The Siblings With Ischemic Stroke Study (SWISS) Protocol
James F. Meschia, M.D.
Department of Neurology, Mayo Clinic, Jacksonville, Florida
meschia.james@mayo.edu

Robert D. Brown, Jr., M.D.
Department of Neurology, Mayo Clinic, Rochester, Minnesota
brown.robert@mayo.edu

Thomas G. Brott, M.D.
Department of Neurology, Mayo Clinic, Jacksonville, Florida
brott.thomas@mayo.edu

Felix E. Chukwudelunzu, M.D.
Luther Midelfort Clinic Mayo Health System, Eau Claire, Wisconsin
chukwudelunzu.felix@mayo.edu

John Hardy, Ph.D.
National Institute on Aging, Bethesda, Maryland
hardyj@mail.nih.gov

Stephen S. Rich, Ph.D.
Department of Public Health Sciences and Department of Neurology, Wake
Forest University School of Medicine, Winston-Salem, North Carolina
srich@wfubmc.edu

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Reprint requests to: James F. Meschia, M.D., Mayo Clinic, 4500 San Pablo Road, Jacksonville, Florida 32224.
Abstract

**Background:** Epidemiologic and twins studies suggest an inherited component to the risk of developing ischemic stroke. Candidate gene association studies have been performed but have limited capacity to identify novel risk factor genes. The purpose of the Siblings With Ischemic Stroke Study (SWISS) is to conduct a genome-wide scan in sibling pairs concordant or discordant for ischemic stroke to identify novel genetic risk factors through linkage analysis.

**Methods:** Screening takes place at various clinical centers to identify patients (probands) with radiographically confirmed ischemic stroke and a family history of at least 1 living full sibling with stroke. After giving informed consent, without violating privacy among other family members, the proband invites siblings concordant and discordant for stroke to participate. Siblings then contact the study coordinating center. The diagnosis of ischemic stroke in potentially concordant siblings is confirmed by systematic centralized review of their medical records. The stroke-free status of potentially discordant siblings is confirmed by validated structured telephone interview. Blood samples for DNA analysis are taken from concordant sibling pairs and, if applicable, from 1 discordant sibling. Epstein-Barr virus–lymphoblastoid cell lines are created, and a scan of the human genome is planned.

**Discussion:** Conducting adequately powered genomics studies of stroke in humans is challenging because of the heterogeneity of the stroke phenotype and the difficulty of obtaining DNA samples from clinically well-characterized members of a cohort of stroke pedigrees. The multicentered design of this study is intended to efficiently assemble a cohort of ischemic
stroke pedigrees without invoking community consent or using cold-calling of pedigree members.
Background

Evidence for Genetic Stroke Susceptibility

Epidemiologic studies support the existence of genetic susceptibility to stroke. A cohort study of 14,371 middle-aged men and women in Finland provided compelling data in support of familial clustering of ischemic stroke [1]. Parental history of coronary disease was not associated with risk of stroke in men and had a borderline significance in women, suggesting that the constellation of genetic risk factors for stroke may be distinct from the genetic risk factors for myocardial infarction.

A strong relationship between ischemic stroke in siblings and ischemic stroke in cohort members was found in the original Framingham Heart Study cohort [2], even after adjusting for age, sex, and sibship size. Multivariate-adjusted analyses showed a nearly twofold increased risk of stroke. One major methodological strength of the Framingham study is that ischemic stroke was diagnosed by a study neurologist by means of a detailed clinical evaluation, rather than by questionnaire, unsubstantiated self-report, or death certificate.

In the Family Heart Study [3], personal and family histories of stroke were assessed by a standardized questionnaire in 3,168 probands and 29,325 of their first-degree relatives. Analysis adjusted for age, sex, and ethnicity showed stroke prevalence to be 4.8% when family history was positive for stroke, compared with 2.0% when family history was negative ($P < 0.01$).

Studies in twins also provide evidence for genetic susceptibility to stroke. In a population of twins from the National Research Council Twin Registry of the National Academy of Science [4], among 1,271 monozygotic
twin pairs, 7 pairs were concordant for stroke. Among 1,128 dizygotic twin pairs, only 1 pair was concordant for stroke. The proband concordance rates were 17.7% for monozygotic pairs and 3.6% for dizygotic pairs (RR = 4.3; \( P < 0.05 \)). Because the study relied on patient self-reporting for diagnosis, it was not possible to draw conclusions about genetic influence on ischemic strokes alone.

**Genetic Studies**

*Linkage Analysis in Large Pedigrees With Mendelian Stroke Syndromes*

Mendelian disorders known to be associated with an increased risk of stroke include hemoglobinopathies (such as sickle cell disease, homocystinuria, and Fabry disease), dyslipoproteinemias (familial hypocholesterolemia and Tangier disease), and cardioembolic disorders (familial atrial fibrillation and familial atrial myxoma) [5]. Most known Mendelian etiologies of stroke present in infancy, childhood, or young adulthood, and collectively represent only a small proportion of stroke cases. Several of these Mendelian disorders were recognized as unique genetic diseases because of striking phenotypic features, such as orange tonsils in Tangier disease and corneal opacities and angiokeratomas of the skin in Fabry disease. Defining the genetic basis for Mendelian stroke syndromes that lack striking phenotypic features is a more daunting task.

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is a Mendelian disorder characterized by recurrent subcortical ischemic strokes, progressive or stepwise subcortical dementia with pseudobulbar palsy, migraine with aura, and mood disorders [6]. The genetics of CADASIL were discovered through model-dependent linkage analysis of large pedigrees [7-10].
However, traditional linkage analysis is unlikely to be the most expedient method of finding novel stroke-susceptibility genes when carrier status cannot be defined on the basis of distinctive clinical, radiographic, or laboratory features.

**The Candidate Gene Approach**

One method to identify genetic risk factors is through a candidate gene association study, in which investigators compare rates of one or more variant polymorphisms of a candidate gene among stroke cases and stroke-free controls. Identifying risk factors depends on selecting the right candidate genes, a daunting task because the human genome harbors about 30,000 genes. A candidate gene is usually selected because the gene product might relate to pathogenesis of disease. Numerous studies have used a candidate gene approach to defining genetic risk factors for stroke, but so far without definitive results.

About 80% of strokes are due to thrombotic occlusion of a blood vessel, and genes related to the coagulation system would therefore seem to be logical candidates for susceptibility to stroke. Although the mutations factor V G1691A (factor V Leiden) and prothrombin (factor II) G20210A are associated with venous thromboembolism and myocardial infarction [11,12], evidence is mounting that neither mutation is strongly associated with risk of stroke. At least 6 case-control studies [13-18] and 1 cohort study [19] have looked for a possible relationship between factor V G1691A and stroke, and all of the studies were negative. In the case of the prothrombin gene variant G20210A, at least 2 negative studies have been reported to date [13,15].
Although a study of British adults found elevated levels of serum homocysteine to be associated with an increased risk of stroke [20], a case-control study of a common polymorphism (methylene-tetrahydrofolate reductase [MTHFR] T677C) that results in increased serum homocysteine concentrations found no difference between patients with stroke and controls in either genotype or allele frequency [21].

