"/> Recurrent atypical haemorrhages (mostly cortical and subcortical) <input type="checkbox"/> Ischemic/haemorrhagic lesions not attributable to a different disorder <input type="checkbox"/> Cerebral MRI consistent with the suspicion of amyloid angiopathy <p>2) At least one of the following signs/symptoms:</p> <ul style="list-style-type: none"> <input type="checkbox"/> Lack of hypertension or well treated hypertension, lack of coagulation abnormalities <input type="checkbox"/> Absence of aneurysms or arteriovenous malformations 	CEREBRAL HERITABLE AMYLOID ANGIOPATHY (H-CAA)
<p>1) Ischemic or haemorrhagic stroke due to arterial dissection, intracranial aneurysms rupture or cardioembolic source</p> <p>2) At least two of the following criteria:</p> <ul style="list-style-type: none"> <input type="checkbox"/> At least two skeletal abnormalities (high stature, pectus excavatum or carinatum, high-arched palate, arachnodactyly, laxity of ligaments with scoliosis or joint hyperextensibility) <input type="checkbox"/> At least two ocular manifestations (strabismus, amblyopia, ectopia lentis, and cataract) <input type="checkbox"/> At least one cardiovascular manifestation (dissection or dilatation of ascending aorta, mitral valve prolapse and cardiac arrhythmias) <input type="checkbox"/> At least one cutaneous manifestation (cutaneous lines, recurrent hernias) <input type="checkbox"/> At least one pulmonary manifestation (spontaneous pneumothorax, apical bubbles) <input type="checkbox"/> Positive familial history for arterial dissections, skeletal abnormalities, typical cardiovascular and ocular manifestations) 	MARFAN SYNDROME

Figure 2. Clinical algorithms for diagnosis of monogenic disorders. MRI indicates magnetic resonance imaging; and TIA, transient ischemic attack.

positive genetic tests occurred in many patients with conventional cardiovascular risk factors.

We calculated the positive predictive value of the disease-associated neurological or systemic clinical features and of positive familial history (Table 4). The positive predictive value was >10% for psychiatric disorders, cognitive disorders, high/low height, and familial history of stroke, migraine, psychiatric disorders, and dementia. However, only family history of stroke was significantly associated with a genetic diagnosis. Multiple logistic regression analysis confirmed the independent statistically significant association between a positive genetic test and a family history of stroke (odds ratio, 4.8; confidence interval, 1.45–15.70).

Discussion

This study found that the adoption of a phenotype-based algorithm for the identification of patients tested for monogenic stroke conditions resulted in a diagnostic of $\approx 7\%$, in contrast to previously reported yields of 1% to 5%.^{1,2}

Genetic testing is expensive, and counseling can be time consuming. Therefore, guidance on the type of patients in whom there is a yield sufficient to merit testing is important. There have been a few previous attempts to develop pregenetic screening strategies for monogenic stroke disorders, but methods were heterogeneous. This study provides some of the most robust data to guide current clinical practice.^{6,24–26}

Our strategy identified 7% of patients affected by monogenic diseases. Our criteria seem to be particularly efficient for CADASIL pregenetic screening because we detected disease-related mutations in 9% of our suspected cases. The strength of this study is that the screening strategy was implemented in a prospectively collected and well-phenotyped series of patients with stroke using novel diagnostic criteria. Many previous studies have been retrospective on populations in whom there may be significant selection bias.^{24–26}

Although our study shows that using our algorithm we achieved a relatively high yield of positive cases, it does not evaluate the effectiveness of the algorithm in diagnosing all

Table 1. Demographic and Clinical Features and Risk Factors of Patients With Suspected Monogenic Disease

Clinical Features	Overall Patients, n=209
Age, y, mean±SD	57.4±14.7
Female sex, n (%)	95 (45)
Lombardia region birth, n (%)	126 (60)
Stroke*	163 (78)
NIHSS mean±SD	4±4.9
First event†	93 (45)
≥2 events‡	54 (51%)
Risk factors	
Hypertension	106 (51%)
Diabetes mellitus	22 (11%)
Current smoker	75 (36%)
Atrial fibrillation	8 (4%)
Hypercholesterolemia	84 (40%)
Ischemic cardiomyopathy	13 (6%)
Peripheral arteriopathy	6 (3%)
Hormone therapy	11 (5%)
Alcohol intake, >20 U/wk	4 (2%)
BMI, >25 kg/m ²	63 (30%)
Physical inactivity	108 (52%)

BMI indicates body mass index; and NIHSS, National Institutes of Health Stroke Scale.

