Title: Intranasal Insulin and Post-stroke Cognition: A Pilot Study

Protocol ID: IRB00029022

NCT02810392

Date Approved: 3.15.18

1. SPECIFIC AIMS

Nearly 800,000 people in the U.S. have strokes each year,[1] and about two-thirds of stroke survivors have cognitive impairment, manifested as memory, language, and judgement problems. Cognitive impairment, even after a mild stroke, impairs survivors' return to gainful employment[2] and social function.[3] It also reduces life satisfaction and reintegration to normal living.[4] Importantly, the degree of a stroke survivor's cognitive deficits is a primary predictor of caregiver burden.[5] Post-stroke cognitive impairment at 2 weeks is a significant predictor of long-term functional outcome,[6] and cognitive impairments impede functional outcome and ability to participate in rehabilitation.[7,8] Inflammation is a likely underlying contributor to cognitive impairment.[9,10] Our preliminary data suggest that the inflammatory biomarker, VCAM-1, is linked to post-stroke cognitive dysfunction, specifically memory.

Currently, no treatments exist that boost cognitive recovery during the early post-acute stroke period.[11] Thus, the National Institute of Neurological Disorders and Stroke (NINDS) has listed the study and treatment of vascular cognitive impairment as one of their highest priorities in stroke research. Intranasally-administered insulin (INI) is a promising new therapy for enhancing memory in patients with mild cognitive impairment (MCI) and Alzheimer's disease (AD). Multiple randomized controlled studies performed by our study team have shown that INI at doses of 20 IU BID for a minimum of 3 weeks improved verbal memory and caregiver rating of functional status in patients with MCI and mild to moderate AD.[12,13] The likely mechanisms of benefit are intranasal insulin's ability to restore normal cerebral insulin signaling.[14] Cerebral insulin resistance[15] is associated with inflammation and oxidative stress,[16] and occurs in both AD and post-stroke cognitive impairment.[15] We hypothesize that intranasal insulin administered in the post-acute period will improve cognition in patients with ischemic stroke or intracerebral hemorrhages (ICH) and reduce caregiver burden. Here we have designed an innovative proof-of-concept, feasibility trial to provide pilot data on the effect of intranasal insulin on: 1) stroke survivor cognitive impairment and caregiver burden; and 2) inflammatory biomarkers, including VCAM-1. Thus, our **Specific Aims** are as follows:

Aim 1. To determine whether patients with ischemic stroke or ICH randomized to INI 20 IU BID for 3 weeks have improved cognition, compared to patients who receive intranasal saline. The primary outcome is the composite of (a) memory and executive function z scores, based on a cognitive battery recommended by the Vascular Cognitive Impairment (VCI) Harmonization;[17] and (b) verbal memory, using story recall.[12,13]

Aim 2. To assess the impact of INI vs saline on inflammatory biomarker levels (VCAM-1, TNF-α, TNFRI and II). (DNA)

Aim 3. To measure differences in burden among caregivers of participants in the INI vs intranasal saline groups.

Exploratory Aim. To develop a repository of DNA on participants to assess any genetic differences in responses for those receiving insulin vs saline.

When insulin is delivered intranasally, it binds to receptors in the hippocampus and frontal cortex within 60 minutes without any systemic absorption,[14] does not cause systemic hypoglycemia, and is safe in elderly patients with MCI or AD.[12,13] In our trial, we will prospectively enroll 20 subjects (10 per group) and randomize to intranasal insulin or saline treatment in blocks of 4. All participants will undergo baseline cognitive testing 6 weeks to 6 months post stroke, then receive the assigned treatment for 3 weeks, followed by a 3-week

washout period, with cognitive testing performed after the treatment and washout periods.As recommended by FDA, the first block of 4 randomized patients (2 patients receive insulin) will undergo treatment, and the safety results from this group will be reviewed prior to proceeding with the study.

2. RATIONALE

Post-stroke cognitive deficits are a public health problem. The prevalence of stroke in the U.S. is estimated at 6.7 million, or 2.8% of the population.[1] About 30% to 40% of patients with stroke will have cognitive impairment 3 to 6 months post-stroke,[18,19] which increases after 12 and 24 months.[20] Overall, almost two-thirds of stroke survivors have cognitive impairment – manifested as memory, language, and judgment problems – and almost one quarter have deficits of higher cortical function without any obvious motor, sensory, or visual deficits.[21] Therefore, at least 4 million people suffer from cognitive impairment due to stroke alone. Stroke-related deficits can be an important cause of early-onset cognitive impairment.[22] Cognitive impairment post-stroke impairs survivors' return to gainful employment[2] and social function,[3] and reduces life satisfaction and reintegration to normal living, even after a mild stroke.[4] In stroke patients, both basic and instrumental activities of daily living (IADLs) are directly influenced by cognitive impairment.[7,8] As a result, it is also a major strain on caregivers because the stroke survivor's cognitive deficits are a primary predictor of caregiver burden.[5]

Inflammation and the association with post-stroke cognitive impairment. Inflammation is a likely candidate mechanism for cognitive impairment and for decline over time after stroke, and a variety of inflammatory cytokines have been implicated in baseline and subsequent cognitive decline.[10,23] One study showed that systemic inflammation was associated with worse memory scores in stroke patients, and independently associated with smaller hippocampal volumes on MRI, suggesting that inflammation may lead to cognitive decline via hippocampal pathways.[9]

Effective interventions to treat post-stroke cognitive impairment are lacking. Treatments that could boost cognitive recovery during the early post-acute stroke period are not established.[11] Thus, the NINDS has listed vascular cognitive impairment as one of their highest priorities in stroke research. In an open-label randomized study, citicholine showed a significant benefit in attention-executive function at 6 and 12 months vs. usual treatment, but no difference in memory, language, spatial perception, or motor speed.[24] Three trials of intensive lifestyle and risk factor management to prevent VCI and dementia are ongoing in Europe.[25]

Treatment for post-stroke cognition: overlap with mild cognitive impairment (MCI) and Alzheimer's disease (AD). We have designed an innovative proof-of-concept study to test whether post-stroke cognitive impairment is improved with intranasal insulin. An overlapping mechanism between ischemic stroke and AD is **cerebral insulin resistance**. An area of the brain important in both stroke and AD is the hippocampus. Thus, this study is designed to test mechanisms at work in a defined area of the brain, in both disorders.

Shared pathology involving insulin resistance and tumor necrosis factor (TNF): Cerebral insulin resistance may occur as a result of decreased response in the setting of normal levels of insulin, or the necessity for more insulin to achieve a normal response.[15] Because insulin receptors are densely localized in the hippocampus, and insulin beneficially modulates toxic Aβ amyloid species, reduced levels of insulin and of insulin activity may contribute to the pathology of AD. Insulin modulates the levels of soluble amyloid-β deposits

(endoproteolytic products of amyloid precursor protein) in the hippocampus, which are synaptotoxic.[26] Cerebral insulin resistance is potentiated by excess TNF in the brain. TNF appears to be regulated by gonadotropins, an imbalance of which leads to excess brain TNF levels.[15] This situation, and the subsequent cytokine cascade, may be viewed as the pre-illness step in the development of AD.[15] TNF also has multiple pleiotropic effects in stroke.[27] It initiates the ischemic cascade, and promotes progression of stroke through inflammation, apoptosis, and necrosis. Increased levels of TNF and the subsequent inflammatory responses during and after ischemic stroke may therefore may be a common pathway for the development of cerebral insulin resistance,[15] and cognitive impairment via hippocampal damage.[9] Similarly, this association fits with the vascular contribution toward the development of AD-related cognitive impairment.[28] Insulin resistance is also associated with inflammation and oxidative stress, both important in acute stroke. In a study of stroke patients, elevated IL-6 and reduced IL-10 levels, oxidative stress, and higher NIH Stroke Scale (NIHSS) scores were all associated with insulin resistance.[16]