Because antiplatelet agents with different mechanisms of action can bring about significant reductions in stroke risk, several platelet receptor genes have been tested as candidate stroke susceptibility genes, including polymorphisms of platelet receptors for fibrinogen (HPA-1, HPA-3), collagen (HPA-5), and von Willebrand factor (HPA-2) [22], and the platelet glycoprotein receptor IIIa polymorphism PIA2 [23]. To date, however, no compelling evidence for an association between any platelet receptor gene polymorphism and risk of stroke has been found.

The angiotensin 1-converting enzyme gene (ACE) has an insertion (I)/deletion (D) polymorphism at intron 16. The ACE D/D genotype is significantly more common in patients with myocardial infarction than in controls, irrespective of blood pressure. To date, results are conflicting among case-control studies seeking to determine whether the D/D genotype is a risk factor for stroke [24-27].

At least 3 cohort studies have assessed the influence of apolipoprotein genotype on risk of stroke [28-30]. Although no association between the presence of either the e3 or e4 allele and the incidence of ischemic stroke was found, the e2 allele was associated with a reduced risk of stroke in patients under age 80 years ($P < 0.01$).
At least 5 case-control studies have assessed the interaction between apolipoprotein E polymorphism and risk of ischemic stroke [31-35], but the relationship remains unclear. Potential confounding factors include the effects of comorbidities and differential mortality rates [35,36].

The degree of stenosis of the cervical internal carotid arteries in symptomatic patients correlates with risk of ipsilateral stroke [37], and various studies have focused on the genetics of carotid atherosclerosis, treating it as an intermediate phenotype. An Austrian study found an association between the LL genotype at position 54 of the paraoxonase gene (PON) and the presence and severity of carotid disease ($P = 0.022$), but no association for a polymorphism at position 191 of the same gene [38]. Another study found no association between carotid disease and a common polymorphism of the endothelial nitric oxide synthase gene (G894T) [39].

**Genome-Wide Screening**

A genome-wide screen can expedite discovery of novel risk factor genes. The basic goal of the genome-wide screen is to identify chromosomal regions linked to a disease phenotype by determining whether polymorphisms in microsatellite markers segregate with disease within a cohort of pedigrees. Microsatellites are not functional; they are noncoding regions of DNA that allow identification of chromosomal regions held in common by members of a pedigree. The collection of sibling pairs and analysis of mean proportion of alleles shared that are identical by descent (IBD) or identical by state (IBS) by use of a highly polymorphic panel of genetic markers has come to be a standard protocol for detecting linkage of a disease susceptibility locus to a chromosomal region. The predominant methods of analysis for determination of risk factor loci use the SPLINK and
Mapmaker/Sibs programs with genotype data from concordant (affected) sibling pairs [40,41]. The technique of genome-wide screening is being applied to a broad range of disorders, including multiple sclerosis [42-45], Alzheimer disease [46-48], type 1 diabetes [49], type 2 diabetes [50], asthma [51], and systemic lupus erythematosus [52]. The use of such linkage-mapping strategies offers the advantage of model-independence, computational speed, and systematic identification of novel loci [42].

**The Siblings With Ischemic Stroke Study (SWISS)**

The candidate gene approach in defining genetic risk factors for stroke has yielded negative or conflicting results for several categories of candidate genes. Furthermore, if important functional mutations should arise in noncoding regions without significant disequilibrium with the site of a screened polymorphism, the association analysis may exclude the true disease susceptibility locus. Thus, linkage analysis using a genome-wide screen may yield positive results more efficiently than testing candidate genes a few at a time. However, we do not consider these approaches to be mutually exclusive. Genome-wide linkage analysis may guide selective candidate gene evaluations within regions of importance. The goal of SWISS is to help to identify the chromosomal regions that should be searched for candidate genes.

**Research Design and Methods**

**Primary Aims**

- To test the hypothesis that human chromosomal regions exist that are associated with ischemic stroke. This hypothesis will be tested by genome-wide screening using DNA samples collected from 300 sibling pairs concordant for stroke.
To establish a cell bank of specimens from these sibling pairs and an accompanying secured database of genetic and phenotypic data.

**Definition of Stroke and Stroke Subtypes**

Stroke is defined according to World Health Organization criteria [53] as rapidly developing signs of a focal or global disturbance of cerebral function, with symptoms lasting 24 hours or longer or leading to death with no apparent cause other than vascular origin. Patients are classified as having an ischemic stroke if they had computed tomographic or magnetic resonance imaging of the brain done within 7 days of onset of symptoms that identified the symptomatic cerebral infarct or failed to identify an alternative etiology for the symptoms. Classification of strokes into subtypes is done according to the validated diagnostic criteria defined by the Trial of ORG10172 in Acute Stroke Treatment (TOAST) [54] investigators: large-artery atherosclerosis, cardioembolism, small-vessel occlusion, stroke of other etiology, and stroke of undetermined etiology. Subtype diagnosis is made on the basis of available and relevant information obtained up to 3 months after the stroke, because initial subtype diagnosis varies from final diagnosis in approximately one-third of cases [55].

**Study Population**

Three groups of subjects will be studied: 1) Probands are patients who have had a recent, verified ischemic stroke. 2) Concordant siblings are full siblings of the proband with a history of at least 1 verified ischemic stroke. 3) Discordant siblings are full siblings of the proband with no history of stroke or stroke warning signs at any time before enrollment. A sample pedigree is shown in Figure 1.
We aim to enroll at least 300 concordant sibling pairs (300 probands plus 300 concordant siblings) and 200 discordant siblings in the study (800 total study subjects). Beginning in 1999, a study was conducted to estimate the number of index cases of ischemic stroke that must be screened to find 1 pair of potentially concordant living siblings [56]. The results showed that, of 310 probands (median age, 75 years; range, 26-97 years; 48% women), 75% had at least 1 living sibling; 10%, at least 1 concordant living sibling; 2%, at least 1 concordant sibling living in the same city; and 7%, at least 1 concordant living and 1 discordant living sibling. The likelihood of having a living concordant sibling did not increase significantly with proband age, even after adjusting for sibship size. Positive family history of stroke was not related to the proband’s stroke subtype or risk factor profile.

On the basis of these results, it appears that at least 10 patients with a recent stroke will have to be screened to find 1 concordant living sibling. SWISS was therefore designed as a multicenter study to enable enrollment of siblings from diverse geographic areas. Enrollment will continue until 300 concordant sibling pairs have been entered into the study. Thus, because it is likely that not all concordant siblings will actually participate, more than 300 probands will be enrolled to obtain DNA from 300 concordant sibling pairs.

**Protecting Subjects in Genetic Research**

Special safeguards must be in place to protect the rights of subjects involved in genetic research. If genetic information is improperly safeguarded, misuse could adversely influence insurability [57] and employability [58] of subjects and could stigmatize individuals or groups.
[59]. For this study, we have adopted many of the practical suggestions of Merz and colleagues [60] regarding the ethical use of human tissue. For linking purposes, we use special study-specific codes, rather than medical record numbers, Social Security numbers, or an easily decoded combination of initials and birth dates. Access to linking files is restricted to an as-needed basis only, and the files are deleted when they are no longer needed. The study mandates strictly unidirectional flow of information. This means that clinical data are used for research purposes, but research data is not used for clinical purposes. This restriction on the use of the SWISS data set is based on our recognition that unique clinical obligations accompany the provision of predictive genetic test results to individual patients [61].

