Published in final edited form as:

Int J Stroke. 2010 April ; 5(2): 117–125. doi:10.1111/j.1747-4949.2010.00420.x.

The Levels of Inflammatory Markers in the Treatment of Stroke (LIMITS) Study: Inflammatory Biomarkers as Risk Predictors after Lacunar Stroke

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Abstract

BACKGROUND—Inflammation is increasingly recognized as playing a central role in atherosclerosis, and peripheral blood markers of inflammation have been associated with incident and recurrent cardiac events. The relationship of these potentially modifiable risk markers to prognosis after ischemic stroke is less clear. The Levels of Inflammatory Markers in the Treatment of Stroke (LIMITS) study will address hypotheses related to the role of inflammatory markers in secondary stroke prevention in an efficient manner using the well-established framework of the Secondary Prevention of Small Subcortical Strokes (SPS3) trial (NCT00059306).

METHODS—SPS3 is an ongoing Phase III multicenter secondary prevention trial focused on preventing recurrent stroke in patients with small vessel ischemic stroke, or lacunes. In SPS3, patients are assigned in a factorial design to aspirin versus aspirin plus clopidogrel, and to usual versus aggressive blood pressure targets. The purpose of LIMITS is to determine if serum levels of inflammatory markers—including high sensitivity C-reactive protein (hsCRP), serum amyloid A (SAA), CD40 ligand (CD40L), and monocyte chemoattractant protein-1 (MCP-1)—predict recurrent stroke and other vascular events among lacunar stroke patients. The project will also

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

S1. Participating Clinical Centers & Study Organization

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determine if these markers predict which people will respond best to dual antiplatelet therapy with clopidogrel and aspirin, as well the relationship to cognitive function.

ANALYSIS PLAN—Multivariable Cox proportional hazard regression modeling will be used to estimate hazard ratios for the effect of marker levels on risk of recurrent stroke and other outcomes after adjusting for additional potential risk factors, including age, gender, ethnicity, treatment arm, and traditional stroke risk factors. Interactions between marker levels and treatment assignment for both arms of the SPS3 study will be assessed. Observations will be censored at the time of last follow-up visit.

CONCLUSIONS—LIMITS represents an efficient approach to the identification of novel inflammatory biomarkers for use in risk prediction and treatment selection in patients with small vessel disease.

INTRODUCTION

Basic and clinical studies provide evidence that inflammation plays a crucial role in atherosclerosis and cardiovascular disease (CVD), but little information is available on serum markers of inflammation as indicators of prognosis after stroke. Despite a growing literature on the role of inflammatory markers, particularly high-sensitivity C-reactive protein (hsCRP), in prediction of outcomes among patients with CVD (1), little is known about their role in predicting outcome in patients with CVD(2). Clinical studies suggest, that some antiplatelet agents may reduce levels of inflammatory markers, and these levels may predict the efficacy of aspirin, clopidogrel, and glycoprotein 2b/3a inhibitors in preventing cardiac disease.

Little is known about the role of anti-platelet therapy in reducing inflammatory markers among stroke patients or the relative efficacy of antiplatelet therapy among those with, and without elevated levels of inflammation.

Lacunes, or small subcortical strokes comprise about 25% of brain infarcts, are especially frequent in US minorities, and are the most common cause of vascular dementia (3) (4) (5) (6) (7). Though infrequently fatal, lacunes are associated with a high risk of recurrence and cognitive impairment. The rate of recurrence among lacunar stroke patients is about 8% per year, slightly higher than other stroke sub-types. (8) (9) (10) (11) (12) Approximately 70% of recurrences in these patients are also lacunes, supporting a distinctive pathomechanism. Associated white matter abnormalities seen on neuroimaging also predict recurrence in these patients. (13) (14) However the variability in prognosis among lacunar stroke patients identifies that risk stratification schemes are needed.

