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WHITE MATTER HYPERINTENSITY VOLUME AS A BIOMARKER OF OXIDATIVE STRESS IN ACUTE ISCHEMIC STROKE

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Oxidative stress contributes to brain injury during ischemic stroke, but antioxidant clinical trials have been unable to demonstrate a benefit to date. The all comers approach to patient selection in these trials may have contributed to their lack of success. We hypothesize that white matter hyperintensity is a biomarker that can identify ischemic stroke patients with increased levels of oxidative stress and improve future trials. White matter hyperintensity represents chronic loss of cellular brain tissue and has been correlated with oxidative stress in humans and animal models. To test our hypothesis, we examined the correlation between white matter hyperintensity volume and molecular biomarkers of oxidative stress in patients with acute ischemic stroke. The patients for our study were a subset of participants in an ongoing prospective biomarker study at Massachusetts General Hospital and the Brigham and Women's Hospital. From all participants in that parent study, we selected patients if they had an analyzable MRI obtained within 72 hours of stroke onset. During the parent study, the plasma Oxygen Radical Absorbance Capacity and urinary 8-hydroxy-2-deoxy-guanosine levels were

measured at baseline (between 0 and 9 hours after stroke onset) and plasma F2-isoprostane levels were measured at baseline and again at 48 hours after stroke onset. White matter hyperintensity volume was determined using a validated semi-automated analysis of brain MRI. Baseline characteristics we examined included demographic features, comorbid illness, and body mass index, and 3-month functional outcomes measured by the modified Rankin Scale score and Barthel Index of Activities of Daily Living. White matter hyperintensity volumes were adjusted for head size using intracranial area measurements and log-transformed prior to statistical analysis. Correlations were performed between the log of the normalized white matter hyperintensity volume and age, functional scores, and biomarkers of oxidative stress. Out of a projected 600 patient cohort in the parent study, an estimated 80% will be eligible for this substudy. At the time of this analysis, measurements had been completed for 158 patients. Mean age was 71±15 years; 56% were male; 71% had hypertension, 44% had hyperlipidemia, 32% had atrial fibrillation, and 20% had diabetes mellitus. Mean log of the normalized white matter hyperintensity volume was 1.38±1.32. Log of the normalized white matter hyperintensity volume was correlated with age ($\rho=0.62$, $p<0.0001$), modified Rankin Scale ($\rho=0.20$, $p=0.04$), and Barthel Index ($\rho=-0.21$, $p=0.04$). White matter hyperintensity volume did not correlate significantly with molecular biomarkers of oxidative stress. In conclusion, our analysis showed that that white matter hyperintensity volume does not correlate with systemic molecular biomarkers of oxidative stress measured at baseline or 48 hours. As expected, white matter hyperintensity volume does correlate with age and functional scores in patients with acute ischemic stroke.

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Introduction

The Burden of Stroke

The very course of history has been changed by cerebrovascular disease.(1) President Woodrow Wilson suffered from a "stuttering course" of strokes believed partially responsible for the United States' refusal to join the League of Nations.(2) Three of the most influential politicians of the twentieth century, Franklin Roosevelt, Winston Churchill, and Josef Stalin, all suffered from severe cerebrovascular disease when they met at Yalta at the end of World War II.(1, 2) President Roosevelt died later of a massive cerebral hemorrhage in 1945, while still president.(3)

Stroke is second only to ischemic heart disease as a cause of death throughout the world and, in wealthy countries, as a cause of lost disability-adjusted life-years.(4, 5) In the United States, someone suffers from a stroke every 40 seconds, on average.(6) Stroke can be broadly defined as "any damage to the brain or the spinal cord caused by an abnormality of the blood supply."(1) Ischemic stroke accounts for 87% of strokes in western societies, with one-month mortality rates as low as 2.5% for a small lacunar infarction and as high as 78% for a large hemispheric lesion.(6-8)

Antioxidants in Stroke

As the blood's supply of oxygen and glucose to an area of the brain is lost, there are degrees of injury related to the extent of oligemia. Some of the tissue suffers a complete lack of blood flow, quickly infarcts, and becomes the "core." In some cases, particularly in larger strokes due to large vessel occlusion, there is a larger area damaged by severely attenuated blood flow, but potentially salvageable with reperfusion. It is termed the "penumbra." Neuroprotection is a term generally applied to an intervention to

prevent the progression from cell injury to death in the ischemic penumbra. Thus far, only one FDA approved pharmacologic intervention exists, recombinant tissue plasminogen activator, or rtPA. More than one thousand agents have been evaluated as interventions in animal models of cerebral ischemia, while only 10% have been tried in clinical trials involving humans.(9)