Recently, there has been intense debate in the United States at the federal level over the privacy rights of pedigree members with respect to genetic research. For this study, we have adopted the position that every member of a pedigree has the right to refuse to have personal information such as name, address, and medical history recorded in a research database. This right cannot be waived by other members of the pedigree [62]. Therefore, probands or their non-investigator surrogates invite other family members to participate. Siblings who are interested in participating voluntarily provide investigators with their name, address, and telephone number.
Selection Criteria

Probands

Inclusion criteria for participation of probands in SWISS include the following:

- The proband has a diagnosis of at least 1 ischemic stroke confirmed by the study neurologist.
- The proband reports having at least 1 living full sibling with a history of stroke.
- The proband has attained his or her 18th birthday at the time of enrollment in the study.

Patients are not excluded from the study for radiographic evidence of hemorrhagic transformation of an ischemic stroke. Exclusion criteria include the following:

- The index stroke is presumed to be iatrogenic; i.e., onset of symptoms occurred within 48 hours after an invasive cerebrovascular or cardiovascular procedure, such as coronary artery bypass grafting, a catheter-based procedure on carotid or coronary arteries, carotid endarterectomy, heart valve surgery, or thoracic or thoracoabdominal aortic aneurysm repair.
- The index stroke is presumed due to vasospasm after nontraumatic subarachnoid hemorrhage; i.e., the onset of symptoms occurred within 60 days after the onset of a nontraumatic subarachnoid hemorrhage. Virtually all delayed cerebral ischemia occurs 5 to 21 days after subarachnoid hemorrhage [63,64].
• The index stroke is presumed due to an autoimmune condition; i.e., the patient has a history of brain-biopsy-proven central nervous system vasculitis.

• The patient is known to have any of the following single-gene or mitochondrial disorders recognized by a distinctive phenotype: CADASIL, Fabry’s disease, homocystinuria, mitochondrial encephalopathy with lactic acidosis and stroke-like episodes (MELAS), or sickle cell anemia. We excluded probands with these disorders because their enrollment might confound future genome scans for novel risk factors.

• The patient had a mechanical aortic valve or a mechanical mitral valve at the time of index stroke onset. We chose this criterion because of the high likelihood that ischemic stroke is iatrogenic in such patients.

• The patient had untreated or actively treated bacterial endocarditis at time of the index stroke onset.

   Probands and concordant siblings have identical exclusion criteria and virtually identical inclusion criteria. Both proband and concordant sibling must be at least 18 years old at the time of enrollment and both must meet the same definition of ischemic stroke.

Concordant Sibling

   Inclusion criteria for participation of concordant siblings in SWISS include the following:

   • The subject has a full sibling enrolled as a proband in SWISS.
   • The subject has a history of ischemic stroke. The definition of ischemic stroke is the same as that used for probands, but the diagnosis
is verified retrospectively by the Stroke Verification Committee (SVC).

- The subject has attained his or her 18th birthday at the time of enrollment.

Exclusion criteria for concordant siblings are identical to those used for probands.

**Discordant Siblings**

Inclusion criteria for discordant siblings are:

- The subject has 2 or more full siblings who each have had an ischemic stroke and who are participating in the study.
- The subject reports having no medical history of stroke or transient ischemic attack (TIA) and denies ever having had symptoms of stroke. Because a SWISS proband might erroneously believe that a sibling never had a stroke, discordance is considered verified only if the sibling can be contacted for a structured telephone interview and gives negative answers to all 8 items on the Questionnaire for Verifying Stroke-Free Status (QVSFS) (Table 1) [65,66].
- The subject has attained his or her 18th birthday at the time of enrollment.

Discordant siblings are excluded if they are deemed unreliable historians in the opinion of the interviewer administering the QVSFS.

**Study Procedures**

Table 2 summarizes the procedures for enrolling subjects into the study and obtaining blood samples for DNA analysis.
Phase I. Enrolling Probands and Recruiting Siblings

At each participating center, a study neurologist screens all patients with a possible diagnosis of ischemic stroke to identify potential SWISS probands. The study neurologist orders or reviews medical tests pertinent to the diagnosis and subtyping of ischemic stroke as part of routine clinical practice and makes a new diagnosis or confirms a previous diagnosis of ischemic stroke in a potential proband. The study neurologist also subtypes the index stroke according to TOAST criteria [54].

The local coordinator or study neurologist conducts a face-to-face interview with patients who meet enrollment criteria to obtain their medical history and to explain the study. If patients agree to participate in the study, they sign and date 2 copies of the informed consent form, retaining 1 copy for themselves. The local coordinator completes the proband case report forms (CRFs), assigns a SWISS study number to the proband, forwards the proband CRFs to the Clinical Coordinating Center, and gives the proband (or surrogate) a set of study invitation letters to be sent to his or her living full siblings. If siblings are interested in participating in SWISS, they complete the contact information section on the study invitation letter and send it to the Clinical Coordinating Center. The Center assigns SWISS numbers to all siblings who provide contact information.

Phase II. Verifying Concordance and Discordance

The goal of Phase II is to confirm that phenotyping of siblings is accurate.

Discordance is confirmed in Phase IIA. The Clinical Coordinating Center contacts potentially discordant siblings who provide contact information, obtains verbal consent for a brief telephone interview,
administers the QVSFS (Table 1), and obtains a standardized medical history in a structured telephone interview. Siblings who give negative answers to the QVSFS medical history items but who give a positive response to 1 or more of the review-of-symptoms items are advised to inform their primary care physician of their symptoms so that they can be evaluated accordingly. Siblings who respond positively to QVSFS item 1 advance to Phase IIB. If all of the QVSFS items are negative, the patient is considered a verified discordant sibling. The discordant sibling CRFs are completed during the telephone interview, and the Clinical Coordinating Center sends 2 copies of the informed consent form to the verified discordant sibling, who returns 1 signed copy to the Center and retains 1 copy. Verified discordant siblings advance to Phase III of the study.

Concordance is confirmed in Phase IIB. The Clinical Coordinating Center sends potentially concordant siblings a Request for Medical Records Form (RMRF) and 2 copies of the informed consent form to sign, date, and return. The RMRF is a slightly modified study-specific version of the official form used by Mayo Clinic for routine patient care. Subjects return 1 copy of the signed form in a pre-addressed, postage-paid envelope provided with the original form and retain the second copy. The Clinical Coordinating Center uses the signed form to request outside medical records pertaining to the index stroke. The Center constructs a file of outside medical records in a standardized, subdivided sequence (hospital admission notes and discharge summaries; neurologic consultation notes; reports of computed tomographic and magnetic resonance imaging of the head; reports of imaging of the heart by transthoracic and transesophageal echocardiography; copies of electrocardiograms; reports of imaging of
cerveocephalic vasculature by angiography using conventional, computed
tomographic, or magnetic resonance techniques or by ultrasonography; and
reports of routine blood work).