Major risk factors for lacunar stroke are advancing age, hypertension, and diabetes. About 70–80% of patients have a history of hypertension, and up to 30% have a history of diabetes mellitus. (15) (16) (17) (18) Other potential risk factors for cerebral small vessel disease include smoking, silent brain infarcts, and white matter abnormalities on MRI. (19) (20) (21) Stroke risk factors were absent in 18% of patients in one large autopsy study. (22) In the Northern Manhattan Stroke Study (NOMASS), the prevalence of hypertension, diabetes, smoking, and hypercholesterolemia did not differ between those with lacunar and non-lacunar stroke. (23)

Inflammatory mechanisms have been associated with lacunes and their prognosis in some studies. A polymorphism of the interleukin-6 gene associated with increased inflammation was an independent risk factor for lacunar stroke in one study. (24) This same polymorphism was found to be associated with carotid artery intima-media thickness in NOMAS, as well, providing evidence that polymorphisms related to inflammation may

relate to both large and small vessel disease. (25) Others also reported that lacunar stroke patients with elevated tumor necrosis factor (TNF)- α and intercellular adhesion molecule 1 (ICAM-1) levels were more likely to experience early neurological deterioration and poor outcome at 3 months. (26) In a case control study, elevated *Chlamydia pneumoniae* antibody titers were associated with increased risk of lacunar stroke, as well as large vessel atherosclerotic stroke. (27) An association between leukocyte count and outcomes after stroke has also been found in those with lacunar stroke, as well as other stroke subtypes. (28) These data provide evidence to support the hypothesis that inflammatory mechanisms may contribute to the risk of lacunar disease and its prognosis.

Based on calls for further data regarding the role of inflammatory markers in stroke prognosis, (1) (2) we designed a prospective observational study to test the hypothesis that inflammatory biomarkers predict recurrence after lacunar stroke. The study is nested within an ongoing secondary stroke prevention trial, the Secondary Prevention of Small Subcortical Strokes (SPS3) trial. (29)(60) The overall aim of the Levels of Inflammatory Markers in the Treatment of Stroke study (LIMITS) is to determine whether serum levels of hsCRP, serum amyloid A (SAA), IL6, CD40 ligand (CD40L), tumor-necrosis factor receptor-1 (TNFR1), and other inflammatory markers predict recurrent stroke and other vascular events among patients with a history of small artery ischemic stroke. Additionally, LIMITS will determine whether these markers predict which patients respond best to dual antiplatelet therapy with clopidogrel and aspirin. Furthermore, LIMITS will assess association of inflammatory markers and cognitive function.

STUDY DESIGN

Overall Plan

LIMITS is designed as an ancillary study to the SPS3 trial, which has been described elsewhere (29). In brief, SPS3 is an ongoing, multicenter, investigator-initiated, NIH/NINDS-funded Phase III trial focused on secondary prevention of stroke recurrence in patients with small vessel ischemic stroke, or lacunes. The study has a 2 X 2 factorial design with one arm being blinded, comparison of monotherapy vs. combination therapy of antiplatelet agents and the other an open label comparison of two targets of blood pressure control: “intensive” versus “usual”. Participants must have had a symptomatic lacune proven on MRI within the prior 6 months prior to randomization. Patients are assigned two interventions: (i) antiplatelet therapy: aspirin (325 mg/day) monotherapy versus aspirin (325 mg/day), plus clopidogrel (75 mg/day) combination therapy (double blind, placebo control); and (ii) two target levels of systolic blood pressure control, ‘usual’ (130–149 mmHg) versus ‘intensive’ (<130 mmHg). The LIMITS study involves collection of plasma and serum samples at baseline and at one-year (up to 18 months), follow-up during the study, and central storage and analysis of samples for inflammatory marker levels.

SPS3 Framework and Organizational Structure

The LIMITS study utilizes the framework of the SPS3 trial to address questions about the role of inflammatory markers in secondary stroke prevention. Of 62 sites presently participating in SPS3, 42 (68%) sites are recruiting patients into LIMITS.