Antioxidants are a promising new class of drugs for treatment for acute ischemic stroke. They have potent neuroprotective effects in animal models of acute stroke. Four antioxidant therapies have been tested in clinical trials enrolling humans.(10) One of the most well-known compounds is NXY-059, which is a spin trap agent, that has been the subject of two highly touted phase III trials, the Stroke-Acute Ischemic NXY Treatment (SAINT) I and SAINT II studies.(11, 12) SAINT I included 1,722 patients and demonstrated an improved distribution of functional outcomes for stroke patients administered NXY-059.(11) SAINT II was a larger trial that enrolled 3,306 patients, for increased power compared to SAINT I. Unfortunately, the second trial showed no benefit for patients receiving the therapy.(12) Much has been written about why the SAINT trials failed; four discussions are cited here.(13-15) One author discusses NXY-059 in the wider context all neuroprotectants, citing specific reasons why this agent in particular might not have been the best choice for clinical use.(16) These reasons include NXY-059's minimal ability to cross the blood-brain-barrier or cellular membranes along with its low potency.(16-19)

Edaravone is a lesser-known antioxidant that is already in clinical use in Japan.(20) This free radical scavenger showed a therapeutic benefit in a Japanese randomized controlled trial.(21) This drug was administered to more than 500,000 patients by the end of 2005 and reported to be "well-tolerated in all patients."(20) In

addition, the authors describe an interim analysis demonstrating that 47% of patients receiving the drug have "a favorable functional outcome (modified Rankin Scale 0 and 1)."(20) In the published data from the SAINT II trial, the fraction of patients in the placebo cohort who had such a functional outcome at 90 days was 28.7%.(12) The efficacy of Edaravone is not widely acknowledged in the U.S., possibly because the trials were conducted in non-western countries. Based on available trial data, however, it seems possible that this agent may increase the proportion of patients with a favorable outcome after acute ischemic stroke from about 25% to 45%. A clinical trial with a sample size of just 254 (127 in each group) would be required to demonstrate this benefit, assuming a two-sided α=0.05 and 90% power.(22) The total cohort would therefore be estimated at only 254. In any case, Edaravone serves as a source of hope for emerging antioxidant therapies.

The goal of our study is to text a potential instrument to expand upon the limited therapeutic options for stroke. Suggestions for improving studies of acute stroke treatments have centered on issues of clinical trial design, and more specifically on improving patient selection.(23) Criteria may be based upon a drug's mechanism of action, or specific patient characteristics, such as clinical evidence of ischemic penumbra.(23, 24) In this vein, we too observe that an all comers approach to stroke therapy has largely failed thus far. We believe that the volume of white matter hyperintensity, which is a chronic neuroimaging finding on MRI, can serve as a novel tool with which to select for patients most amenable to potential antioxidant therapies in stroke.

Oxidative Stress in Stroke

To understand the basis for employing antioxidants as a stroke therapy, it is important to understand the evolution of oxidative stress at the cellular level in the ischemic brain. In an encyclopedic review, Lipton provides a detailed description of the known cellular events of ischemic neuronal death.(25) Cherubini et al. discuss the process with more specific reference to oxidative stress as follows: Briefly, as the cell's supply of oxygen and glucose diminish, production of adenosine triphosphate ceases, which disrupts ion gradients and leads to an outflow of glutamate and inflow of calcium ions. Increased intracellular calcium levels lead to activation of many enzymes, producing nitric oxide and free radicals (including reactive oxygen species), which are both important causes of injury to the cell and its organelles.(26) Mitochondria are noted contributors to the increased levels of free radicals, especially superoxide, during ischemia, because of damage to the electron transport chain.(26, 27)

Each cell is endowed with a supply of endogenous antioxidants and antioxidant enzymes with which they regulate the natural occurrence of low levels of reactive oxygen species.(28) When these oxidative species overwhelm the antioxidant factors in the cell, the resulting imbalance is termed oxidative stress.(29) Persistent oxidative stress is a significant cause of cellular injury, including damage to lipids and DNA, and eventually cell death. Of note, brain tissue is unusually susceptible to oxidative injury secondary to its high oxygen needs and high concentrations of lipid such as arachidonic acid, among other factors.(26, 28) The stable interplay between antioxidants and oxidants over the long term can be thought of as a cell's chronic level of oxidative stress. A change to this level in the short term, for example, in the face of acute ischemic injury, is called acute oxidative stress. With the goal of monitoring oxidative stress levels in ischemic brain

tissue, biomarkers of oxidative stress has become an area of particular interest in stroke research.(26)