The Clinical Coordinating Center submits completed files on
potentially concordant siblings to the SVC on a weekly basis. A neurologist
member of the SVC reviews the files and attempts to confirm the diagnosis
of ischemic stroke, using a standard stroke work-up checklist to assist with
and document a systematic review of the outside medical records. If the
SVC physician cannot confirm the diagnosis of stroke, the potentially
concordant sibling does not advance in the study. If concordance is
confirmed, the physician classifies the TOAST stroke subtype, completes the
concordant sibling CRFs, and forwards the forms to the Clinical
Coordinating Center. Although as many as 10% of the concordant siblings
in SWISS may have a history of 2 or more strokes, the SVC confirms the
diagnosis of ischemic stroke only for the most recent stroke for which there
are records sufficient to confirm the diagnosis (the “sibling index stroke”).
The verified concordant sibling then advances to Phase III.

Phase III. Acquiring Blood for Genetic Analysis

Blood samples are taken only when a study pedigree is complete,
i.e., clinical data and ICF are available from 1 proband and at least 1 verified
concordant sibling, with or without 1 verified discordant sibling. If the
diagnosis of ischemic stroke cannot be verified for any sibling of a proband,
the clinical data from that proband are saved, but no blood samples are
collected. When a pedigree is complete, the Clinical Coordinating Center
instructs the home health agency to collect blood samples from all pedigree
members. A phlebotomist from the home health agency visits the subjects at
their homes, obtains a blood sample, and ships it to the DNA Bank. The use of a nationwide home health agency for phlebotomy service solves the logistic challenge of obtaining blood from siblings living far away from one another. Whereas a requirement that study subjects travel to a local study center could result in failure to enroll patients for logistic reasons, a home health agency can obtain blood from study subjects rendered homebound by stroke or other ailments.

Phase IV. Genome-wide Scan

The DNA Bank notifies the Genetics Laboratory when 300 concordant sibling pair specimens are ready for analysis. The Genetics Laboratory then performs the genome-wide screen.

Measures of Outcome

The primary outcome is the degree of linkage between the stroke phenotype and genetic markers as measured by the proportion of alleles shared by concordant sibling pairs (accumulated over all pairs at each marker).

Clinical Database

For each proband, we collect name, date of birth, gender, race, home address, home phone number, e-mail address, and alternative contact information. We record the enrolling investigator’s study number and the study center number to assure accurate attribution of efforts and to make it possible to verify entries in CRFs with source documents. Data are collected on stroke risk factors and medical history, date of onset of stroke symptoms, TOAST stroke subtype, and the total number of living full siblings.

The following information is collected on all living full siblings who return sibling response letters: name, date of birth, gender, name of the
proband they are related to, twin status, home address, home phone number, e-mail address, alternative contact information, and standardized risk factor and medical history.

In addition, for each concordant sibling, we record date of review of outside medical records, a stroke work-up checklist addressing medical reports reviewed by the physician member of the SVC who confirms stroke concordance, date of onset of index stroke (and of first stroke, if sibling had more than one), TOAST subtype of index stroke, and responses to all items contained in the QVSFS. For discordant siblings, we record responses to all items contained in the QVSFS.

**Data Analysis**

**Statistical Methods**

One difficulty of mapping stroke genes is that the phenotype may be etiologically heterogeneous. Stroke is a chronic disorder with both genetic and environmental risk factors contributing to susceptibility, and risk factors (such as hypertension or diabetes) may be undetected and untreated. Because stroke is a late-onset disorder, the parents of concordant sibling pairs (CSPs) almost certainly are deceased. This suggests that undiagnosed risk in siblings, increased morbidity due to lack of access to care or treatment, and competing mortality due to increased prevalence of other atherosclerotic risk factors may prevent discovery of sibling pairs concordant for stroke.

To address these issues, we propose to collect concordant siblings along with the probands, and we also propose to collect an additional (unaffected) sibling, when available. By more clearly defining the sharing of alleles, data pertaining to this additional sibling will assist in the
molecular and statistical genetic analyses. For the CSP design, the proportion of alleles shared by the CSP (accumulated over all pairs at that marker) is the statistic that determines evidence for linkage between the stroke phenotype and the genetic marker. If only CSPs are collected, only 4 alleles (at most) can be identified. The third (discordant) sibling is collected for purposes of determining potential nonpaternities in a sibship (more than 4 alleles) and for better estimating the proportion of alleles shared that are identical by state (in the case of absence of parents). For certain combinations of genetic parameters, collection of discordant sibling pairs (DSPs) can also serve as a powerful complementary approach for gene mapping. As demonstrated in the case of diabetic nephropathy, the discordant sibling pair approach can have a 4-fold greater power than the CSP design [67]. Although our primary analytic methods will focus on the concordant pair design, we will be able to use both CSP and DSP approaches, if needed.

Traditional applications of gene mapping have used families in which the trait (disease) is transmitted in a clearly Mendelian fashion. For more complex traits, the inheritance pattern does not fit a single-gene model, and methods that assume a genetic model may provide erroneous results [68]. Ischemic stroke clearly demonstrates familial aggregation, yet no single-gene model of transmission is consistent with the family data. In this project, we propose to use model-independent (relative pair) analysis, a method that is designed to detect linkage without the specification of an underlying genetic model and that is robust to contributions by environmental variation.
To test formally for significance of deviations from expectation in sibling pairs, we will use the maximum likelihood statistic (MLS) proposed by Risch [69]. In this formulation, the form of the likelihood remains the same whether CSPs or DSPs are used, and only the components of the likelihood are changed. The likelihood for $N$ sibling pairs can be written as:

$$L = \Pi_j \{ \Sigma_i w_{ij} y_i \}, \quad i = 1, 2; \quad j = 1, N$$

where: $w_{ij} = P(\text{marker phenotype} | \text{i marker alleles IBS for sib pair j})$ and $y_i = P(\text{sibs share i marker alleles IBS} | \text{sib pair type})$,

and where sibling pair type can be either CSP or DSP. The values of $y_i$ that maximize $L$ represent the maximum likelihood estimates of $y_i$, and the ratio of the maximized likelihood to the likelihood based upon expectation of “no linkage” represents the MLS, where large values of MLS are indicative of linkage. Since deviations in sharing under linkage for CSP are in the direction of an excess of sharing 2 alleles, and deviations in sharing under linkage for DSP are in the direction of an excess of sharing 0 alleles, the collection of CSP and DSP families will require separate analyses.

When parents are available for study, $w_{ij}$ can be easily calculated and is then independent of marker allele frequencies. In our study, because parents will not be available for genetic analysis, $w_{ij}$ must be estimated. In this case, issues relating to marker allele frequency within ethnic groups and across sites become important, particularly because the families we plan to genotype are not likely to be well characterized. While allele frequencies are generally available for whites in databases, this is not usually true for African-Americans or other racial and ethnic groups. A series of sensitivity
analyses to vary the marker allele frequencies can be performed so that the effect of a rare marker allele on evidence for linkage is minimized.