Shared pathology involving the hippocampus as a target: Cerebrovascular pathology may contribute to the pathology of AD and is thus often referred to as "co-pathology".[28] In addition, the cerebral circulation disturbances related to small vessel disease and hypoxia caused by systemic vascular disease likely lead to hippocampal and brain atrophy noted in stroke patients.[29] In patients who died after large ischemic stroke in the middle cerebral artery (vs age-matched controls), cerebral ischemia led to up-regulation of cyclin-dependent kinase 5 (Cdk5),[30] a pathogenic process also important in AD because of its role in tau phosphorylation and amyloid plaque formation.[31] Another study showed that elevated systemic inflammation in patients with first-ever mild to moderate ischemic stroke was associated with hippocampal atrophy and worse cognitive performance, particularly memory.[9] **These shared pathologies between ischemic stroke and AD suggest the possibility of designing of treatments with benefits to both disorders.**

A novel treatment for post-stroke cognitive impairment. Intranasal administration of insulin is delivered directly to the brain and binds to receptors in the hippocampus and frontal cortex within 60 minutes without systemic absorption.[14] In multiple randomized controlled studies, our team showed that intranasal insulin (20 IU BID to 40 IU BID) for a minimum of 3 weeks improved verbal memory and caregiver rating of functional status in patients with MCI and mild to moderate AD.[12,13] The likely mechanisms of benefit are intranasal insulin's ability to restore normal cerebral insulin signaling, and thus synaptic survival in the hippocampus.[14] **Given the beneficial actions of insulin in the brain in both AD and ischemic stroke, we hypothesize that intranasal insulin administered in the post-acute period will lead to improved cognition in patients with ischemic stroke.**

Post-stroke cognitive problems determine, in large part, both the patient's ability to return to previous activities and the degree of caregiver burden. The mechanisms behind post-stroke cognitive impairment and/or decline are not well-established, but there is an overlap with mechanisms in Alzheimer's disease-related cognitive dysfunction. **Gaps this project fills and relevance to the AHA mission:** Our project builds on what is known about post-stroke cognition and the role of inflammation, and applies a promising and novel intervention for treatment of post-stroke cognitive dysfunction. Multiple overlapping pathologies and pathways, which culminate in abnormal insulin activity in the brain, make the testing of intranasal insulin administration in stroke patients a logical next step. If successful, this study will provide the first evidence that intranasal delivery of insulin could improve cognition if administered soon after a stroke. This is an extremely important and relevant goal matching the AHA mission to improve the lives of stroke patients.

Neurologic deficits of ischemic and hemorrhagic stroke are identical. Patients with hemorrhagic stroke commonly have cognitive deficits post-stroke and are therefore being included in this study.

3. METHODS

Our hypothesis is that INI could improve post-stroke cognitive impairment if administered in the early post-acute stroke phase. The same cohort of participants in this randomized controlled trial will be analyzed for all 3 aims (shown in **Figure 4**).

Methods/procedures common to all aims:

Design: Block randomized, double-blind placebo-controlled trial of 20 patients, 10 per group.
Study population: Ischemic or ICH stroke patients with complaints of cognitive impairment post-stroke and a Montreal Cognitive Assessment (MoCA) score above the 5th but at or below the 50th percentile of norms based on age, race, and education (Table 1) OR a MOCA score of 2 out of 5 in the delayed recall portion and between the ages of 21 and 89 [Dr. Kaycee Sink, personal communication based on >9,000 diverse participant scores]. Patients will be asked permission for the study team to administer the 5-minute MoCA over the phone if they do not have a MoCA documented in their chart, or if they do not have an upcoming appointment where a full MoCA can be performed to assess eligibility. Patients who score ≤21 or ≤2 out of 5 on the delayed recall on the 5-minute MocA over the phone will be eligible.

Table 1. Distribution of MoCA scores for eligibility based on race, age, and education.

	<70 years	70-79	80+
Whites <12 years education			
5th to 50th	17-23	16-22	14-20
Blacks < 12 years education			
5th to 50th	14-20	13-19	11-17
Whites 12 years education			
5th to 50th	18-24	17-23	15-21
Blacks 12 years education			
5th to 50th	15-21	14-20	12-18
Whites >12 years education			
5th to 50th	21-26	19-25	17-23
Blacks >12 years education			
5th to 50th	18-23	16-22	14-20

Inclusion criteria: Patients as above with clinical ischemic stroke or ICH and measurable deficit who are able to sign informed consent, have a caregiver (for patients with deficits that would require them to have assistance with carrying out study tasks), and live within a reasonable driving distance from WFBMC.
Exclusion criteria: Patients under age 21 or 90 years or older, those transferred to skilled nursing facility, or with severe stroke deficits that prohibit participation in cognitive testing (global or receptive aphasia, or severe expressive aphasia,[32] or those with diabetes taking insulin, unstable or severe psychiatric disorders that would impair the ability to undergo cognitive testing; severe head trauma, alcoholism, neurologic disorders other than stroke, end stage kidney disease that requires dialysis, acute renal failure that is causing delirium or other kidney disease that would impair the ability to undergo cognitive testing, end stage liver disease that is associated with hyperammonemia or metabolic encephalopathy, chronic obstructive pulmonary disease severe enough that the patient has known hypoxia or hypercarbia, is on chronic oxygen or their lung function is severe enough to impair the ability to undergo cognitive testing; unstable angina or poorly controlled

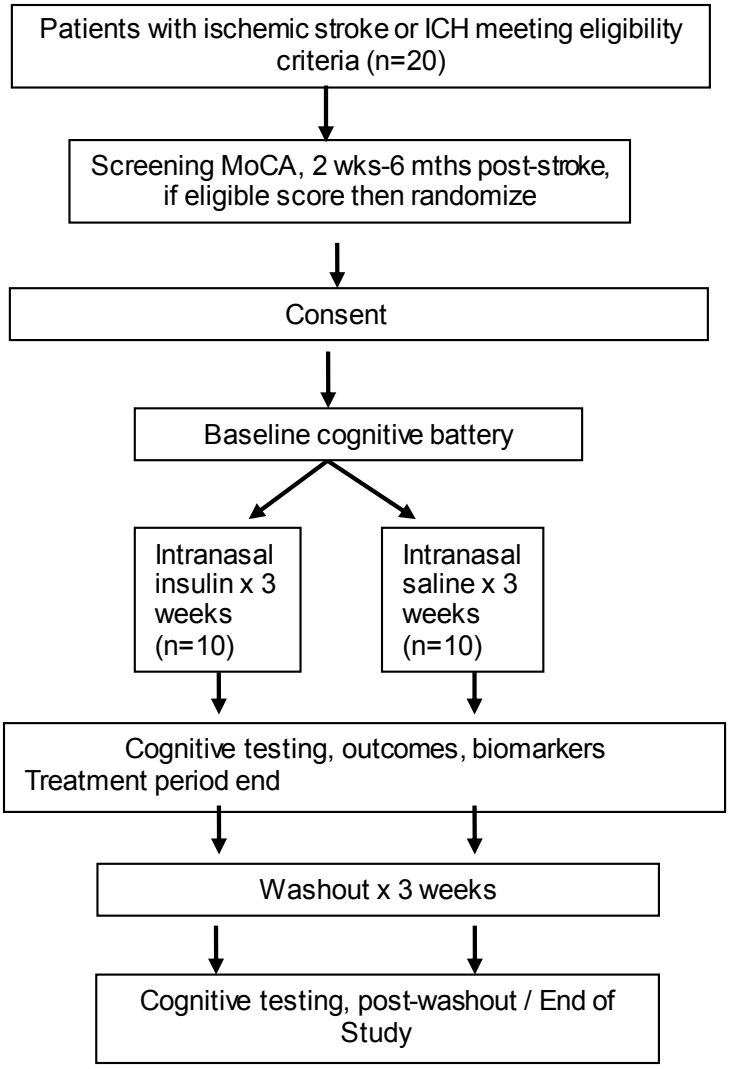


Figure 1. Flow diagram of the study procedures

heart failure that would limit the patient's ability to undergo cognitive testing,[12] and those with prior deficits in ADLs. We have not pre-specified a maximum NIHSS at onset for exclusion, since waiting for at least 6 weeks allows patients with severe deficits to recover naturally, and still potentially be able to participate. It also allows most patients to complete inpatient rehabilitation.