Inclusion and Exclusion Criteria

The population for this ancillary study consists of patients enrolled into the SPS3 trial at sites participating in LIMITS. Patients with a clinical diagnosis of MRI-proven lacunar stroke within 6 months, and absence of cortical or large subcortical stroke, carotid stenosis or cardioembolic source, are eligible. All patients eligible for SPS3 are eligible for this ancillary study; there are no additional exclusion criteria. The inclusion and exclusion

criteria for SPS3 are listed in Table 1. Patients are enrolled into SPS3 and have the blood sample drawn for LIMITS at least 3 weeks after stroke.

Informed consent

LIMITS utilizes the same informed consent being used for the parent study. Some sites utilize a separate consent form.

Blood Collection Kits, Phlebotomy and Local Processing

Materials for collection and shipping of blood specimens are provided to the participating sites by the Columbia University Center for Advanced Laboratory Medicine (CALM). CALM provides blood collection, aliquoting, and shipping kits to the study sites at the onset and as needed during the course of the project. A 10 cc blood sample in ethylenediaminetetraacetic acid (EDTA) and a 9.5 ml gel serum separator tube are drawn on the day of randomization, prior to the initiation of therapy. Samples are centrifuged locally at 3000g at 4°C for 20 minutes and the samples are then aliquoted into 12 1.5 cc cryovials. Tubes are labeled with the subject's SPS3 study number using labels pre-printed by the CALM laboratory using standard freezer-safe materials. Aliquots are frozen locally at -70°C until shipping.

Shipping of samples to central laboratory

Frozen samples are shipped by overnight courier on dry ice to CALM on a quarterly schedule for central processing. The initial two shipments from each site were done on a shorter time scale to make sure that sites perform proper collection and storage of specimens. Upon receipt by CALM, the samples are placed in freezer bags, registered into a specimen tracking system (Freezerworks, Dataworks Development, Inc, Mountlake Terrace, WA), bar-coded, and stored in a -70°C freezer.

Rationale for choice of inflammatory biomarkers

The biomarkers planned for initial analysis in LIMITS are hsCRP, serum amyloid A (SAA), IL6, CD40 ligand (CD40L), and tumor-necrosis factor receptor-1 (TNFR1). It is anticipated, however, that with the creation of this blood specimen repository, further opportunities for testing other markers will become available.

HsCRP—CRP is an acute phase reactant in the pentraxin family of proteins, and is part of the innate, non-specific immune response. It is produced in the liver on stimulation by IL6, and plays an important role in the innate immune response. The high sensitivity assay for CRP has several features that make it ideal both as an epidemiological tool and for clinical purposes, including stability after freeze-thaw cycles, limited diurnal and seasonal variability, and the absence of a need for it to be measured in the fasting state. (1) Several studies have demonstrated its utility as a measure of future risk of atherosclerotic coronary artery disease. (1) Relatively few studies, however, have examined hsCRP as a risk factor for ischemic stroke. (2) (30) (31) (32)(33)(34) In the prospective Northern Manhattan Study, hsCRP predicted MI and death, but not first ischemic stroke, after adjusting for potential confounders. In a follow-up study after first ischemic stroke, hsCRP measured predominantly within the first 72 hours post stroke and was associated with increased risk of mortality over the following 5 years after adjusting for confounding factors. (36) There was no increased risk of recurrent stroke, however; which probably reflects the elevations in hsCRP seen with early collection of samples and their relationship to stroke severity, a major predictor of post-stroke mortality.