It is likely that some families in SWISS will have more than 2 living concordant siblings. Blackwelder and Elston [70] compared the performance of several sibling-pair linkage tests and evaluated the need for weighing sibling pairs when data from large sibships are available. Three basic tests were evaluated: (a) 2-allele test (proportion of sibling pairs with 2 marker alleles that are IBD); (b) mean test (the mean number of marker alleles that are IBD); and (c) chi-square goodness-of-fit test (comparing observed and expected IBD). The 2-allele test was shown to produce significance levels most widely dispersed from theoretical values, often less significant than expected. Depending on the true underlying genetic model, either the 2-allele test or the mean test would have superior power. In SWISS, therefore, both the 2-allele test and the mean test will be used in evaluating evidence for linkage with ischemic stroke. Because the mean test statistic is usually unaffected by the inclusion of concordant sibships 3 or more in size, and because the pairs are considered “pair-wise independent,” all concordant pairs can be used in the analyses as long as the total number of sibling pairs is greater than 100.

Data from our proposed genetic studies will consist of marker genotypes at approximately 20-cM intervals along each chromosome. Because the genetic markers are evaluated not by chromosome but by multiplexed panels, with markers from different chromosomes evaluated simultaneously, the initial statistical analyses of sibling-pair data will be pairwise linkage analyses. We have used a variety of software packages for this purpose in the past, including SIBPAL, CSPEX, GeneHunter and
MapMaker/Sibs. Most recently, we have used GeneHunter successfully in a number of our genome screen studies with larger family structures (asthma, SLE, and diabetic nephropathy), and we feel that this package provides reasonable estimates of marker sharing statistics and diagnostics.

Estimates of Power to Detect Linkage

For concordant sibling pair studies, Risch [71,72] demonstrated that the fraction $KR/KP$, defined as the risk ratio $\lambda R$ for a type-R relative, can be used to model the probable modes of transmission for a complex disease. Thus, under a given model, the value of $\lambda R$ should decrease in a model-specific manner for each decreasing degree of unilineal relationship, and this expected value can then be contrasted with recurrence risks obtained from a set of relatives (monozygotic twins, dizygotic twins, siblings, offspring, second-degree relatives, etc.). For a single-locus model, therefore, the value of $(\lambda R-1)$ should decrease by a factor of 2, and a multiplicative model predicts risk on the basis of the product of the individual factors.

Risch [72] extended the approach of Suarez et al [73] to include any relative pair. On the basis of this formulation, the power to detect linkage can be obtained for relative (sibling) pairs. For CSPs, assuming that the candidate locus is near a stroke susceptibility locus ($\theta > 0$), power depends upon $\lambda S$ (sibling recurrence risk) and $\lambda O$ (offspring recurrence risk). If there is little dominance effect, then $\lambda S = \lambda O$, and hence the power can be computed on the basis of sibling recurrence risk. For other pairs of relatives, Risch [72] has shown that the single parameter $\lambda O$ is sufficient to specify power (and $\theta$ if $\theta > 0$). As noted earlier, the recurrence risk data in relatives are sparse for stroke. Data from Framingham suggest that a reasonable estimate of $\lambda S$ for stroke may range from 2 to 5.
We assume that the genetic markers used have polymorphic information content (PIC) equivalent to that of an equiprobable 4-allele system, yielding a PIC of about 70%. The sample size required to determine a given power is inversely proportional to the PIC of the markers; thus, a sample of 300 CSPs genotyped at a marker with PIC of 70% would be equivalent to a fully informative marker typed on 210 concordant pairs. In our consideration of power, therefore, we consider a marker with incomplete information, and the initial analyses will comprise pairwise analyses. Application of multipoint (interval) mapping methods will further increase power [74]. Our formal analyses with MapMaker/Sibs or GeneHunter will add further power by means of the multipoint method. Using this approach, we can estimate power for a set of 300 CSPs with stroke (equivalent to 210 pairs with fully informative markers but without parents), and should have ample power to detect linkage between a marker and a moderately strong susceptibility locus, especially for locus-specific sib risks greater than 3. We are including recombination fractions of 0.075 in the computation, since the screening set to be used averages about 15-20 cM between markers.

For a homogeneous single disease susceptibility locus, the power to detect linkage with our expected 300 CSPs generally approaches 100% (Table 3). If stroke susceptibility is attributable to several loci, the risk becomes dependent upon the nature of the contributions (additive or epistatic), and the loci are more difficult to identify. Recent efforts utilizing analysis of genome scan data conditional on the evidence for linkage of a major susceptibility factor show promise [75]. As was demonstrated in the discordant sibling-pair analyses, we may have substantial power to detect linkage using a complementary analytic approach, so that the addition of
even 100 discordant siblings to the CSPs may provide additional insight on linkage.

The expected results and what constitutes evidence of linkage have been discussed in detail in the literature [76-78]. The empirical goal of our project is to map genes that contribute to ischemic stroke. We believe the highest priority is to choose a strategy that will not overlook genes of importance. Empirical results of whole genome scans of other complex diseases [49,50,79] suggest it is not likely that overwhelming evidence of linkage consistent with the stringent criteria suggested by Lander and Kruglyak [76] will emerge in an initial genome survey with markers at 15-20 cM intervals. A more pragmatic approach to genome-wide screens has been suggested by Elston et al. [80], who proposed use of a relatively lenient cutoff for evidence of linkage (such as $P = 0.05$), or an even looser criterion ($P = 0.10$), as a first pass at identifying regions of interest. A second-pass criterion (using additional markers in the region) would require a highly significant type 1 error rate ($P = 0.001$) to deliver an overall genome-wide appropriate type 1 error. In our study, we propose that a statistical equivalent to lod 1.9 or greater be defined as “suggestive” evidence for linkage.

Although evidence for linkage using this nominal cutoff will include locations that do not include stroke-susceptibility genes, the approach offers a high probability that any likely regions will be selected for further analysis. A particularly interesting application of this approach has identified a possible celiac disease locus on chromosome 6q [81]. In that investigation, the genome screen included only 45 sibling pairs. Study of these data identified several regions of possible linkage for detailed analysis.
From the detailed analysis, a single location on 6p with a lod score above 4.5 was identified, suggesting the presence of a celiac disease-susceptibility gene. Although we will be genotyping a much larger number of concordant and discordant sibling pairs, we expect our study to progress in a similar manner—the genome screen will identify regions of possible linkage, which will be ultimately evaluated in detail by means of linkage disequilibrium analysis and testing in independently collected data sets.

Genotyping

Local centers receive blood shipping kits, including a Vacutainer for blood, by mail at the start of SWISS, and the Clinical Coordinating Center will restock the supply on a continuing basis. Used kits are shipped overnight to the DNA Bank for processing. Lymphoblastoid cell lines will be generated from peripheral blood leukocytes and DNA extracted using routine methods. DNA analysis will begin after the 300th concordant sibling pair is enrolled, which we anticipate to be at the end of year 4. At that time, the DNA Bank will ship at least 50 µg of DNA to the Genetics Laboratory.

At the Genetics Laboratory, the DNA will be plated onto 384-well plates for marker genotyping. The ABI Genescan/Genotyper system will be employed in semiautomated fluorescent genotyping, comparing fragment sizes to an internal standard of CEPH DNA. An ABI377 with 96 wells generates the marker data. All genotypes will be scored blind to phenotype. Two hundred thirty-seven microsatellite markers, obtained from Genethon, CHLC, and GDB (106 di-, 21 tri-, and 110 tetranucleotides), will be typed in all sibling pairs. These 237 markers have been sorted into 30 panels. We will run 92 samples per gel (with 4 lanes for controls); estimating ~920
samples at 30 panels, 300 gels will be needed. Allowing for reruns and data loss, we estimate that 400 gels will be required to complete this task and extract greater than 90% of the genetic data. The average distance between adjacent markers in this panel series is 16.3 cM (1-40 cM). Average heterozygosity will be calculated. The CRI-MAP program will determine intermarker distances and will also be used to form the study-specific genetic map. A genotype database (Megabase) will be used to check the binning of alleles, convert allele sizes to whole numbers, and (where possible) to test for non-Mendelian inheritance. Megabase will store all relevant genotypic/phenotypic data and produce all files needed for statistical analysis.