Recruitment: WFBMC admits approximately 460 ischemic stroke patients and approximately 400 ICH patients per year. We anticipate 40% (n=270) of these patients will be eligible after excluding for aphasia, severe stroke, and insulin-dependent diabetes; of those, a pool of 180 patients per year could be enrolled. Patients will be identified while hospitalized on the WFBMC stroke service. Recruitment strategies have been developed to identify and approach potential participants. The study coordinator will screen the stroke service census and communicate with the study team if a potential patient is identified. Patients that are deemed 1.) not eligible at the time of initial screening and 2.) do not meet exclusion criteria, will be followed and approached when/if they become eligible. An invitation letter and/or recruitment phone call script will be used if patients were not able to be approached during their inpatient stay or follow up clinic visit. Patients may also be approached by phone and/or during other WFBMC clinic visits (i.e. rehab). Any MoCA performed as part of inpatient or outpatient assessments prior to enrollment will be used for screening eligible patients. If there is no recent MoCA assessment, the screening MoCA will be performed by the study team either during the phone screen or during a visit day and time that is convenient for the patient. If the patient scores within the range of eligibility OR a score of ≤2 out of 5 in the delayed recall portion of the MoCA, the coordinator will obtain consent from the patient or a legal representative, and the initial cognitive testing/blood draw will be scheduled. We will aim to enroll patients that are between 6 weeks and 6 months post-stroke onset. We will also advertise the study on the WFBMC web site, in local newspapers, and possibly TV and radio. Patients may also be referred to the study from local Neurology practices. The recruitment letter and recruitment phone call script and phone screen MoCA have been written to allow patients outside of WFBH to contact the study team.

Randomization and Blinding: The participant will be randomized and undergo the full baseline cognitive battery. Caregivers will also sign a separate consent to complete the Caregiver Strain Index. Subjects will be randomized to intranasal insulin (20 IU BID) vs saline BID according to a stratified permuted block randomization of 4 as per our statistician, stratified by age 40 to 69 years vs ≥ 70 years) and presence of language deficit (based on NIHSS) at the time of randomization. Insulin and saline will be packaged in identical single-dose ampules that will be opened and inserted into the chamber of the Vianase™ device. Ampoules will be dispensed in 3 week supplies at each study visit. Package labels will instruct participants to administer each dose 30-60 minutes after breakfast and dinner. If a dose is missed, it will not be replaced. The insulin and saline will be identical in packaging except for randomization code. Patients/caregivers, investigators, and outcome assessors will be masked to the group assignment. All subjects will receive their first dose in the clinic and wait for 30 minutes to determine any adverse effects of the inhaled dose, while remaining blinded to the treatment. The glucose level will be measured with the glucometer to monitor for hypoglycemia and documented. All subjects will additionally check peak dose blood sugar levels with a glucometer, 3 times per week during insulin or saline treatment. The patients and caregivers will receive a handbook which describes the method for loading the device, inhalation, methods for fingersticks, use of the glucometer and test strips, a blood sugar log, and the signs and symptoms of hypoglycemia. They will also document any other adverse effects on the weekly log.

Timing for the procedures (Figure 1). Cognitive impairment measured early after stroke predicts functional outcomes in stroke patients at 13 months.[6] Furthermore, waiting until 6 weeks after the stroke to conduct baseline cognitive testing and treatment allows enough recovery and completion of intensive rehabilitation for those patients who may not be able to complete cognitive testing immediately after stroke, but takes advantage of the potential benefits of insulin in the early phase, shown in animal studies.[33] In addition, we potentially reduce the variability of the biomarkers by avoiding the acute phase of stroke. The frequency of cognitive testing is based on trials of intranasal insulin for MCI and AD.[12,13]
Patients with a history of psychiatric medication use for the management of mental and emotional disorders prior to their stroke will not be excluded from the study. However, as these medications may have an effect on cognitive testing, initiation of psychiatric medications should be avoided from the time of study enrollment through the post wash out period unless it is clearly indicated to be in the patient's best interest. Patients will be informed of the prohibited medications during consent and will be asked to immediately inform the study team of any new medications or changes in existing medications.

Baseline Data collection. We will collect the baseline data described below as in the VCI Harmonization standards:[17]
Demographics: Birth date, sex, race/ethnicity, years in current country of residence, number of years of education, occupation, literacy, living situation and level of independence, type of residence, marital status, and name and contact information for a family member or caregiver will be collected.
Proxy/informant information collected: Birth date, sex, race/ethnicity, relationship and length of time of relationship with the patient, education, and living status with respect to the subject.
Family history information collected: First-degree relatives with stroke, myocardial infarction, and dementia, approximate age at onset, and age at death.
Stroke hospital data collected: Severity (NIHSS) at onset, location of stroke, vascular territory, modified Rankin score at discharge, short physical performance battery score, length of stay,

stroke complications, stroke prevention medications at discharge, stroke risk factors (diabetes, hypertension, hyperlipidemia, smoking, alcohol/substance abuse, atrial fibrillation, carotid stenosis, hypercoagulable state), and stroke subtype (NINDS classification).

Intranasal insulin administration: We will use an innovative investigational device developed by Kurve Technology (Fig 5A). Typical spray bottle administration results in large droplets that penetrate only within the first 20% of the lower nasal cavity, and due to gravity and insufficient airflow, ~90% of the droplets wind up in the stomach (Fig.5B). The ViaNase™ device delivers a substance throughout the nasal cavity, to the olfactory region and paranasal sinuses, thereby maximizing access to nose-to-brain channels (Fig.5C). This distribution occurs because droplet size is adjusted according to the weight of the substance, through an individually optimized droplet generator resulting in maximal vortical distribution (Fig.5D). We have used the ViaNase™ device with excellent results in two pilot trials (described above).[12,13] A total volume of 20IU of insulin or placebo (saline) will be administered each time. Caregivers will supervise participants in administering intranasal treatment 2 times per day, after breakfast and dinner. Participants and caregivers will be trained in use of the delivery device. In previous studies, participants with aMCI/AD have found the device to be easy and pleasant to use, with compliance rates of 95 to 97%.

Cognitive battery:

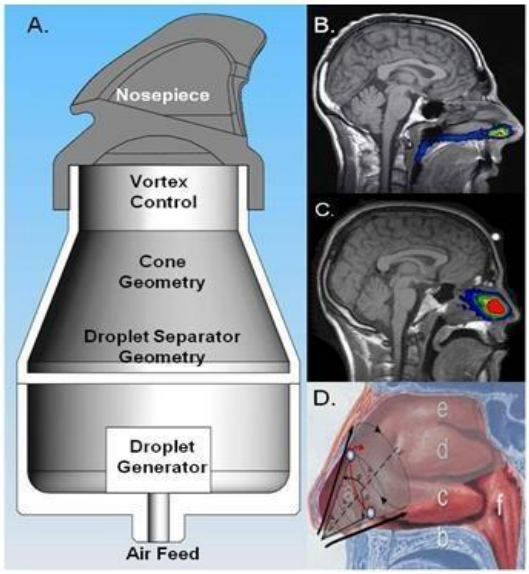
Aim 1 focuses on two primary outcomes: (a) cognitive tests, including a summary memory score and a summary executive function score, and (b) the story recall test, which was sensitive to intranasal insulin effects in MCI/AD patients within 3 weeks of treatment.[12] Since participant fatigue can be a major factor with these cognitive tests in stroke participants, we have designed a testing administration procedure that can be completed in fewer than 40 minutes. Our battery and time for administration include:

Montreal Cognitive Assessment (MoCA):[34] The MoCA is a cognitive screening instrument which provides information about overall mental status. It is useful for identifying overall cognitive impairment, and also provides information about core cognitive domains, such as visuospatial abilities, attention, and memory.