Biomarkers of Oxidative Stress

A biomarker is defined by the Biomarkers Definitions Working Group as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention."(30) In the case of oxidative stress, biomarkers have traditionally been the products of oxidative reactions with molecular components of the cellular architecture.(26) With regard to ischemic stroke, an ideal biomarker will represent a property of the ischemic brain that can be quantified or measured indirectly, given the clear practical difficulties in obtaining direct measurements within brain tissue in the living human.(26) For example, one might measure the level of a molecular biomarker in the urine or blood to detect oxidative stress in the brain, though these relationships have yet to be fully explored.(31) Notably, biomarkers need not be a laboratory-style test or molecular species. Observations such as physical exam maneuvers or findings such as patterns in neuroimaging fall well within the definition of a biomarker.

As mentioned above, lipids are subjected to damage from oxidative stress. F2-isoprostanes are products of free radicals and arachidonic acid that have shown particular utility as biomarkers of oxidative stress in humans.(32) Because the majority of arachidonic acid is present as part of the phospholipid components of plasma membranes, it is thought that F2-isoprostanes are made as part of phospholipids and then enzymatically released.(32, 33) F2-isoprostanes are particularly attractive as biomarkers of oxidative stress for reasons including their stability and specificity, their detectable

levels in all body fluids, and their independence of dietary fat(34) They have been found elevated in animal models of oxidative stress as well as animal stroke models.(35, 36) An earlier study measuring F2-isoprostane in stroke patients found no relationship between F2-isoprostane levels and stroke.(37) More recently, several groups have found F2-isoprostane levels elevated with acute ischemic stroke in humans.(38-41) One current study based on laboratory models reports evidence that F2-isoprostanes may be involved in the pathogenesis of ischemic neuronal death rather than a passive product of the process.(42)

Another target of oxidative insult is the cell's DNA. This process is suspected to consist of two distinct steps, the first of which is early-onset DNA oxidation followed by later-onset DNA destruction by endonucleases.(26, 43) 8-hydroxy-2-deoxy-guanosine is one molecular result of oxidative DNA damage.(26, 43) Repair of DNA lesions such as 8-hydroxy-2-deoxy-guanosine likely ensues after the injury, but the question is raised whether the energy devoted to these repairs is an appropriate allocation of resources in damaged tissue or may actually contribute further harm.(43) 8-hydroxy-2-deoxy-guanosine levels have been monitored in the peripheral plasma of animal models of stroke and were associated with levels at the site of infarction.(44) Another group found that 8-hydroxy-2-deoxy-guanosine levels were more prominent in areas surrounding the infarct and proposed that oxidative DNA damage may be dependent on some preservation of oxygen supply to the tissue.(45)

The Oxygen Radical Absorbance Capacity assay quantifies oxidative stress from a different perspective and measures the total antioxidant ability of a sample.(46, 47) The basis of this assay was originally the use of a source of oxidative species that reacted with a class of fluorescent proteins called phycoerythrins. The fluorescence of these proteins

decreased as they were exposed to this oxidative damage. The sample was added to this reaction, and the total antioxidant abilities in the sample – including molecular antioxidants and antioxidant enzymes – stopped, or quenched, the oxidative damage. The amount of damage the antioxidants prevented was then quantified and was the Oxygen Radical Absorbance Capacity of the solution.(46, 47) The chemical fluorescein was later proposed as an alternative to phycoerythrin proteins because of several specific limitations of the protein reagent.(48, 49) The Oxygen Radical Absorbance Capacity assay has been used extensively in nutrition research to quantify the antioxidant capacities of foods and supplements in vitro.(50) The method's technique of taking into account all antioxidant capacity make it particularly useful in biological fluids, where measurement of any one antioxidant may be less informative.(51) Three examples of studies measuring the Oxygen Radical Absorbance Capacity in human plasma are cited here.(52-54) One group found the Oxygen Radical Absorbance Capacity decreased in patients with stroke.(55)

As mentioned previously, chronic oxidative stress and acute oxidative stress exist in concert. One way to conceptualize oxidative stress is to visualize oxidative stress level graphed over time. In this imaginary graph, acute oxidative stressors would appear as peaks stemming from a baseline of chronic oxidative state. Studies discussed above generally concern oxidative stress biomarkers of stroke in the acute phase. However, fine distinctions for timeframes of various studies are not always easily made. For example, one group reports collecting serum "between 5 and 10 days of the stroke onset."(55) Chronically increased markers of oxidative stress after stroke, as well as decreased antioxidants, have also been found.(56-58)