**Cell Lines**

We regard banking of samples to be a central aim of this study. Collection of clinical samples is extremely expensive and time consuming, and it is probable that progress in identifying genes involved in stroke will be incremental. For genes of smaller effect, very large sample sizes are likely to be needed. Having these resources available will ensure that future work can build effectively on the work we present here. Epstein-Barr virus–transformed lymphoblastoid cell lines will be used.

**Statistical Methods**

The program SPLINK will be used to compute single-point maximum lod scores under the “possible triangle” restrictions and to calculate allele frequency estimates for each marker. These will be used in the multipoint analyses, which will be carried out using Mapmaker/Sibs (or, for larger families, GeneHunter), on the whole sample of concordant sibling pairs. A multipoint exclusion map for the whole sample will be created with
Mapmaker/Sibs. For the purposes of this analysis, the disease-susceptibility model will be parameterized in terms of $\lambda_S$, the relative risk to siblings of a case. Genome-wide significance levels will be derived by simulation in the following manner: 500 replicate samples of each chromosome will be simulated under the null hypothesis of no linkage, using the observed allele frequencies and ensuring that the individuals typed at each locus are the individuals in the original dataset. Genome-wide significance of a given lod score will be estimated by simulating a genome scan, choosing 1 replicate of each chromosome at random. The number of peaks exceeding the required level in any of the analyses will be counted, and the process repeated 10,000 times. The average number of such peaks per genome scan will be used to estimate the true genome-wide occurrence rate, and hence the significance, of the lod score.

**Discussion**

Genomics has been applied to animal models of stroke and yielded discoveries about loci that correlate with stroke risk. For example, Rubattu and colleagues [82] identified 3 major quantitative trait loci in the F2 cross of stroke-prone and spontaneously hypertensive rats. Conducting adequately powered genomics studies of stroke in humans is considerably more challenging. Part of the challenge comes from the heterogeneity of the stroke phenotype. Another daunting task is simply obtaining DNA samples from clinically well-characterized members of a cohort of stroke pedigrees.

Assembly of genetic material from several stroke pedigrees can be easily achieved in places with centralized medical records systems and human studies policies that permit so-called community consent. An example of such a resource is the Icelandic Gene Database [83].
Icelandic approach taken by the private firm deCODE is not possible in the United States because of decentralization of medical care and records and concerns over privacy and autonomy rights [84,85]. There has been a steady evolution in multicentered clinical trials in the field of stroke research [86]. SWISS is designed as a multicenter clinical trial, and the use of a nationwide home health agency solves the logistic challenge of obtaining blood from siblings living far away from one another. Thus, it is hoped that this study design will efficiently assemble a cohort of ischemic stroke pedigrees without invoking community consent or using “cold-calling” of pedigree members.
List of Abbreviations

CADASIL, cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy
CEPH, centre d’Etude du Polymorphism Humain
CHLC, Cooperative Human Linkage Center
CRF, case report form
CSP, concordant sibling pair
DSP, discordant sibling pair
GDB, Genome Data Base
IBD, identical-by-descent
IBS, identical-by-state
ICF, informed consent form
MLS, maximum likelihood statistic
PIC, polymorphic information content
QVSFS, Questionnaire for Verifying Stroke-Free Status
RMRF, Request for Medical Records Form
RR, relative risk
SVC, Stroke Verification Committee
SWISS, Siblings With Ischemic Stroke Study
Acknowledgments

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Competing Interests

None are declared.
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Legend

Fig. 1. Sample study pedigree for the Siblings With Ischemic Stroke Study (SWISS). Solid symbols indicate ischemic stroke; CS, concordant sibling; and DS, discordant sibling.
Table 1.—Questionnaire for Verifying Stroke-Free Status (QVSFS)

<table>
<thead>
<tr>
<th>Medical history</th>
</tr>
</thead>
<tbody>
<tr>
<td>Were you ever told by a physician that you had a stroke?</td>
</tr>
<tr>
<td>Were you ever told by a physician that you had a TIA, ministroke, or transient ischemic attack?</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Review of symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Have you ever had sudden, painless weakness on one side of your body?</td>
</tr>
<tr>
<td>Have you ever had sudden numbness or a dead feeling on one side of your body?</td>
</tr>
<tr>
<td>Have you ever had sudden, painless loss of vision in one or both eyes?</td>
</tr>
<tr>
<td>Have you ever suddenly lost one half of your vision?</td>
</tr>
<tr>
<td>Have you ever suddenly lost the ability to understand what people were saying?</td>
</tr>
<tr>
<td>Have you ever suddenly lost the ability to express yourself verbally or in writing?</td>
</tr>
</tbody>
</table>

From Meschia et al. [65]. By permission of the American Heart Association.
Table 2.—Summary of Study Procedures

Phase I—Enrolling probands and recruiting siblings

Study neurologist identifies patient who meets criteria for SWISS.
Proband gives written informed consent and receives study invitation letters for siblings.
Interested siblings send contact information to Clinical Coordinating Center.

Phase IIA—Verifying discordance

Clinical Coordinating Center staff conducts structured telephone interview with
presumed discordant sibling to verify absence of stroke (discordance) and sends
informed consent form to verified discordant siblings.
Verified discordant sibling sends written informed consent to Clinical Coordinating
Center.

Phase IIB—Verifying concordance

Clinical Coordinating Center staff sends request forms for medical records and informed
consent forms to presumed concordant siblings.
Clinical Coordinating Center staff uses returned, signed forms to request external medical
records.
Physician member of Stroke Verification Committee reviews medical records to verify
stroke diagnosis (concordance).

Phase III—Acquiring blood for genetic analysis

Home health agency phlebotomist visits homes of proband, verified concordant sibling,
and (if applicable) verified discordant sibling to obtain blood samples.
Home health agency forwards blood samples to DNA Bank.

Phase IV—Genome-wide screen

DNA Bank stores blood samples and notifies Genetics Laboratory when samples from
300 concordant sibling pairs have been collected.
Genetics Laboratory performs genome-wide screen.
Table 3.—Power to Detect Linkage as a Function of $\lambda s$, $\theta$, and Type I Error Rate ($\alpha$)

<table>
<thead>
<tr>
<th>$\lambda s$</th>
<th>$\theta = 0.050$</th>
<th>$\theta = 0.075$</th>
<th>$\theta = 0.050$</th>
<th>$\theta = 0.075$</th>
<th>$\theta = 0.050$</th>
<th>$\theta = 0.075$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0</td>
<td>0.98</td>
<td>0.95</td>
<td>0.96</td>
<td>0.90</td>
<td>0.40</td>
<td>0.24</td>
</tr>
<tr>
<td>2.5</td>
<td>1.00</td>
<td>0.99</td>
<td>1.00</td>
<td>0.98</td>
<td>0.74</td>
<td>0.52</td>
</tr>
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<td>3.0</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>0.92</td>
<td>0.73</td>
</tr>
<tr>
<td>4.0</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>0.92</td>
</tr>
</tbody>
</table>

$\alpha$, type I error; $\theta$, recombination fraction (which is related to the distance between the marker and trait loci); $\lambda s$: locus-specific relative risk to siblings.
Appendix

Executive Committee: James F. Meschia, MD (chair); Thomas G. Brott, MD; Robert D. Brown, MD; John Hardy, PhD; Stephen S. Rich, PhD.