Memory Tasks.

Hopkins Verbal Learning Test-Revised (HVLT-R)[35] is a measure of rote verbal learning and memory. After a 20-minute delay, participants are asked to name all items in a 12-item word list they recall. There are 6 lists available to facilitate repeat administration in future testing (10 minutes with delay and recognition).

Figure 5. (A) ViaNase™ device schematic; (B) Particle dispersion with typical spray bottle application; (C) Particle dispersion with ViaNase™ application; (D) Schematic showing ViaNase™ vortical distribution.



Brief Visual Memory Test-Revised (BVMT-R)[36] is a measure of nonverbal learning and memory captured with immediate and delayed free recall trials, and a recognition memory task (10 minutes with delay and recognition).

Executive Function Tasks

Trail Making Test-A & B (public domain): Part A is a measure of visuomotor speed and sequencing, where the subject is asked to connect numerical dots (1-25) in ascending order as quickly as possible. Part B, administered immediately afterwards, is a measure of executive functioning (working memory, mental flexibility) whereby the subject must connect dots to sequence consecutive

numbers and letters (1, A, 2, B, etc.→12, L, 13). Each task is scored in terms of time to completion, and number of errors (4 to 7 minutes).

WAIS-III Digit Span subtest[37] is comprised of two portions. Digits Forward is a measure of simple attention that requires the participant to repeat increasingly longer strings of digits in order, starting with 2 digits and progressing to 9. The task is discontinued when both strings of a specific length are incorrect. Digits Reverse is considered a measure of working memory, and requires repetition of increasingly longer strings of digits in the reverse order, starting with 2 and progressing to 8 (1 to 5 minutes).

WAIS-III Digit-Symbol Coding.[37] A sensitive measure of visuomotor processing speed, this task requires involves rapidly coding geometric symbols given a number, by using a legend of number-symbol pairs at the top of the page (2.5 minutes).

Verbal fluency: Animal naming and FAS (public domain): These tasks of verbal fluency provide the subject one minute to say as many words as possible. For Animal Naming, any living creature is counted (1.25 minutes). For FAS, words beginning with a given letter (F, A, and then S) are counted excluding proper nouns, numbers, and variations of the same word (3.5 minutes).

Story Memory recall. A narrative of 44 informational bits is read and the recalled information is recorded immediately and after 20 minutes. Information retained over the delay will be calculated as delayed recall/ immediate recall. The participant is asked to recite it immediately, and then following a 20-minute delay. This measure of verbal memory is less affected by executive difficulties experienced in those with history of stroke or vascular disease. Multiple passages exist to allow for repeat testing.[12] Total time for administration is 20 minutes with delayed recall.

Other outcome scales:

Functional outcome Instrumental Activities of Daily Living (IADL) scale: We will use the Stroke Impact Scale ADL/IADL scale,[38] and the SIS-16,[39] both of which has been extensively used and validated in the stroke population. This scale is based on self-reported information about functional skills necessary to live in the community, is easy to administer, can be administered by proxy, and takes about 10-15 minutes.[40]

Modified Caregiver Strain Index: This survey includes 13 questions related to caregiver strain (sleep, finances, time, inconvenience, etc.); a score of 7 or greater is considered significant caregiver strain.[41] Multiple studies of stroke and the elderly have measured caregiver burden with this scale.[42,43] Coordinators will administer the scale to caregivers separately from the stroke patient.

Table 2. Measures and the timing of assessments

Measure/Task	Study start	Treatment period end	3 wks post-washout End of Study
Randomization	X		
Fasting glucose and insulin	X	X	
Cognitive testing	X	X	X
Depression (PHQ-9)	X	X	X
Biomarkers	X	X	
DNA collection	X	X	
Caregiver strain index	X	X	X
IADL and SIS-16	X	X	
Modified Rankin score (mRS)	X	X	

Blood collection for cytokines and DNA: Following consent, blood will be collected from fasting subjects on the day of baseline cognitive testing. One tube will be used to measure basal levels of cytokines; this blood will be processed immediately and stored at -80°C. One additional tube will be used for DNA analysis; the blood will be placed in a refrigerator (4°C) until personnel from the Center for Genomics & Personalized Medicine Research can transport the

sample to the laboratory. At the end of the study, all frozen blood samples collected pre- and post- insulin treatment will be shipped on dry ice to Assaygate (Ijamsvsille, MD) for cytokine quantification of VCAM-1, TNFα, sTNFRI, sTNFRII.

Cytokine quantification: All samples in the study will be stored and sent for analysis in one batch. Plasma cytokine concentrations will be quantified by custom bead-based suspension array for human cytokines by Assaygate as previously described.[44] Assaygate provides multiplex protein analysis services for a wide range of human and mouse inflammatory proteins.

DNA analysis: Genomic DNA will be isolated from whole blood (yellow-top, ACD tubes). DNA will be isolated using the AutoPure LS (Qiagen, Inc.), and then bisulfite-converted using the EZ DNA Methylation Gold kit (Zymo, Irvine, CA). To determine the proportion of DNA methylation at each of over 485,000 CpG sites, we will use the HumanMethylation450 BeadChip (Illumina, Inc.) along with the iScan Reader (Illumina, Inc.).

Aim 1 Analysis

Cognitive Analysis: The analysis will be based on intention to treat. The primary outcomes are the executive function (Trails B, Digit Symbol, Digit Span) and Memory (HVLT and Brief Visual Memory Test-Revised) composite z scores at 3 weeks of treatment in both groups.

Secondary outcomes: Story Recall and MoCA score (out of 30), and IADLs. Pre-specified confounders include age, sex, education, presence of language deficit, and depression, measured with the Patient Health Questionnaire-9 (PHQ-9). The PHQ-9 has been validated as a measure of depression severity (PHQ-9)[45] including stroke,[46] and these measures will be obtained from the stroke subject (not proxy).

IADL analysis: The summary scores of the SIS IADL scale and SIS-16 will be compared from baseline to 3 weeks post treatment in both groups.

Functional covariates: Disability and physical function measures will be collected to understand the trajectory of motor recovery and will be mapped to the cognitive trajectory from baseline (2 weeks to 20 weeks post-stroke). *Modified Rankin Scale (mRS)*: A disability/dependence scale designed as an outcome measure for stroke,[47,48] and is now the most commonly used endpoint in therapeutic clinical trials of stroke interventions, such as thrombolytic therapy.[49] The score ranges from 0 (no symptoms at all) to 6 (death). Exploratory analyses will be performed to determine whether INI leads to an improvement in IADLs, SIS-16, and mRS.

Aim 1 Statistical Analysis: The 3 week intervention effect of INI on the cognitive outcomes will be assessed using analysis of covariance (ANCOVA) adjusting for age, language deficit, and baseline cognitive score. We will assume a Type I error rate $\alpha = 0.05$ for all analyses. Adjusting for baseline characteristics will help attenuate the potential effect of differential dropout,[50] but we will conduct a sensitivity analysis using multiple imputation to ensure dropout does not unduly affect our results.[51] The observed estimates, standard deviations, and dropout rates will be used for the design of future studies of INI treatment for stroke patients.