*Information missing in 1 case.

†Information missing in 9 cases.

‡Information missing in 84 cases.

cases of monogenic strokes in a stroke population. This would have required genetic testing in patients in whom the algorithm did not suggest a high probability of monogenic stroke. However, such study design, which has never been applied

in previous studies, would require huge funding support. Moreover, the results of recent studies screening systematically patients with stroke for monogenic diseases did not find higher disease frequencies,²⁷ supporting the idea that the use of more narrow selection criteria is more favorable for identifying an higher number of positive patients.

Potential further limitations of the study are that we used a hospital-based sampling frame and the relatively small number of cases in whom genetic testing was performed. Another limit is that we only tested patients for 5 monogenic causes of stroke, whereas more recently described causes, such as COL4A1 and CARASIL, have not been included in our screening.²⁸ Furthermore, there was a relatively low National Institutes of Health Stroke Scale score although this is similar to that in previous studies^{29,30} and may partly reflect the lower National Institutes of Health Stroke Scale associated with lacunar strokes in diseases, such as CADASIL.

Differently from previous series, no patient presenting with TIA as index event resulted carrier of a monogenic disease. This finding may be explained by chance alone although it cannot be excluded that patients included in the study as stroke might have presented previously with a TIA.

Our results also highlight many important elements that should be considered in the investigation of monogenic causes of stroke. First, common cerebrovascular risk factors, in particular hypertension, diabetes mellitus, hypercholesterolemia, and smoke, should not be considered as exclusion criteria for genetic screening. Furthermore, the concomitant presence of cerebrovascular risk factors may be important to take into account because recent evidence suggests that they may interact to increase phenotype severity even in monogenic stroke patients.^{31,32} Second, familial history, particularly of stroke, should probably be considered as mandatory for genetic screening unless familial history is unavailable or first-degree relatives died of other causes at an age before they might have

Table 2. Results of Genetic Tests

Disease	n (%) of Tests, n=227	n (%) of Positive Tests, n=14	Gene	Type of Event	Mutation (cDNA)	Exon
CADASIL	103	9 (9)	<i>NOTCH3</i>	Left carotid TIA (aphasia)	c.268C>T (Shahien et al ¹⁶)	3
				Lacunar stroke	c.328C>T (Joutel et al ¹⁷)	3
				Lacunar stroke	c.349T>C (Wang et al ¹⁸)	4
				Lacunar stroke	c.752G>A (Mykkänen et al ¹⁹)	5
				Lacunar stroke	c.1732C>T (Joutel ¹⁷ et al)	11
				Vertebrobasilar TIA	c.1819C>T (Escary et al ²⁰)	11
				Lacunar stroke	c.2953C>T (Joutel ¹⁷ et al)	18
				Vertebrobasilar lacunar stroke	c.2953C>T (Joutel et al ¹⁷)	18
				Lacunar stroke	c.3691C>T (Joutel et al ¹⁷)	22
Fabry disease	33	1 (3)	<i>GLA</i>	Ischemic cortical–subcortical stroke	c. 187T>G (Shabbeer et al ²¹)	6
H-CAA	70	1 (1)	<i>APP</i>	Hemorrhagic stroke	c.2077G>A (Bugiani et al ²²)	17
MELAS	16	2 (12)	<i>MT-TL1</i>	Ischemic cortical–subcortical stroke	m.3243A>G (Goto et al ²³)	//
Marfan syndrome	5	1 (20)	<i>FBN1</i>	Hemorrhagic stroke	c.1185T>G*	10

CADASIL indicates cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy; H-CAA, hereditary cerebral amyloid angiopathy; MELAS, mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes; and TIA, transient ischemic attack.

*New mutation.