Operations Committee: James F. Meschia, MD (chair); Thomas G. Brott, MD; Jagjit Gill, PhD; Brett Kissela, MD; Kristin Simonson, RN; Tammy Olson.

Stroke Verification Committee: James F. Meschia, MD (chair); Thomas G. Brott, MD; Marc Lojacono.

Genetics Laboratory: NIH/NIA Laboratory of Neurogenetics, John Hardy, PhD, director.

Statistical Center: Wake Forest University School of Medicine, Stephen S. Rich, PhD, director.

Clinical Coordinating Center: Mayo Physician Alliance for Clinical Trials (MPACT), Rochester, MN, Stephen L. Kopecky, MD, medical director; Jagjit Gill, PhD; Kristin Simonson, RN; Tammy Olson; Patricia Mikesh; Virginia Bruce.

DNA Bank: Coriell Cell Repository, Camden, NJ; Jeanne Beck, PhD, director.
Phlebotomy Service: Hooper Holmes Incorporated, Healthdex Division, Newark, NJ.

Project Officer: John Marler, MD, NINDS.

Participating Centers

Buffalo General Hospital, Buffalo, NY. Principal Investigator: F.E. Munschauer, MD; Coordinator: Sharon Harrington, RN; Sub-Investigators: Peterkin Lee-Kwen, MD; Richard Chan, MD; Patrick Pullicino, MD, PhD.

Centre Hospitalier Affilie Universitaire de Quebec, Quebec City, PQ. Principal Investigator: Denis Simard, MD; Coordinator: Annette Hache, RN; Sub-Investigator: Ariane Mackey, MD.

Charles R. Drew University of Medicine and Science, Los Angeles, CA. Principal Investigator: George Locke, MD; Coordinators: Marcia Montenegro, RN; Norma Dapo, RN; Sub-Investigator: Lowell Nelson, MD, PhD.

Chattanooga Neurology Associates, Chattanooga, TN. Principal Investigator: Thomas Devlin, MD; Coordinators: Patty Wade-Hardie, RN; Tammy Owens, RN; Sub-Investigators: Adele Ackell, MD; Sharon Farber, MD; Ravi Chander, MD; G. Hagan Jackson, MD; Kadrie Hytham, MD; Bruce Kaplan, MD; David Rankine, MD.
Cleveland Clinic Florida, Weston, FL. Principal Investigator: Virgilio Salanga, MD; Coordinator: Rosa Patino Paul Picirillo, MD; Sub-Investigators: Eduardo Locatelli, MD; Nestor Galvez-Jimenez, MD, FACP.

Clinical Stroke Research Center, Syracuse, NY. Principal Investigator: Antonio Culebras, MD; Coordinators: Linda Schad, LPN; Therese Dean, BS.

East Bay Region Associates in Neurology, Berkeley, CA. Principal Investigator: Brian C. Richardson, MD; Coordinators: Catherine W. Ndungu; Karen Ashikeh, RN; Julie S. Ginsburg; Lynn S. Jehle, RN; Sub-Investigators: Joanna A. Cooper, MD; Heidi M. Shale, MD; Bradley T. Wrubel, MD.

Emory University School of Medicine, Atlanta, GA. Principal Investigator: Barney Stern, MD; Coordinator: Bethany Lane, RN; Sub-Investigators: Michael Frankel, MD; Marc Chimowitz, MD; Owen Samuels, MD.

Field Neurosciences Institute, Saginaw, MI. Principal Investigator: Faith Abbott, DO; Coordinator: Richard Herm, RN, BSN, CEN; Sub-Investigators: Malcolm Field, MD; Debasish Mridha, MD.

Florida Neurovascular Institute, Tampa, FL. Principal Investigator: Erfan Albakri, MD; Coordinators: Marina Pierce, MD, PhD; Beth Bertoldi, SNA.
**Helen Hayes Hospital, West Haverstraw, NY.** Principal Investigator: Laura Lennihan, MD; Coordinator: Laura Tenteromano, RN.

**Hospital Charles Le Moyne, Greenfield Park, PQ.** Principal Investigator: Leo Berger, MD; Coordinator: Martin Caplette, RN, BSC; Sub-Investigator: Andre Bellavance, MD, PhD.

**Indiana University School of Medicine, Indianapolis, IN.** Principal Investigator: Linda Williams, MD; Coordinator: Alison Sears, RN, BSN; Sub-Investigators: Askiel Bruno, MD; William Jones, MD; Alfredo Lopez, MD; James Fleck, MD; Jose Biller, MD.

**Jewish General Hospital, Montreal, PQ.** Principal Investigator: Jeffery Minuk, MD; Coordinator: Claudia Schanz.

**Johns Hopkins Bayview Medical Center, Baltimore, MD.** Principal Investigator: Rafael Llinas, MD; Coordinator: Janice Alt; Sub-Investigator: Christopher Earley, MD.

**Luther-Midelfort Clinic, Eau Claire, WI.** Principal Investigator: Felix Chukwudelunzu, MD; Coordinators: Tonya Kunz, RN; Karen Snobl, RN; Sub-Investigators: James Bounds, MD; Rae Hanson, MD; David Nye, MD.

**Maine Line Health – Stroke Program, Bryn Mawr, PA.** Principal Investigator: Gary Friday, MD; Coordinator: Charlotte Baker, RN, BSN, CCRC.
Maine Medical Center, Portland, ME. Principal Investigator: John Belden, MD; Coordinator: Diane Diconzo-Fanning, RN; Sub-Investigator: Paul Muscat, MD.

Marshfield Clinic, Marshfield, WI. Principal Investigator: Percy Karanjia, MD; Coordinator: Lyn Stephani, CCRC; Sub-Investigator: Kenneth Madden, MD.

Mayo Clinic, Jacksonville, FL. Principal Investigator: Thomas Brott, MD; Coordinators: Marc Lojacono, CRC; Linda Hall, CCRC; Sub-Investigators: James F. Meschia, MD; Frank Rubino, MD; Benjamin Eidelman, MD.

Mayo Clinic, Rochester, MN. Principal Investigator: Robert Brown, MD; Coordinator: Colleen Albers, RN; Sub-Investigators: George Petty, MD; Eelco Wijdicks, MD; Irene Meissner, MD; Bruce Evans, MD; Kelly Flemming, MD; Robert Fealey, MD; Jimmy Fulgham, MD; David Wiebers, MD.

Mayo Clinic, Scottsdale, AZ. Principal Investigator: David Dodick, MD; Coordinators: Judy Tiede, RN; Nadine Lendzior, RN; Barbara Cleary, RN; Sub-Investigator: Timothy Ingall, MD.