Exploratory analyses will examine response by subgroup for evidence of an enhanced treatment effect, including subgroups defined by participant age (<70 vs. ≥70 years), baseline cognitive impairment and the presence or absence of any degree of language deficit (aphasia). Covariates will be utilized so that they do not overlap with clinical subgroups. Because of the relatively small sample size, if the prespecified subgroups based on continuous characteristics are not sufficiently large, new clinically meaningful subgroups may be formed for exploratory analyses.

Power Analysis: The primary purpose of the pilot study is to demonstrate feasibility and safety. The proposed study will collect data to estimate power in future trials, identify instruments most

sensitive to the proposed cognitive effects, and anticipate recruitment and retention rates in the target population. However, the proposed study will have 83% power to detect a 1 standard deviation difference in cognitive scores between groups at 3 weeks (i.e. effect size = 1.0), assuming normally distributed outcomes, $\alpha = 0.05$, and a 10% dropout rate.

Potential Problems and Alternative Strategies

1) Recruitment is slower than anticipated. Recruitment will be monitored very carefully to ensure we are on target with the study timeline. However, if distance from the medical center is the only reason that some patients are excluded, we will amend the protocol to include in-home cognitive testing. We will also extend recruitment to Moses Cone, advertise in the Greensboro newspaper, and the local radio stations.

2) Study compliance may be lower than anticipated. We will consider additional phone monitoring and problem-solving with participants to overcome compliance barriers. Compliance was 95% to 97% in all prior studies.

Aim 2 Rationale

Aim 2 will test the hypothesis that INI's effects on post-stroke cognition will suppress proinflammatory cytokines and enhance immune responsiveness. In addition to improving cognition, INI down-regulates systemic inflammation associated with obesity and diabetes by altering hypothalamic insulin growth factor signaling mechanisms. We hypothesize that INI will improve post-stroke systemic inflammatory responses by similar mechanisms.[52–54] Our primary goal in Aim 2 is to assess differences in inflammatory biomarkers between the two groups. The secondary goal is to assess the relationship between these biomarkers and the primary cognitive outcomes (memory and executive function z scores, and story recall). We will also assess whether the relationship between biomarkers and cognition is mediated by the treatment group.

Biomarker panel: *VCAM-1*, which is exclusively expressed in endothelial cells, is induced by TNFα and IL-1.[55] VCAM-1 levels likely reflect advanced atherosclerosis and endothelial dysfunction.[56] *TNFα* is the putative cause of cerebral insulin resistance,[15] one of the presumed mechanisms through which INI may improve memory, and was associated with cognitive impairment in the Framingham cohort.[57] The soluble TNF receptor I (*sTNFRI*) was associated with stroke mortality in the Northern Manhattan study;[58] therefore, we will measure sTNFR I and II as a reflection of TNF pathway activity.

Approach and outcomes: Biomarkers and DNA will be measured 4weeks- 3 months after stroke onset (at baseline cognitive testing) and at 6-9 months post stroke.

Aim 2 Statistical Analysis. All biomarker assay results will be based on mean values of duplicate assay measurements. The distribution of each biomarker will be assessed, and log-transformations will be performed as appropriate. Non-parametric tests will be used to compare medians (Wilcoxon rank sum or Spearman rank correlation) and parametric tests (Student t test, Pearson correlation) to compare means. We will assess the randomization effect using the same ANCOVA model described for Aim 1, adjusting for randomization allocation factors and baseline scores. When log-transformed biomarkers are used, the treatment effect will be assessed on the log-scale values. We will test for the association between cognition and serum biomarkers, both using the composite memory and executive function z scores and each of the component parts, as well as story recall and MoCA scores. We will use a linear regression approach adjusting for age, education, and baseline cognitive scores. Secondarily, we will perform a mediation analysis to determine the potential role of intranasal insulin in the relationship between inflammatory biomarkers and cognition relationship. The long-term cognitive randomization effect will be explored using a mixed

model analysis controlling for within-subject variability including both the post-intervention and long-term follow-up time points, further adjusting for randomization factors and baseline cognitive score. Treatment effects at 20 weeks will be assessed using contrast statements. The results of these analyses will provide a new understanding of the relationships between inflammatory biomarkers and cognitive outcomes in stroke patients. These results can then be used to study a broader population of cognitive impairment after stroke and/or VCI.

Potential problems and alternative strategies:

1) Multiple comparisons of biomarkers, outcomes, and covariates could be an issue, but the pilot nature of this small study does not require adjustment of the significance.
2) Technical issues: Dr. Vidula Vachharajani has extensive experience in sample preparation, shipment, data analysis and interpretation. Assaygate's methods for cytokine detection are highly sensitive and published performance characteristics show a low coefficient of variation. Thus, we do not anticipate any problems executing these studies.

Aim 3 Rationale

For caregivers of stroke patients, more than 60% of caregivers have significant strain at 28 days after discharge, although this decreased over the 1 year after stroke.[59] The goal of Aim 3 is to assess whether intranasal insulin treatment improves cognitive impairment after stroke, and also improves caregiver burden. This is an important outcome because a similar caregiver rating of functional status in the pilot studies of intranasal insulin for MCI/AD was significantly improved with this treatment.[12,13]

Aim 3 Analysis

For each outcome, we will analyze mean difference from baseline to post-treatment and other time points; therefore, two-sample t-tests will likely be used to compare mean differences in the 2 treatment groups. Alternatively, the outcomes could be categorized as positive, negative, or no change using a nonparametric approach. Specific analyses will include the following comparisons in the 2 groups: CSI post-treatment , and atend of study, before and after adjusting for baseline CSI, NIHSS, and cognitive scores. We will perform multivariate linear regression analyses of each outcome adjusting for treatment group, age, sex, race, stroke severity, and co-morbidities (e.g. diabetes, hypertension, atrial fibrillation, CAD, CHF) at baseline.

Potential Problems and Alternative Strategies

Retention of caregivers will be a potential issue, and the goal is to identify a primary caregiver at the start of the trial and continue measures with the same caregiver throughout the trial. However, caregivers may change during the trial due to unforeseen circumstances. We will therefore identify a secondary caregiver at enrollment for each stroke participant.

Exploratory Aim Analysis

The DNA will be processed and stored until additional funding is obtained to cover the costs of the analyses. Participants will be given the opportunity to opt out of this portion of the blood collection on the consent form. When funding has been obtained, we will submit an amendment to the protocol.

Table 3. Timeline for the pilot study and procedures

	Y1			Y2	
Start-up					
Enrollment					
Data collection					

Biomarker analyses					
Analysis, manuscript prep					

Ethical aspects of the application Safety monitoring phone calls will occur at weeks 1 and 2 to ensure proper administration techniques, monitor blood glucose changes, and assess for any early adverse events. In-person visits will occur at each cognitive testing time frame as shown in Table 1. Potential risks with intranasal insulin: There are no known serious risks associated with INI without absorption enhancers as will be administered in our study. In our prior studies of INI, there were no episodes of severe hypoglycemia, and no adverse effects or functional disturbances following intranasal insulin administration. No other serious adverse effects of treatment were observed.[12,13] It is possible, albeit unlikely, that increased glucose levels may occur. If participants show fasting glucose levels above 126 mg/dl or random glucose levels above 200 mg/dl on two occasions, they will be referred to their primary care physician for evaluation. We will use the standing institutional Data Safety Monitoring Committee (DSMC). The investigators and the DSMC will develop a plan for interim data analyses to monitor adverse events and preliminary analyses.