SAA—SAA, similar to CRP, is an acute phase protein synthesized by hepatocytes in response to cytokine activation, but SAA levels reach higher plasma concentrations and may have different responses to stimuli than CRP. (37) SAA has been associated with risk of coronary and stroke events in several epidemiological studies. (1) In the Women's Health Study, SAA in the upper quartile was associated with an elevated risk of cardiovascular events (RR 3.0, 95% CI 1.5 to 6.0). (38) In another study, SAA had a marginal effect on future cardiovascular outcomes (adjusted HR=1.03, 95% CI, 1.02 to 1.05) when assessed as a continuous measure. (39) SAA level has also been linked to increased risk of early mortality in unstable coronary syndrome patients. (40)

IL-6—IL6, like hsCRP, has been associated with risk of vascular events in several studies (1) In a nested case-control study, each increase in quartile of IL-6 was associated with a 38% increase in risk of MI ($p=0.001$). (41) In the Women's Health Study (WHS), IL-6 was associated with incident cardiovascular events, including stroke. (42) (38) As noted above, an interleukin-6 gene polymorphism was an independent risk factor for lacunar stroke. (24)

Soluble CD40L—CD40 ligand (CD40L) is a pro inflammatory molecule produced by endothelial cells, macrophages, lymphocytes, platelets and smooth muscle cells. It is involved in leukocyte interactions with one another and with endothelium, as well as platelet-platelet interactions, and promotes a cascade of events associated with atherosclerosis and acute coronary syndromes and potentially stroke, including increasing production of metalloproteinases, tissue factor, cytokines, and adhesion molecules. (43) Soluble CD40L (sCD40L) is shed from stimulated lymphocytes as well as activated platelets, and is biologically active. Blockage of CD40L can reduce lesion formation. (44) (45) (46) (47) In clinical studies, sCD40L predicted a first vascular event. (48) In post-hoc analysis of a trial of abciximab, a glycoprotein 2b/3a inhibitor, in unstable cardiac patients undergoing angioplasty ($n=1088$), patients with sCD40L levels in the fourth and fifth quintiles had significantly elevated risk of subsequent cardiovascular events over 6 months. (49) Furthermore, the effect of abciximab on lowering the risk of a recurrent event was seen only in those with elevated levels of CD40L, indicating that CD40L could be used as a marker of response to therapy. In a similar post-hoc analysis of another clinical trial among patients with acute coronary syndromes, sCD40L above the median (drawn within 72 hours of the initial event) predicted increased risk of recurrent MI and death after adjusting for other risk factors, including hsCRP. (50) In the Myocardial Ischemia Reduction with Aggressive Cholesterol Lowering (MIRACL) study of early, high-dose atorvastatin therapy after MI, high sCD40L predicted recurrent cardiovascular events, again independently of aspirin therapy. (51) sCD40L has also been demonstrated to be up regulated in patients with acute ischemic stroke, and this up regulation continues for at least 3 months after the infarction. (52) sCD40L may be an appropriate and useful marker of future risk of recurrent cardiovascular events in stroke patients, and potentially for response to antiplatelet or other therapy.

TNF receptor 1—In cross-sectional analysis, TNF α levels were independently associated with prevalent MI and with subclinical measures of atherosclerosis. (30) (53) Other studies have suggested soluble TNF receptor levels may be a better marker of atherosclerotic burden and vascular events than TNF α (54) (55) TNFR1 is also emerging as an important marker of poor outcome after both stroke and myocardial infarction, (56) and we have found associations between TNFR1 and mortality in the northern Manhattan population (unpublished data). Recent animal studies have also shown that TNFR1 may be associated with decreased neuronal proliferation after stroke, and that abrogation of this activity may enhance recovery. (52) (57)

MCP-1—Monocyte chemoattractant protein-1 (MCP-1) is the major mediator of the recruitment of monocytes and other leukocytes into the arterial wall in atherosclerosis, but has been less well studied in large-scale epidemiological studies than other markers. MCP-1 deficient mice have reduced infarct volume (approximately 30% reduction) after experimental middle cerebral artery occlusion. In epidemiological studies, MCP-1 correlated strongly with carotid intima media thickness, a subclinical marker of atherosclerosis ($r=0.59$, $p<0.0001$). (58) MCP-1 levels are also increased in patients with unstable angina and acute MI but not in those with stable angina, indicating that it may be a marker of instability or plaque activity. (59)

Sample assays

Plasma samples will be analyzed in batches blinded to treatment and outcome. HsCRP and SAA assays are performed using the Dade-Behring BN-II nephelometric assay system (Dade-Behring, Deerfield, IL). This nephelometric method to measure hsCRP is the FDA-approved, national standard assay for this marker. Enzyme linked immunosorbent assays (ELISA) are performed for the measurement of IL6 (R and D Systems, Minneapolis, MN), TNFR1, MCP1 and CD40L (Biosource, Invitrogen, Carlsbad, CA).