Table 3. Clinical Features of Patients Positive and Negative at Genetic Analysis

Clinical Features	Positive Patients, n=14	Negative Patients, n=195	P Value
Age, y, mean±SD	50.6±14.8	57.8±14.7	0.08
Female sex	7 (50%)	88 (45%)	ns
Lombardia region birth	11 (78%)	115(59%)	ns
Stroke*	10 (71%)	153 (78%)	ns
Ischemic stroke*	8 (57%)	104 (53%)	ns
Hemorrhagic stroke	2 (14%)	49 (25%)	ns
TIA	4 (29%)	38 (19%)	ns
NIHSS, mean±SD	4.4±5.2	3.9±4.9	ns
First event†	7 (50%)	89 (45%)	ns
≥2 events‡	5 (36%)	42 (21%)	ns
Hypertension	6 (43%)	100 (51%)	ns
Diabetes mellitus	3 (21%)	19 (10%)	ns
Current smoker	6 (42%)	69 (35%)	ns
Atrial fibrillation	0	8(4%)	ns
Ischemic cardiomyopathy	2 (14%)	11 (6%)	ns
Hypercholesterolemia	8 (57%)	76 (39%)	ns
Peripheral arteriopathy	1 (7%)	5 (2%)	ns
Hormone therapy	0	11 (6%)	ns
Alcohol intake	0	4(2%)	ns
BMI, >25 kg/m ²	4(2%)	54(28%)	ns
Physical inactivity	6 (43%)	102 (52%)	ns
Migraine	7 (50%)	83 (43%)	ns
Seizures	1 (7%)	40 (20%)	ns
Psychiatric disorders	4 (29%)	47 (24%)	ns
Mood disorders	4 (29%)	34 (17%)	ns
Cognitive disorders	5 (36%)	51 (26%)	ns
Deafness	0	14 (7%)	ns
Renal failure	0	11 (6%)	ns
High/low height	2 (14%)	19 (10%)	ns
Miscarriages	0	9 (5%)	ns
Acroparesthesia	1 (7%)	18 (9%)	ns
Dysmorphism	0	3 (1%)	ns
Ligament laxity	0	5 (21%)	ns
Myopathy	0	4 (2%)	ns
Skin changes	0	0	ns
Family history			
Stroke	12/13 (92%)	72/154 (47%)	0.002§
Migraine	4/12 (33%)	42/149 (28%)	ns
Epilepsy	0/12	9/145 (6%)	ns
Psychiatric disorders	2/12 (16%)	20/150(13%)	ns
Dementia	3/13 (23%)	37/148(25%)	ns

BMI indicates body mass index; NIHSS, National Institutes of Health Stroke Scale; ns, nonsignificant; and TIA, transient ischemic attack.

*Information missing in 1 case.

†Information missing in 9 cases.

‡Information missing in 84 cases.

§Significant association.

Table 4. PPV of Clinical Features and Family History

	Overall Patients, n=209	Positive Cases at Genetic Analysis, n=14	PPV
Symptoms			
Migraine	90 (43%)	7	0.5
Seizures	41 (19%)	1	0.07
Psychiatric disorders	51 (24%)	4	0.28
Cognitive disorders	56 (27%)	5	0.35
Deafness	14 (7%)	0	0
Renal failure	11 (5%)	0	0
Low/high height	21(10%)	2	0.14
Miscarriages	9 (4%)	0	0
Acroparesthesia	19 (9%)	1	0.07
Dysmorphism	3 (1%)	0	0
Ligament laxity	5 (2%)	0	0
Miopathy	4 (2%)	0	0
Skin changes	0	0	0
Family history			
Stroke	84/167 (50%)	12/13	0.86
Migraine	46/161 (29%)	4/12	0.28
Epilepsy	9/157 (6%)	0/12	0
Psychiatric disorders	22/161 (14%)	2/12	0.14
Dementia	40/161 (25%)	3/13	0.21

PPV indicates positive predictive value.

expressed the disease. In contrast, other specific clinical and familial factors (ie, familial history of migraine, epilepsy, and psychiatric disorders) were not predictive of an underlying genetic diagnosis.

We found a low prevalence of H-CAA, Fabry disease, or Marfan syndrome in our series. This may be because of 2 reasons. First, these diseases are really rare in stroke populations. Consistent with this, although Fabry disease was suggested as an important cause of younger onset stroke in 1 study,^{33–35} further studies have failed to replicate this finding. Second, the diagnostic algorithms may not adequately address these diseases.³⁶

Conclusions

To make the diagnosis of monogenic diseases is important not only for the individual patient but also for family members to allow the possibility of predictive testing. However, the diagnosis is often challenging because of the overlapping phenotypes between each disorder and the heterogeneity of phenotypes within families. The current study, in which genetic testing was performed in cases identified using a clinical algorithm, found that 7% of patients were affected by monogenic diseases. Family history is a key feature for a clinical suspicion of monogenic disease, whereas stroke in the absence of cardiovascular risk was not a useful marker of a monogenic diagnosis.