Medical University of South Carolina, Charleston, SC. Principal Investigator: Timothy Carter, MD; Coordinator: Feng Liu, MSN.
Mercy General Hospital, Sacramento, CA. Principal Investigator: Paul Akins, MD; Coordinator: Deidre Wentworth, RN; Sub-Investigator: Richard Atkinson, MD.

Metro Health Medical Center, Cleveland, OH. Principal Investigator: Joseph Hanna, MD; Coordinator: Alice Liskay, RN; Sub-Investigators: Marc Winkelman, MD; Nimish Thakore, MD, DM.

Neurological Associates, Inc., Richmond, VA. Principal Investigator: Francis McGee, Jr., MD; Coordinators: Janet McGee, REPT, CCRC; Christine Seaton, RN; Sub-Investigators: Alan Zacharias, MD; Stephen Thurston, MD; Thomas Smith, MD; Robert White, MD; Philip Davenport, MD; Vincent Calabrese, MD; John Brush, MD; Susanna Mathe, MD; Robert Cohen, MD; J. Kim Harris, MD; John O’Bannon III, MD; Andrew Worthington, MD; John Blevins, MD.

Rhode Island Hospital, Providence, RI. Principal Investigator: Janet Wilterdink, MD; Coordinator: Carol Cirillo, RN.

Roberts Research Institute Stroke Prevention and Atherosclerosis Research Centre, London, ON. Principal Investigator: David Spence, MD; Coordinator: Nancy Richer, RN; Sub-Investigator: Claudio Munoz, MD.

Royal University Hospital, Saskatoon, SK. Principal Investigator: Ali Rajput, MD; Coordinator: Aileen Schultz, RN; Sub-Investigator: Alexander Rajput, MD.
Ruan Neurology Clinic and Clinical Research Center, Des Moines, IA. Principal Investigator: Michael Jacoby, MD; Coordinator: Pam McManus, RN; Sub-Investigators: Bruce Hughes, MD; Randall Hamilton, MD; Paul Babikian, MD; Mark Puriccelli, DO.

Rush Presbyterian, Chicago, IL. Principal Investigator: Sean Ruland, DO; Coordinator: Karen Whited, RN; Sub-Investigators: Michael Schneck, MD; Michael Sloan, MD; Phillip Gorelick, MD, MPH.

Scripps Clinic, La Jolla, CA. Principal Investigator: Mary Kalafut, MD.

Shands Jacksonville/University of Florida, Jacksonville, FL. Principal Investigator: Scott Silliman, MD; Coordinators: Barbara Quinn, RN; Cicely Bryant, RN; Sub-Investigator: Jose Guillermo Merino-Juarez, MD.

Thomas Jefferson University Hospital, Philadelphia, PA. Principal Investigator: Rodney Bell, MD; Coordinator: Lisa Bowman, RN, BSN, CNRN; Sub-Investigators: David Brock, MD; J. Javier Provencio, MD.

University of California-Davis School of Medicine, Sacramento, CA. Principal Investigator: Piero Verro, MD; Coordinator: Nancy Rudisill, RN, MS.

University of California-Los Angeles Stroke Center, Los Angeles, CA. Principal Investigator: Jeffery Saver, MD; Coordinator: Rinat Masamed,
BA; Sub-Investigators: Chelsea Kidwell, MD; Megan Leary, MD; Margaret Tremwel, MD; Bruce Ovbiagele, MD.

University of California San Diego Stroke Center, San Diego, CA. Principal Investigator: Patrick Lyden, MD; Coordinator: Nancy Kelly, RN; Sub-Investigators: Thomas Hemmen, MD; Brett Meyer, MD.

University of Cincinnati, Cincinnati, OH. Principal Investigator: Brett Kissela, MD; Coordinators: Kathleen Alwell, RN; Rosemary Miller, RN, CCRC; Sub-Investigators: Joseph Broderick, MD; Daniel Woo, MD; Daniel Kanter, MD.

University Health Network, Toronto, ON. Principal Investigator: Frank Silver, MD; Coordinator: Relu Wiegner, RN; Sub-Investigator: Cheryl Jaigobin, MD.

University of Illinois at Chicago, Chicago, IL. Principal Investigator: Cathy Helgason, MD; Coordinator: Elizabeth Brennan, RN, BSN, CCRC.

University of Iowa Hospital, Iowa City, IA. Principal Investigator: Patricia Davis, MD; Coordinator: Kathryn Niehus, RN, BSN; Sub-Investigator: Harold P. Adams, Jr., MD.

University of Kentucky, Lexington, KY. Principal Investigator: L. Creed Pettigrew, MD; Coordinator: Deborah Taylor, MS; Sub-Investigators: Stephen Ryan, MD; Michael Hoffman, MD.
University of Pennsylvania Medical Center, Philadelphia, PA. Principal Investigator: Scott Kasner, MD; Coordinators: Simone Shaw, RN, BSN; Katherine Mack, RN, BSN; Sub-Investigators: David S. Liebeskind, MD; Brett L. Cucchiara, MD; Michael L. McGarvey, MD.

University of South Alabama, Mobile, AL. Principal Investigator: Richard Zweifler, MD; Coordinators: Robin Yunker, RNC, MSN; Debra Alday, RN, BSN; Sub-Investigators: Amelito Malapira, MD; John Rothrock, MD; M. Asim Mahmood, MD.

University of Texas Southwestern Medical Center at Dallas, Dallas, TX. Principal Investigator: D. Hal Unwin, MD; Coordinators: Anne Redhead, RN; Jennifer Stanford, RN, MSN, CCRC; Sub-Investigators: Dion Graybeal, MD; Mark Johnson, MD.

University of Virginia, Charlottesville, VA. Principal Investigator: Bradford Worrall, MD, MSc; Coordinator: Kay Maupin, RN; Sub-Investigators: Dean Kindler, MD; E. Klarke Haley, Jr., MD; Karen Johnston, MD, MSc; Devin Brown, MD.

University of Wisconsin, Madison, WI. Principal Investigator: Robert Dempsey, MD; Coordinator: Jamie Kish, BA; Sub-Investigator: Douglas Dulli, MD.
Wake Forest University School of Medicine, Winston-Salem, NC. Principal Investigator: David Lefkowitz, MD; Coordinator: Jean Satterfield, RN; Sub-Investigators: Charles Tegeler IV, MD; Patrick Reynolds, MD.

Washington University School of Medicine, St. Louis, MO. Principal Investigator: Chung Hsu, MD, PhD; Coordinators: Carol Hess, RN; Jacqueline Epps-Wilbanks; Sub-Investigators: Aninda Bhattacharyya, MD; Fernanda Santiago, MD; Jin-Moo Lee, MD, PhD; Abdullah Nassief, MD; Lisa Yanase, MD; Evan Allen, MD, MBA.

Wayne State University, Detroit, MI. Principal Investigator: Seemant Chaturvedi, MD; Coordinators: Flicia Mada, RN; David Wiseman, RN; Elizabeth Berlow, RN, MPH; Sub-Investigator: Bradley Jacobs, MD.
SWISS proband identified by a study neurologist

Other potential SWISS study subjects