Outcomes and Safety Monitoring

The primary outcome for SPS3 is all recurrent stroke, ischemic and hemorrhagic. Ischemic strokes are clinically defined as a focal neurological deficit persisting for greater than 24 hours, and are ascertained via clinical evaluation and use of CT or MRI. (60) Hemorrhagic strokes are clinically defined as neurologic deficits associated with intraparenchymal or subarachnoid hemorrhagic lesions confirmed by CT, MRI, or autopsy. (60) Safety monitoring will be handled by the SPS3 CC and Medical Safety Monitor (MSM), and is routinely performed by an independent DMSB. Because the present proposal is non-interventional, there are no specific safety concerns for LIMITS.

Sample Size Calculations and Statistical Analysis Plan

The SPS3 study utilizes a factorial design to simultaneously assess the impact of two therapies. A total of 2500 patients will be enrolled into the trial. LIMITS has as a goal to recruit 1440 patients (57%) of the total in SPS3. The sample size of 1440 was chosen based on feasibility, and assumes that about 40 enrolling centers will enroll at least 12 patients annually for 3 years. We computed the detectable hazard ratios based on a required power of 80% and a significance level of 0.05 for comparing the outcome rates in the first and fourth quarters. Where possible, sample size calculations were based on the same assumptions used in the initial SPS3 grant application. Specifically, we assumed an annual 7% rate of recurrent stroke and an annual 10% rate of recurrent ischemic stroke, MI, or death. In addition, a 10% 3-year loss-to-follow up was assumed, which had minimal impact on the results. Figure 1 illustrates the power versus the detectable hazard ratio for the LIMITS study. The solid line in the figure represents the power curve for the endpoint of recurrent ischemic stroke. Similarly, the dashed line represents the power curve for the combined endpoint of recurrent ischemic stroke, MI, or death. If event rates are lower than expected due to improvements in other preventive strategies, the sample sizes of the parent SPS3 trial and the LIMITS study may need to be increased.

All hypothesis tests performed during the analysis of the primary and secondary endpoints will be two-sided and will use an alpha level of 0.05. Alpha allocation could be determined independently of the parent study. The study population is a representative subgroup of the parent study, but the hypotheses are independent. The testable hypotheses of LIMITS are shown in Table 2.

The statistical analyses fall into two general categories. For the first set of analyses, values of hsCRP (baseline, one-year, and change from baseline to one year) will be considered the independent variable of primary interest for hypothesis testing. The null hypothesis is that marker levels are not related to the risk of recurrent ischemic stroke. To test this hypothesis Cox proportional hazard regression will be used to estimate the unadjusted hazard ratio for the marker level of interest (e.g. baseline, 1-year follow-up or the change between the two time points) with time-to-event for IS as dependent variable. Multivariable Cox proportional hazards regression will be used to estimate the adjusted hazard ratio for the marker level after adjusting for additional potential risk factors, including age, sex, race-ethnicity, and traditional stroke risk factors defined either dichotomously (hypertension, cardiac disease, diabetes mellitus, smoking) or continuously (HDL, LDL). Observations will be censored at the time of last follow-up visit. Finally, additional analyses will utilize quarters of the inflammatory markers as independent variables, using those in the lowest quarter for each marker as the reference group.