Summary and future research
Our translational pilot study will provide important and highly relevant preliminary data on a novel therapy, INI, for cognitive improvement after ischemic stroke or ICH, while measuring physical function simultaneously. We will accumulate early efficacy data, in addition to feasibility and safety in stroke patients. At the same time, we will determine the impact of the intervention on systemic inflammation using markers that we and others have shown to influence post-stroke cognition (VCAM-1) and cerebral insulin resistance (TNF). This project will be the first step to addressing 1) a significant need in the quality of life of ischemic stroke patients, ICH patients and their caregivers, and 2) a research priority for AHA and NINDS, and will therefore form the basis for larger therapeutic trial applications. Our multidisciplinary team includes a vascular neurologist focused on stroke outcomes, a neuroscientist with expertise in neuro-inflammation, a cognitive neuroscientist with expertise in INI trials, and a statistician with experience in stroke and aging clinical trials. We therefore have the expertise to conduct and complete this research and make a contribution to the lives of stroke survivors and their caregivers.

4. PROTECTIONS FOR HUMAN SUBJECTS

4.1 Risks to Human Subjects

There are 3 potential risks of participation in this trial. 1) Hypoglycemia: Insulin given intranasally with the nose-to-brain device used in these studies has not been associated with low blood sugar in previous studies. In diabetic patients on oral hypoglycemic, we do not expect any additional risks. In a recent study testing intranasal insulin in this population, Intranasal insulin administration appeared safe and did not affect systemic glucose control. [60] Nevertheless, the study staff will train participants and caregivers to recognize signs of low blood sugar such as shaking, fast heartbeat, sweating, dizziness, anxiousness, hunger, impaired vision, weakness, headache, and irritability. If the participant develops these symptoms, they will be instructed to eat a snack, call the study staff at (336) 716-4101 and call his or her physician. 2) Nasal irritation: A few participants in previous studies complained of nasal irritation after being given the study drug, but these symptoms did not persist beyond the initial use of the study drug. As with any drug, there may be unexpected side effects to nasal insulin. Although insulin has been safely administered intravenously, the potential toxicity of

the proposed clinical formulation to the nasal cavity and other locally exposed tissues has not been evaluated in animal studies. 3) Blood Draw: There is no additional risk associated with the blood draw for this study compared with routine clinical care. All blood draws will be performed by study personnel in a monitored setting (Neurology clinic or the CRU).

There are no risks to performing the cognitive testing, although participants may become fatigued. If this occurs, the participant can stop testing at any time. Participants may experience some distress if they are aware of the results of the caregiver strain index (CSI), completed by their caregiver. In addition, caregivers may not feel comfortable providing truthful answers to the questions in the presence of the participant. Therefore, the CSI will be completed on paper by the caregiver while the stroke participant is completing the cognitive testing.

5. RISK PROTECTION

We have minimized the risk of adverse effects from the inhaled study drug by monitoring participants in the clinic or CRU for at least 30 minutes after the first dose. Also, we will review the blood sugar logs of the first block of 4 participants (2 insulin and 2 saline) prior to enrolling the next block (as per FDA recommendations). In addition, the participants will monitor peak dose blood sugars 2 to 3 hours after the study drug and record blood sugars at least 3 times per week for the first week of the study treatment.

To minimize the number of sticks for blood draws, blood collection will occur at the same time as a scheduled blood draw as often as possible. To minimize the possible psychological stress of the participant's caregiver in completing the CSI, this will not be shared with the stroke survivor unless the caregiver allows sharing of the index with the participant.

Potential benefits to the subjects and others

There are two potential benefits to participation in this study. If there is indeed an improvement in cognition with intranasal insulin, the subjects in this group will reap the benefits of improved memory, cognitive function, and presumably, better quality of life and functional status. All participants will likely benefit from contact with study personnel who will be able to monitor for any changes in their functional status or depression after stroke and provide referrals, as needed.

5.1. Data Safety Monitoring Board

All adverse and serious adverse events will be monitored by the study coordinator and reported to the WFUHS IRB and the Data Safety Officer, William Applegate, MD. Each event will be reviewed to determine whether it may be related to the intranasal insulin, to the natural history of an individual's stroke, or to other existing conditions. The safety officer will have scheduled assessments of the data from the trial, after the first block of 4 randomized subjects, after 20 subjects, and then at the end of enrollment of 40 subjects (about 18 months total). In compliance with the FDA IND, we will submit all adverse events to FDA and a yearly progress report within 60 days of the "may proceed" date of October 17, 2014.

6. INFORMED CONSENT & HIPAA AUTHORIZATION FORM

All study personnel who screen medical records will have completed Human Subjects Protection Education, have secure access to records, and have completed the WFBMC security and data protection requirements for accessing patient records. All admissions to the

WFBMC stroke service will be screened by the study coordinator and/or PI for eligibility. Eligible participants must be at least 6 weeks post-stroke. After a potential participant is identified, the study coordinator will contact the potential participant, explain the study and provide him or her with a copy of the consent and other study documents. If the patient chooses to participate, written consent will be obtained prior to randomization. All participants (and their caregivers) will sign the consent before they are enrolled in the study. The consent form will be approved by the WFUHS Institutional Review Board prior to study start.

6.1. Confidentiality and Privacy

Personal information and such information that directly identifies the participants will be collected during this study. This includes names, addresses, telephone numbers and dates of birth. Personal health information includes all health information created as related to the study and information that is maintained in the medical records at this institution. Examples of this personal health information include: health history, family health history, how the participant responds to study questions, and information obtained during study visits and phone calls.

All study information will be secured under lock and key by the program manager and the primary investigator at WFUSM will be responsible for maintaining this secure file of the study information. We will emphasize to subjects that our interest is in average responses as opposed to individual data. No individual data will be released without prior consent of the individual(s) in question. All data collected will be coded with all HIPAA identifiers removed and transferred electronically using encrypted data transfer techniques. We will abide by recently published HIPPA regulations, which are part of the review process for Human Subjects Research at our institution. The signed consents and information that directly identifies the participants (names, addresses, phone numbers and other contact information required for the follow-up telephone calls and for mailing additional information to participants) will be maintained separately from the Case Report Forms and the institutional IRB approval documents.

The Case Report Forms will identify participants by a unique study ID and the list that links the study ID to the personal information will be maintained in a separate file from the Case Report Forms. Paper copies of the data forms will be stored in a secure, locked location and all data collected as part of the study will be entered into a database to which only specific study personnel will be allowed access. This system is encrypted and password protected. Data will be uploaded into a web-based database housed within Public Health Sciences and maintained by the statistician (Dr. Beavers). Data entry is completed through a secure, encrypted login, by the study coordinator. Data elements are stored in a secure database with access limited to user access. Data will be stored for at least six years following completion of the study. No personal information that directly identifies the participant (name, address, phone number) will be entered into the study database. Participants will not be directly identified in any publication or presentation that may result from this study.

The subject, study coordinator, PI, and study personnel will be blinded to the intervention assignment until the end of the study. The biostatistician will be the only unblinded study team member, and will be in charge of generating the reports for the DSMB review. The study coordinator will maintain frequent contact with each participant throughout the treatment period.

If necessary, the personal health information and/or information that identifies the participants will be shared with the WFSM IRB, sponsor representatives at the American Heart Association, FDA and/or representatives from government agencies such as the Department of Health and Human Services (DHHS) for the purposes of: determining the results of the study, making sure that the study is being conducted correctly or providing the required reports.

Importance of the knowledge to be gained

From this trial we will determine whether intranasal insulin leads to cognitive improvement vs. placebo, with memory and executive function composite scores as the primary outcomes. This study is important because there are currently no other therapies available to improve post-stroke cognition. The data obtained in this trial will be used to design a larger clinical trial that will provide more definitive evidence of benefit and perhaps FDA approval for the indication of cognitive recovery after stroke.

7. INCLUSION OF WOMEN & MINORITIES

Based on the average distribution of gender in our stroke population at WFBMC, we anticipate about half of the participants will be women, and 25% will be African American. Less than 1% of our stroke population is of Hispanic ethnicity, so we do not expect to enroll more than 1 in this trial. Children will not be enrolled because the age requirement is over age 18 years.