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Stroke

Clinical Pre-genetic Screening for Stroke Monogenic Diseases: Results From Lombardia GENS Registry

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SUPPLEMENTAL MATERIAL

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Supplemental Table I. Clinical algorithms for diagnosis of monogenic disorders

CADASIL

- 1) Subcortical lacunar T2 sequence lesions at MRI.
- 2) At least one of the following sign/symptoms
 - History of recurrent stroke /TIA
 - Migraine with aura
 - Dementia
 - Major mood disorders
 - Familial history of stroke/mood disorders and/or migraine and/or dementia

FABRY DISEASE

- 1) Not atherosclerotic stroke (mostly vertebrobasilar and lacunar)
- 2) At least one of the following sign/symptoms:
 - Acroparesthesia/ neuropatic pain (recurrent hands and feet pain)
 - White matter hyperintensities detected on MRI
 - Angiokeratoma
 - Corneal lesions: "Cornea verticillata"
 - Seizures
 - Renal involvement (proteinuria, chronic renal failure)
 - Cardiac involvement

MELAS

- 1) Stroke-like episodes (mostly cortical and not related to a vascular territory) in patients younger than 45 years
- 2) At least one of the following sign/symptoms:
 - Miopathy with lactic acidosis
 - Seizures
 - Migraine
 - MRI typical lesions (bitemporal and basal ganglia calcification)
 - Cardiomyopathy
 - Progressive dementia
 - Mental retardation
 - Short stature
 - Diabetes

FAMILIAL OR SPORADIC HEMIPLEGIC MIGRAINE (FHM/SHM)

- 1) At least two aura migraine attacks associated with motor weakness (hemiparesis, paresis, plegia) lasting >5 min e <24 h
- 2) At least one of the following signs/symptoms:
 - Fully reversible visual symptoms including positive features (i.e, flickering lights, spots or lines) and/or negative features (i.e, loss of vision)
 - Fully reversible sensory symptoms including positive features (ie, pins and needles) and/or negative features (i.e numbness)
 - Fully reversible dysphasic speech disturbance
 - Supplementary clinical features:
 - Migraine following aura.
 - Childhood onset migraine (<30 years)
 - At least one first- or second-degree relative has had attacks fulfilling these criteria
 - Progressive ataxia e/o nystagmus
 - Attacks triggered by fever, trauma, pleiocytosis

CEREBRAL HERITABLE AMYLOID ANGIOPATHY (H-CAA)

1) At least one of the following signs

- Recurrent atypical haemorrhages (mostly cortical and subcortical)
- Ischemic/haemorrhagic lesions not attributable to a different disorder
- Cerebral MRI consistent with the suspicion of amyloid angiopathy

2) At least one of the following signs/symptoms:

- Lack of hypertension or well treated hypertension), lack of coagulation abnormalities
- Absence of aneurysms or arteriovenous malformations
- Positive familial history for haemorrhagic and ischemic stroke
- Cognitive impairment
- Occipital calcifications

MARFAN SYNDROME

1) Ischemic or haemorrhagic stroke due to arterial dissection, intracranial aneurysms rupture or cardioembolic source

2) At least two of the following criteria:

- At least two skeletal abnormalities (high stature, pectus excavatum or carinatum, high-arched palate, arachnodactyly, laxity of ligaments with scoliosis or joint hyperextensibility)
- At least two ocular manifestations (strabismus, amblyopia, ectopia lentis, and cataract)
- At least one cardiovascular manifestation (dissection or dilatation of ascending aorta, mitral valve prolapse and cardiac arrhythmias)
- At least one cutaneous manifestation (cutaneous lines, recurrent hernias)
- At least one pulmonary manifestation (spontaneous pneumothorax, apical bubbles)
- Positive familial history for arterial dissections, skeletal abnormalities, typical cardiovascular and ocular manifestations)

Supplemental Methods: Genetic analysis methodology

CADASIL (#MIM 125310)

DNA was extracted from whole blood samples using standard procedures. Genetic test were performed by direct automated sequencing of the coding and flanking regions of the disease genes. CADASIL DNA amplification was performed by using primers, kindly provided by Prof. Dichgans (Ludwig Maximilians University, Munich, Germany), designed to amplify the 2-23 exons of the NOTCH3 gene, including the intron-exon boundaries. The PCR reactions were performed using AmpliTaq Gold® DNA polymerase (Applied Biosystems). Direct sequence was performed on an automated sequencing system (Applied Biosystems 3730 DNA Analyzer) using the BigDye™ Terminator Cycle Sequencing Kit Version 1.1 (Applied Biosystems). Sequencing of exons 3, 4, 6 and 8 was done first; screening of the 18 remaining exons encoding the EGF repeats was pursued until a mutation creating or deleting a cysteine residue was identified. The nucleotide position of mutation present in the coding regions refers to the mRNA sequence (NM_000435).