The second set of analyses will assess whether the change in markers from baseline to one year differs by treatment group using a linear regression model. The primary independent variables of interest will include covariates for each of the treatment arm dichotomies: aspirin vs. aspirin plus clopidogrel and aggressive vs. standard blood pressure treatment. These analyses will be adjusted for the baseline value and the use of statin treatment as a concomitant medication, as both may influence marker levels.

In addition, for hsCRP, different ranges of standardized levels of risk have been recommended by consensus in the literature for primary prevention of cardiac disease (1) and post-stroke (2) (61) (62). These thresholds are: (i) Primary prevention: low, medium and high-risk (CRP < 1, 1–3 and >3 mg/L, respectively); (ii) Post-stroke: <15 and >15 mg/L. These potentially clinically meaningful thresholds will be assessed as exposure variables in secondary analyses. We will also explore the additional value of inflammatory markers to models based on clinical variables, using reclassification methods, depending on the findings in our primary analyses. (63)

Source of Funding

The SPS3 trial is funded by a contract with the NIH/NINDS, and this ancillary study is funded through an ancillary R01 (NINDS NS050724) from the NINDS. Supplemental funding is provided by the BMS-Sanofi Pharmaceutical Partnership.

DISCUSSION

Although peripheral blood inflammatory markers have been found in prospective studies to predict incident vascular events, including stroke, there are several limitations to the existing knowledge base. First, not all prospective studies have confirmed that hsCRP is associated with incident cardiac disease or stroke. (64) (65) (66) (67) The choice of study population may play a role in these discrepancies, as inflammation may be unimportant among those who are older or have other risk factors. Many studies included predominantly healthy middle-aged individuals without a significant burden of risk factors. Second, levels of inflammatory markers may change after stroke (36) and thus levels used in primary prediction may differ from those used in secondary prognostication. Third, management implications of elevations in inflammatory biomarkers remain uncertain. Recent results from a large primary prevention trial using rosuvastatin provide indirect evidence of the utility of measuring hsCRP as a determinant of whether to treat patients with statins; the relevance of this finding to patients with stroke is unclear. (68)

It is uncertain regarding measurement of inflammatory markers after stroke. Among studies conducted to assess the predictive significance of inflammatory markers, most were small, conducted at single centers, included multiple stroke subtypes (including TIA), utilized measurements at very short intervals after stroke, had short follow-up periods (up to 1 year), incompletely adjusted for other predictors of outcome, and involved post-hoc analyses. These studies provide little evidence of the value of these markers in predicting response to therapy. The present study will address many of these limitations.

A strength of LIMITS is that it will examine the role of inflammatory markers in relation to a specific stroke subtype. Most research related to inflammation in atherosclerotic disease has focused primarily on cardiac disease. Although stroke has similarities to ischemic heart disease, important differences necessitate independent study of stroke. The etiologic categories of ischemic stroke are more diverse than those of coronary artery disease. Stroke patients are older on average than cardiac patients, and risk factor profiles differ. The present study will allow the determination of the prognostic significance of inflammatory markers in lacunar stroke, a specific, common, important, and well-characterized stroke subtype.

Second, by nesting LIMITS within a secondary prevention trial, we will be able to address the question of the effect of anti-inflammatory therapies and the ability to modify these marker levels in stroke patients. Specifically, it is unknown whether combination antiplatelet therapy is more effective than single antiplatelet therapy in reducing levels of inflammatory markers in stroke patients. Furthermore, it is unknown whether some patients have a greater response to antiplatelet therapies. If it were found, for example, that elevations of certain markers identify a subgroup with greater benefit from combination antiplatelet therapy, these patients could be treated with a lower risk-to-benefit ratio.

Third, it is unknown if inflammatory marker levels are risk factors in both genders and all ethnicities. Limited research is available about the race-ethnic distribution of hsCRP, SAA, and CD40L. (30) (69) (70) While some recent data provides evidence for stability across populations, Hispanics, the fastest growing subpopulation within the US, were not included in these studies. (71) (72) (35) Data from a Japanese cohort provide evidence that there may be significant differences in marker levels among race-ethnic groups and between men and women. (35) Few studies have focused on the elderly. A recent NHLBI workshop on risk assessment specified the importance of further research on different race-ethnic groups, particularly Hispanics. (1) We will address the role of these markers in stroke prediction among Hispanics, who are expected to comprise 20% of the SPS3 population, and in men and women.