8. PARTICIPATION OF ADULT WOMEN WHO ARE PREGNANT OR POSSIBLY PREGNANT

We will not recruit any women who are pregnant or who believe they may possibly be pregnant into the proposed trial due to insufficient research on the safety profile of intranasal insulin on pregnant women. Pregnancy tests are administered as standard of care at hospital admission to women of childbearing age who suspect that they may be pregnant or could have become pregnant. Our rationale for omitting children is that they would require an entirely different intervention.

Literature Cited

1. Go AS, Mozaffarian D, Roger VL, Benjamin EJ, Berry JD, Borden WB, Bravata DM, Dai S, Ford ES, Fox CS, Franco S, Fullerton HJ, Gillespie C, Hailpern SM, Heit JA, Howard VJ, Huffman MD, Kissela BM, Kittner SJ,

Lackland DT, Lichtman JH, Lisabeth LD, Magid D, Marcus GM, Marelli A, Matchar DB, McGuire DK, Mohler ER, Moy CS, Mussolino ME, Nichol G, Paynter NP, Schreiner PJ, Sorlie PD, Stein J, Turan TN, Virani SS, Wong ND, Woo D, Turner MB. Heart Disease and Stroke Statistics—2013 Update A Report From the American Heart Association. *Circulation*. 2013;127:e6–e245.

2. Hofgren C, Björkdahl A, Esbjörnsson E, Stibrant-Sunnerhagen K. Recovery after stroke: cognition, ADL function and return to work. *Acta Neurol. Scand.* 2007;115:73–80.

3. Hommel M, Miguel ST, Naegele B, Gonnet N, Jaillard A. Cognitive determinants of social functioning after a first ever mild to moderate stroke at vocational age. *J. Neurol. Neurosurg. Psychiatry*. 2009;80:876–880.

4. Edwards DF, Hahn M, Baum C, Dromerick AW. The Impact of Mild Stroke on Meaningful Activity and Life Satisfaction. *J. Stroke Cerebrovasc. Dis.* 2006;15:151–157.

5. Rigby H, Gubitz G, Eskes G, Reidy Y, Christian C, Grover V, Phillips S. Caring for stroke survivors: baseline and 1-year determinants of caregiver burden. *Int. J. Stroke Off. J. Int. Stroke Soc.* 2009;4:152–158.

6. Wagle J, Farner L, Flekkøy K, Bruun Wyller T, Sandvik L, Fure B, Stensrød B, Engedal K. Early Post-Stroke Cognition in Stroke Rehabilitation Patients Predicts Functional Outcome at 13 Months. *Dement. Geriatr. Cogn. Disord.* 2011;31:379–387.

7. Zinn S, Dudley TK, Bosworth HB, Hoenig HM, Duncan PW, Horner RD. The effect of poststroke cognitive impairment on rehabilitation process and functional outcome. *Arch. Phys. Med. Rehabil.* 2004;85:1084–1090.

8. Patel M, Coshall C, Rudd AG, Wolfe CD. Natural history of cognitive impairment after stroke and factors associated with its recovery. *Clin. Rehabil.* 2003;17:158–166.

9. Kliper E, Bashat DB, Bornstein NM, Shenhar-Tsarfaty S, Hallevi H, Auriel E, Shopin L, Bloch S, Berliner S, Giladi N, Goldbourt U, Shapira I, Korczyn AD, Assayag EB. Cognitive Decline After Stroke Relation to Inflammatory Biomarkers and Hippocampal Volume. *Stroke*. 2013;44:1433–1435.

10. Rafnsson SB, Deary IJ, Smith FB, Whiteman MC, Rumley A, Lowe GDO, Fowkes FGR. Cognitive Decline and Markers of Inflammation and Hemostasis: The Edinburgh Artery Study. *J. Am. Geriatr. Soc.* 2007;55:700–707.

11. Hoffmann T, Bennett S, Koh C-L, McKenna K. A Systematic Review of Cognitive Interventions to Improve Functional Ability in People Who Have Cognitive Impairment Following Stroke. *Top. Stroke Rehabil.* 2010;17:99–107.

12. Reger MA, Watson GS, Green PS, Wilkinson CW, Baker LD, Cholerton B, Fishel MA, Plymate SR, Breitner JCS, DeGroodt W, Mehta P, Craft S. Intranasal insulin improves cognition and modulates β-amyloid in early AD. *Neurology*. 2008;70:440–448.

13. Craft S BL. Intranasal insulin therapy for alzheimer disease and amnestic mild cognitive impairment: A pilot clinical trial. *Arch. Neurol.* 2012;69:29–38.

14. Francis GJ, Martinez JA, Liu WQ, Xu K, Ayer A, Fine J, Tuor UI, Glazner G, Hanson LR, Frey WH, Toth C. Intranasal insulin prevents cognitive decline, cerebral atrophy and white matter changes in murine type I diabetic encephalopathy. *Brain*. 2008;131:3311–3334.

15. Clark I, Atwood C, Bowen R, Paz-Filho G, Vissel B. Tumor Necrosis Factor-Induced Cerebral Insulin Resistance in Alzheimer's Disease Links Numerous Treatment Rationales. *Pharmacol. Rev.* 2012;64:1004–1026.

16. Ozkul A, Ayhan M, Akyol A, Turgut ET, Kadikoylu G, Yenisey C. The effect of insulin resistance on inflammatory response and oxidative stress in acute cerebral ischemia. *Neuro Endocrinol. Lett.* 2013;34:52–57.

17. Hachinski V, Iadecola C, Petersen RC, Breteler MM, Nyenhuis DL, Black SE, Powers WJ, DeCarli C, Merino JG, Kalaria RN, Vinters HV, Holtzman DM, Rosenberg GA, Wallin A, Dichgans M, Marler JR, Leblanc GG. National Institute of Neurological Disorders and Stroke–Canadian Stroke Network Vascular Cognitive Impairment Harmonization Standards. *Stroke*. 2006;37:2220–2241.

18. Barba R, Martínez-Espinosa S, Rodríguez-García E, Pondal M, Vivancos J, Ser TD. Poststroke Dementia Clinical Features and Risk Factors. *Stroke*. 2000;31:1494–1501.

19. Sachdev PS, Chen X, Brodaty H, Thompson C, Altendorf A, Wen W. The determinants and longitudinal course of post-stroke mild cognitive impairment. *J. Int. Neuropsychol. Soc. JINS*. 2009;15:915–923.

20. Serrano S, Domingo J, Rodríguez-Garcia E, Castro M-D, Ser T del. Frequency of Cognitive Impairment Without Dementia in Patients With Stroke A Two-Year Follow-Up Study. *Stroke*. 2007;38:105–110.

21. Hoffmann M. Higher Cortical Function Deficits After Stroke: An Analysis of 1,000 Patients from a Dedicated Cognitive Stroke Registry. *Neurorehabil. Neural Repair*. 2001;15:113–127.

22. Röding J, Glader E-L, Malm J, Eriksson M, Lindström B. Perceived impaired physical and cognitive functions after stroke in men and women between 18 and 55 years of age--a national survey. *Disabil. Rehabil.* 2009;31:1092–1099.

23. Narasimhalu K, Lee J, Leong Y-L, Ma L, De Silva DA, Wong M-C, Chang H-M, Chen C. Inflammatory markers and their association with post stroke cognitive decline. *Int. J. Stroke Off. J. Int. Stroke Soc.* 2013;

24. Alvarez-Sabín J, Ortega G, Jacas C, Santamarina E, Maisterra O, Ribo M, Molina C, Quintana M, Román GC. Long-Term Treatment with Citicoline May Improve Poststroke Vascular Cognitive Impairment. *Cerebrovasc. Dis.* 2013;35:146–154.