White Matter Hyperintensity

As discussed above, the purpose of our study is to investigate a novel method of patient selection in acute stroke trials using white matter hyperintensity volume. We believe white matter hyperintensity volume can aid in the selection of stroke patients most amenable to antioxidant therapies because it is an imaging biomarker of chronic oxidative stress and may have implications for acute oxidative injury as well. White matter hyperintensity, also known as leukoaraiosis or white matter changes, denotes areas of increased signal on MRI in the periventricular and/or subcortical areas of the deep white matter that are usually symmetric and bilateral.(59, 60) As an historical note, white matter hyperintensity and leukoaraiosis – by definition – refer to imaging phenomena that are not completely synonymous with Binswanger's disease, which signifies neuropathology first described in 1894.(59, 61) Consensus has evolved that Binswanger's disease is an inappropriate label for a disease defined by neuroimaging.(59, 61) The term leukoaraiosis was first coined in 1986 to describe hypodensities seen on CT and changes in the signal on MR.(59, 62) For the purposes of this discussion, white matter hyperintensity will refer to the imaging phenomenon seen on both CT and MRI and its associated neuropathology.

White matter hyperintensity is a common disease that is even more common in patients who suffer strokes, and it is thought to be mainly vascular in origin.(63) The pathophysiologic basis of white matter hyperintensity is complex and remains incompletely understood.(64) Briefly, the areas of disease include infarcted tissue at different stages of progression as well as vascular damage called periventricular venous collagenosis, which likely causes venous stenosis and eventually occlusion.(60, 63, 65) Areas of white matter around the lesion show apoptosis and gliosis and demonstrate

"progressive cell and axonal loss with loosening of the WM structure."(60) The disease increases in prevalence with age as well as cardiovascular risk factors, including hypertension, diabetes, smoking, c-reactive peptide, homocysteine, and atherosclerosis.(59, 60, 63, 66-72) In the elderly, white matter hyperintensity is quite common. At 85 years old, the incidence is close to 100%.(63) It has been linked to dementia, cognitive impairment, falls, and urinary incontinence.(59, 60, 63, 73) White matter hyperintensity itself has been shown to be a predictor of stroke, both ischemic and hemorrhagic, and volume of white matter hyperintensity has been shown to predict growth of an ischemic infarct in patients with acute stroke.(60, 64, 74) Furthermore, though not all studies agree, larger cohorts have shown that white matter hyperintensity predicts worse functional outcome after stroke.(75-77)

White Matter Hyperintensity and Oxidative Stress

Many groups have demonstrated evidence from chronic cerebral hypoperfusion models in animals that oxidative stress may be involved in the development of white matter hyperintensity, and five recent studies are cited here.(78-82) This model is certainly not specific for white matter hyperintensity as a disease, and the animal studies link the hypoperfusion model to Alzheimer's Disease, vascular dementia, and cerebral palsy.(80, 83) While certain conclusions from these data are helpful, differences in the neuroanatomy of rodents and humans underscore the specificity of white matter hyperintensity to the human species. As one review notes, approximately 90% of the rodent brain is grey matter, while the human brain is equal parts grey matter and white matter.(60)

To the best of our knowledge, only two groups have demonstrated a correlation between indicators of oxidative stress and white matter hyperintensity severity in humans. In 1996, the Austrian Stroke Prevention Study examined plasma antioxidant levels and observed decreased levels of lycopene and α-tocopherol in subjects with higher grades of white matter hyperintensity. The finding for α-tocopherol remained significant even after correcting for known risk factors of white matter hyperintensity, such as age, hypertension, hypercholesterolemia, and heart disease. Moreover, this group calculated an adjusted odds ratio of 3.70 (90% CI, 1.69 to 8.10) for higher grades of white matter hyperintensity using quartiles of α-tocopherol serum concentration.(84) In 2004, a group at the Shimane Institute of Health Science in Japan examined nitric oxide metabolites and F2-isoprostane in subjects with grades of periventricular hyperintensity, which is a component of white matter hyperintensity (they specifically excluded subcortical infarcts in their analysis). Interestingly, this group found an inverse correlation with nitric oxide metabolites and periventricular hyperintensity. F2-isoprostane did not correlate with periventricular hyperintensity, but F2-isoprostane levels had a significant effect on worsening periventricular hyperintensity grade in a regression analysis.(85)