Fourth, LIMITS will assess whether inflammatory biomarkers remain stable over one year, and whether increases over time portend increased risk independently of single baseline measurements. Few studies have reported stability of inflammatory markers over time, (73) (74) and it remains uncertain whether a measurement at a single point in time is an accurate reflection of levels. It may also be that relative increases over time are important in predicting risk.

A final advantage of our design is practical. By nesting our study within SPS3 we will be able to minimize additional costs and take advantage of an existing clinical trial infrastructure, as was also done in the Warfarin Aspirin Recurrent Stroke Study. (75)

Our study design has notable limitations, as well. The LIMITS study is nested in an ongoing secondary prevention trial (SPS3), which may lead to selection bias due to the inclusion and exclusion criteria inherent in a clinical trial. We will also have limited data about chronic inflammatory diseases or clinical infections. Because our study is nested within a clinical

trial, we also limited the amount of additional data collected about chronic inflammatory conditions. The proportion of patients with clinically apparent conditions is likely to be low, however. Rates of infection at the time of stroke range from 6% to 25%, depending upon the population studied and methods used to detect infection. (76) (77) (78) (79) Pneumonia is the most common infection, with urinary tract infection next most common. (55) (80) The likelihood of infection at time of stroke is related to stroke severity, (81) (82) however, with a rate of 14% among those with milder strokes in one study. (81) Higher rates of infection were also seen in population-based studies (80) (81) than in secondary analyses of clinical trials, (55) (83) probably because patients in clinical trials tend to have fewer comorbidities. Patients with permanently disabling strokes will be excluded from SPS3, and all patients will have exclusively lacunar infarcts. Rates of infection are therefore likely to be <10%. In addition, most patients will be enrolled at a time after acute infections are likely to have resolved.

Similarly, we are limited to 2 time points (baseline and 1 year) because of the nested design and desire to limit impact on the parent trial. The limited number of time points is also based on the feasibility of completing all blood draws in all participants in LIMITS.

We also limited the number of serum markers to be tested. We recognize that many other markers could be tested, and we may miss testing an important marker. We selected these markers because of their record in the literature as being predictive of incident and recurrent cardiovascular events, our experience performing these tests, cost, and feasibility. We also did not want to increase the likelihood of finding positive associations by chance alone (increased type 1 error). Additional assays can be tested on the stored specimens, however, when funds become available to perform additional hypothesis-driven studies.

In conclusion, LIMITS provides the opportunity for identification of novel risk factors for prognosis after stroke that will help the medical community plan better prevention and treatment strategies. High risk patients may be identified and target areas may be revealed if specific risk factors are found or if they are found to be more important in specific ethnic groups.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Supported by the National Institutes of Health/National Institute of Neurological Disorders and Stroke (NINDS U01 NS038529, NINDS R01 NS050724) and BMS-Sanofi Pharmaceutical Partnership. Dr. Elkind receives honoraria for speaking from BMS-Sanofi Pharmaceutical Partnership.

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Figure 1.

Plot of Power vs. Detectable Hazard Ratio for examining the effect of clopidogrel plus aspirin versus aspirin alone on inflammatory markers in stroke patients.