25. Dichgans M, Zietemann V. Prevention of Vascular Cognitive Impairment. *Stroke*. 2012;43:3137–3146.

26. Felice FGD, Vieira MNN, Bomfim TR, Decker H, Velasco PT, Lambert MP, Viola KL, Zhao W-Q, Ferreira ST, Klein WL. Protection of synapses against Alzheimer's-linked toxins: Insulin signaling prevents the pathogenic binding of Aβ oligomers. *Proc. Natl. Acad. Sci.* 2009;106:1971–1976.

27. Hallenbeck JM. The many faces of tumor necrosis factor in stroke. *Nat. Med.* 2002;8:1363–1368.

28. Breteler MM. Vascular risk factors for Alzheimer's disease:: An epidemiologic perspective. *Neurobiol. Aging*. 2000;21:153–160.

29. Kalaria RN. Cerebrovascular Disease and Mechanisms of Cognitive Impairment Evidence From Clinicopathological Studies in Humans. *Stroke*. 2012;43:2526–2534.

30. Mitsios N, Pennucci R, Krupinski J, Sanfeliu C, Gaffney J, Kumar P, Kumar S, Juan-Babot O, Slevin M. Expression of Cyclin-Dependent Kinase 5 mRNA and Protein in the Human Brain Following Acute Ischemic Stroke. *Brain Pathol.* 2007;17:11–23.

31. Simpkins JW, Gatson JW, Wigginton JG. Commentary on "A roadmap for the prevention of dementia II. Leon Thal Symposium 2008." Rationale and recommendations for first evaluating anti-Alzheimer's disease medications in acute brain injury patients. *Alzheimers Dement.* 2009;5:143–146.

32. Srikanth VK, Thrift AG, Saling MM, Anderson JFI, Dewey HM, Macdonell RAL, Donnan GA. Increased Risk of Cognitive Impairment 3 Months After Mild to Moderate First-Ever Stroke A Community-Based Prospective Study of Nonaphasic English-Speaking Survivors. *Stroke*. 2003;34:1136–1143.

33. Zhu CZ, Auer RN. Optimal blood glucose levels while using insulin to minimize the size of infarction in focal cerebral ischemia. *J. Neurosurg.* 2004;101:664–668.

34. Nasreddine ZS, Phillips NA, Bédirian V, Charbonneau S, Whitehead V, Collin I, Cummings JL, Chertkow H. The Montreal Cognitive Assessment, MoCA: a brief screening tool for mild cognitive impairment. *J. Am. Geriatr. Soc.* 2005;53:695–699.

35. Brandt J, Benedict R. Hopkins Verbal Learning Test--Revised. Lutz, FL: PAR; 2001.

36. Benedict R. Brief Visuospatial Memory Test. Odessa, FL: PAR; 1994.

37. Wechsler D. Wechsler Adult Intelligence Scales-Third Edition. San Antonio, TX: The Psychological Corporation; 1997.

38. Duncan PW, Wallace D, Lai SM, Johnson D, Embretson S, Laster LJ. The Stroke Impact Scale Version 2.0 Evaluation of Reliability, Validity, and Sensitivity to Change. *Stroke*. 1999;30:2131–2140.

39. Duncan PW, Lai SM, Bode RK, Perera S, DeRosa J. Stroke Impact Scale-16 A brief assessment of physical function. *Neurology*. 2003;60:291–296.

40. Duncan P, Lai S, Tyler D, Perera S, Reker D, Studenski S. Evaluation of proxy responses to the Stroke Impact Scale. *Stroke*. 2002;33:2593–2599.

41. Thornton M, Travis SS. Analysis of the reliability of the modified caregiver strain index. *J. Gerontol. B. Psychol. Sci. Soc. Sci.* 2003;58:S127–132.

42. Wilkinson PR, Wolfe CDA, Warburton FG, Rudd AG, Howard RS, Ross-Russell RW, Beech RR. A Long-term Follow-up of Stroke Patients. *Stroke*. 1997;28:507–512.

43. Marchi-Jones S, Murphy JF, Rousseau P. Caring for the caregivers. *J. Gerontol. Nurs.* 1996;22:7–13.

44. Brown CM, Mulcahey TA, Filipek NC, Wise PM. Production of Proinflammatory Cytokines and Chemokines During Neuroinflammation: Novel Roles for Estrogen Receptors α and β. *Endocrinology*. 2010;151:4916–4925.

45. Kroenke K, Spitzer R. The PHQ-9: A new depression diagnostic and severity measure. *Psychiatr. Ann.* 2002;32:1–7.

46. Williams L, Brizendine E, Plue L, Bakas T, Tu W, Hendrie H, Kroenke K. Performance of the PHQ-9 as a screening tool for depression after stroke. *Stroke*. 2005;36:635–638.

47. Van Swieten J, Koudstaal P, Visser M, Schouten H, van Gijn J. Interobserver agreement for the assessment of handicap in stroke patients. *Stroke*. 1988;19:604–607.

48. Rankin J. Cerebral vascular accidents in patients over the ag of 60: II. Prognosis. *Scott. Med. J.* 1957;2:200–215.

49. The National Institute of Neurological Disorders; Stroke rt-PA Stroke Study Group. Tissue plasminogen activator for acute ischemic stroke. *N. Engl. J. Med.* 1995;333:1581–1587.

50. Groenwold RHH, Donders ART, Roes KCB, Harrell FE Jr, Moons KGM. Dealing with missing outcome data in randomized trials and observational studies. *Am. J. Epidemiol.* 2012;175:210–217.

51. Little R, Rubin D. Statistical Analysis with Missing Data. John Wiley and Sons; 1987.

52. Zhang G, Li J, Purkayastha S, Tang Y, Zhang H, Yin Y, Li B, Liu G, Cai D. Hypothalamic programming of systemic ageing involving IKK-β, NF-κB and GnRH. *Nature*. 2013;497:211–216.

53. Henkin RI. Inhaled insulin-intrapulmonary, intranasal, and other routes of administration: mechanisms of action. *Nutr. Burbank Los Angel. Cty. Calif*. 2010;26:33–39.

54. McCusker RH, Kelley KW. Immune–neural connections: how the immune system's response to infectious agents influences behavior. *J. Exp. Biol.* 2013;216:84–98.

55. Wang Q, Tang XN, Yenari MA. The inflammatory response in stroke. *J. Neuroimmunol.* 2007;184:53–68.

56. Iiyama K, Hajra L, Iiyama M, Li H, DiChiara M, Medoff BD, Cybulsky MI. Patterns of vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 expression in rabbit and mouse atherosclerotic lesions and at sites predisposed to lesion formation. *Circ. Res.* 1999;85:199–207.

57. Jefferson AL, Massaro JM, Beiser AS, Seshadri S, Larson MG, Wolf PA, Au R, Benjamin EJ. Inflammatory markers and neuropsychological functioning: the Framingham Heart Study. *Neuroepidemiology*. 2011;37:21–30.

58. Luna JM, Moon Y, Liu K, Spitalnik S, Paik M, Sacco R, Elkind MSV. Tumour necrosis factor receptor 1 and mortality in a multi-ethnic cohort: the Northern Manhattan Study. *Age Ageing*. 2013;42:385–390.

59. Bhattacharjee M, Vairale J, Gawali K, Dalal PM. Factors affecting burden on caregivers of stroke survivors: Population-based study in Mumbai (India). *Ann. Indian Acad. Neurol.* 2012;15:113–119.

60. Novak V, Milberg W, Hao Y, Munshi M, Novak P, Galica A, Manor B, Roberson P, Craft S, Abdulijalil A. Enhancement of Vasoreactivity and Cognition by Intranasal Insulin in Type 2 Diabetes. *Diabetes Care.* 2014; 37:751-759.