Fabry disease (#MIM 300644)

The 7 exons of the GLA gene were amplified from peripheral blood-derived genomic deoxyribonucleic acid by means of polymerase chain reaction. The polymerase chain reaction fragments were analyzed by means of denaturing high-performance liquid chromatography using the Wave deoxyribonucleic acid fragment analysis system (Transgenomic, San Jose, California). Heteroduplex fragments were purified (QIAquick Kit, Qiagen, Santa Clarita, California) and then sequenced using a BigDye-terminator cycle sequencing system (ABI PRISM, Applied Biosystems, Foster City, California). Reference sequence used in the study is NM_000169.

MELAS (#MIM540000)

The presence of the m.3243A>G mutation was investigated by PCR amplification (primers: FOR3150, RC3380) followed by restriction fragment length polymorphisms (RFLP) analysis using the *HaeIII* restriction endonuclease. The quantification of mtDNA heteroplasmy was calculated by densitometry after gel electrophoresis or using a Taqman-based quantitative PCR protocol (FOR: 5'-CCACACCCACCCAAGAAGACAG-3'; RC: 5'-AGGAATTGAACCTCTGACTGTAAAGTT-3'; probe 6-FAM-CCGGGCCCTGCCAT-MGB). Other mtDNA regions were analyzed by direct sequencing using the MitoSeq Resequencing system (Applied Biosystems).

MARFAN SYNDROME(#MIM 174700):

FBN1 gene (MIM 134797): Sanger sequencing of the exons and exon-intron boundaries of FBN1 was performed on genomic DNA. Genomic DNA was isolated from peripheral blood leukocytes by the QIAmp DNA blood kit (Qiagen Inc., Valencia, CA, USA). The analysis was performed by direct automated sequencing using the BigDye Terminator Cycle sequencing kit V 3.1 (Applied Biosystems) on an ABI 3100 Genetic Analyzer, following the manufacturer's directions.

TGFBR2 gene (MIM 190182): The seven exons and flanking regions of the TGFBR2 were amplified using previously reported intron-specific primers, with the exception of three amplicons (exons 1, 2 and 5) for which the two primers were newly designed using the Amplify 1.0 software: (1F:5'TCCGGGAAGGCGCCGTCCTCGCT3', 1R: 5'CGACTGTCAAGCGCAGCGGAGAG3'; 2F:5'GCTGCCTGGCAGTTGGATAAT3', 2R:5'ACACTGACTGTGTGTACTATG3'; 5F:5'AATGATGGGCCTCACTGTCT3', 5R: 5'ACACAATGATGCTGGTCCAC3'). The analysis was performed by direct automated sequencing. Direct sequencing analysis was performed using the BigDye Terminator Cycle sequencing kit V 3.1 (Applied Biosystems) on an ABI 3100 Genetic Analyzer, following the manufacturer's directions

HCAA(#MIM 605714, 105150, 176300)

The following coding regions were analyzed by direct Sanger sequencing: 1) exon 17 of APP gene; 2) exons 2, 3, and 4 of TTR gene; exon 2 of CST3 gene. Primers designed in flanking intronic regions were used for PCR amplification. After purification of PCR products, sequencing reactions were performed by dye-terminator cycle sequencing according to manufacturer's instructions and loaded on an ABI Prism 3500 DNA analyzer.

FHM/SHM (#MIM 601011, 602481, 141500): The following genes have been analysed by direct Sanger sequencing of coding regions and flanking intron sequences as described previously [Condliffe SB, Fratangeli A, Munasinghe NR, Saba E, Passafaro M, Montrasio C, Ferrari M, Rosa P, Carrera P, The E1015K variant in the synprint region of the Cav2.1 channel alters channel function and is associated with different migraine phenotypes, The J of Biological Chemistry, 2013, 288:33837-33883]: CACNA1A (FHM1-OMIM 141500), ATP1A2 (FHM2-OMIM 602481), SCN1A (FHM3-OMIM 609634). (<http://genome.ucsc.edu/cgi-bin/hgGateway>).

The PCR products and sequencing reactions were purified on Multiscreen 96 PCR plates (Millipore) and G50 Multiscreen TM-HV plates (Millipore) respectively, using the automated liquid handling system Biomeck FX

(Beckmann Coulter). Dye-terminator cycle sequencing reactions were set up following the manufacturer's instructions and loaded on a ABI Prism 3730 DNA Analyzer (Applied Biosystems). Called sequences were assembled and compared with the reference sequences in genomic databases (CACNA1A, LRG_7; ATP1A2, LRG_6; SCN1A, LRG_8).