(a) Solid line represents curve for endpoints of recurrent ischemic stroke

(b) Dashed line represents curve for combined endpoint of ischemic stroke, MI, or death

Table 1**Inclusion and Exclusion Criteria**

Inclusion criteria		
1.	One of the following lacunar stroke clinical syndromes (adapted from Fisher) lasting > 24 hrs:	
	Pure motor hemiparesis (PMH)	PMH with facial sparing
	Pure sensory stroke	PMH with horizontal gaze palsy
	Sensorimotor stroke	PMH with contralateral III palsy
	Ataxic hemiparesis	PMH with contralateral VI palsy
	Dysarthria-clumsy hand syndrome	Cerebellar ataxia with contralateral III palsy
	Hemiballism	Pure dysarthria
2.	Subcortical TIA with positive DWI on MRI	
3.	Absence of signs or symptoms of cortical dysfunction such as aphasia, apraxia, agnosia, agraphia, homonymous visual field defect, etc.	
4.	No ipsilateral cervical carotid stenosis ($\geq 50\%$) by a reliable imaging modality done in an approved laboratory since the qualifying S3, if hemispheric.	
5.	No major-risk cardioembolic sources requiring anticoagulation or other specific therapy. Minor-risk cardioembolic sources will be permitted if anticoagulation is not prescribed by the patient's primary care physician.	
6.	MRI evidence of S3, specifically A and B:	
7.	A. Presence of an S3 (≤ 2.0 cm in diameter) corresponding to the qualifying event (required for all brainstem events) or multiple S3s (the size limit of lacunes has been increased to 2.0 cm to facilitate recruitment).	
	B. Absence of cortical stroke and large subcortical stroke (recent or remote).	
Exclusion criteria		
1.	Disabling stroke (Modified Rankin Scale ≥ 4)	
2.	Previous intracranial hemorrhage (excluding traumatic) or hemorrhagic stroke	
3.	Age under 40 years	
4.	High risk of bleeding (e.g. recurrent GI or GU bleeding, active peptic ulcer disease, etc)	
5.	Anticipated requirement for long-term use of anticoagulants (e.g. recurrent DVT) or other antiplatelets	
6.	Prior cortical stroke (diagnosed either clinically or by neuroimaging), or prior cortical or retinal TIA	
7.	Prior ipsilateral carotid endarterectomy	
8.	Impaired renal function: serum creatinine > 2.0 mg/dl and GFR > 60	
9.	Intolerance or contraindications to aspirin or clopidogrel (including thrombocytopenia, prolonged INR)	
10.	A score < 24 (adjusted for age and education; adapted from Crum et al, 1993; n = 18,056) on the Folstein Mini Mental Status Examination	
11.	Medical contraindication to MRI	
12.	Pregnancy or women of child-bearing potential who are not following an effective method of contraception	
13.	Geographic or social factors making study participation impractical	
14.	Unable or unwilling to provide informed consent	
15.	Unlikely to be compliant with therapy / unwilling to return for frequent clinic visits	
16.	Patients concurrently participating in another study with an investigational drug or device	
17.	Other likely specific cause of stroke (e.g. dissection, vasculitis, prothrombotic diathesis, drug abuse).	

Table 2**Hypotheses of the Levels of Inflammatory Markers In Treatment of Stroke (LIMITS) Study**

The LIMITS study, a multi-center prospective cohort study among survivors of small vessel stroke within the context of the SPS3 randomized trial, is proposed to test the following:

Hypotheses:

- A.** Elevated levels of serum inflammatory markers measured after a first small subcortical stroke increase the risk of (a) recurrent ischemic stroke (IS) and (b) recurrent IS, myocardial infarction (MI), or death.
 - B.** Dual antiplatelet therapy with clopidogrel and aspirin is more effective than aspirin alone in reducing the risk of (a) recurrent IS and (b) recurrent IS, MI and death among those with elevated levels of hsCRP and other inflammatory markers.
 - C.** Elevated levels of serum inflammatory markers measured after a first small subcortical stroke increase the risk of cognitive decline and dementia.
 - D.** Clopidogrel therapy plus aspirin reduces levels of hsCRP and other inflammatory markers more than aspirin therapy alone in patients with a recent history of small subcortical infarcts.
 - E.** Aggressive lowering of blood pressure will reduce hsCRP and other inflammatory markers, as compared to reference normal hypertension regimen.
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