These studies provide compelling evidence that white matter hyperintensity volume correlates with oxidative stress, and endothelial dysfunction serves as a satisfying explanation for the process. However, the relationships described above were demonstrated in healthy populations instead of stroke patients. The studies also used white matter hyperintensity (or periventricular hyperintensity) grading such as the Fazekas criteria, which is an ordinal scale that has been found to be less reliable than volumetric analysis.(86) In order to truly demonstrate that white matter hyperintensity

volume may serve as a tool to aid in patient selection within stroke clinical trials, we embarked on a study to explore whether oxidative stress correlates with white matter hyperintensity volume in acute ischemic stroke patients.

One mechanistic link between white matter hyperintensity and reactive oxygen species revolves around the idea of endothelial dysfunction. The mechanics of endothelial dysfunction, how it relates to white matter hyperintensity, and its underpinning molecular events are well described in a recent review.(87) Briefly, endothelial dysfunction can cause blood brain barrier damage, reduced blood flow, and change the interactions of glial cells with the endothelium.(87) Reactive oxygen species may be involved in the changes in perfusion brought about by endothelial dysfunction, perhaps mediated by angiotensin II and nicotinamide adenine dinucleotide phosphate oxidase.(88)

A more general theory to link oxidative stress and white matter hyperintensity revolves around thinking of white matter hyperintensity as simply ‘sick brain’ – given that its areas involve much cellular and structural loss. Potentially, in this tissue depleted of its natural complement of cells and a healthy milieu, oxidative insults that are a normal part of biological processes can inflict substantial harm. Furthermore, oxidatively mediated diseases such as true brain ischemia, may inflict much more damage than would be seen otherwise. Thus, an individual experiencing chronically elevated levels of oxidative stress, as in smokers, for example, may have a predisposition to white matter hyperintensity as well as ischemic strokes that are more significant than they would be otherwise.(31)

A "Gold Standard" for Oxidative Stress

The relationship between white matter hyperintensity and oxidative stress is a tempting target for study and has exciting potential implications. As these processes are both somewhat diffuse, however, we must first ensure that we are able to measure both variables as best we can. For white matter hyperintensity, measurement is largely described in the definition: the areas of bright signal on MRI. Therefore, volumetrically quantifying the white matter hyperintensity in a reproducible manner seems to be the finest measure possible.

The best way to quantify oxidative stress is less obvious. Several kinds of oxidative stress measurements have been described above. The label of "gold standard" for measuring oxidative stress in living systems has been given to F2-isoprostanes.(32) They earned this moniker due to their performance in the Biomarkers of Oxidative Stress Study II, which was a validation study designed to find the best biomarker for in vivo measurement of oxidative stress. In this study, F2-isoprostanes were mentioned as "promising" measures in both plasma and urine.(89) Another review summarized the established data regarding various biomarkers, and classified plasma F2-isoprostanes as having "met with confidence" evaluation of validity, relationship to disease, and variation with oxidative dose, though questions remained about their specificity and stability.(90) Interestingly, the Oxygen Radical Absorbance Capacity performed well regarding specificity and variation with dose, and urinary 8-hydroxy-2-deoxy-guanosine had stellar marks in each category except its specificity.(90) Taken together, the strengths of the different biomarkers complement each other.

More to the point, oxidative stress is such that measurement of multiple biomarkers in parallel is almost certain to be more powerful that measuring one

individually. When considering the many different tissue structures, physical states, and biochemical composition of even one organ of the body, it seems to go without saying that measuring biomarkers that represent different kinds of damage will reveal the clearest picture.(91) That being said, we do know that the brain contains a large supply of arachidonic acid, which will evolve F2-isoprostanes in the setting of oxidative stress. Thus, F2-isoprostanes seem to be an excellent marker of oxidative stress that is only further strengthened by simultaneous assays of the Oxygen Radical Absorbance Capacity and 8-hydroxy-2-deoxy-guanosine.

Statement of Purpose, Specific Hypothesis, and Specific Aims

Statement of Purpose and Specific Hypothesis

The goal of this study is to examine the performance of white matter hyperintensity volume as a biomarker of oxidative stress in acute ischemic stroke patients. As discussed above, evidence by others demonstrates that white matter hyperintensity severity and oxidative stress levels are correlated in the general population.(84, 85) We hypothesize that this relationship remains true in the stroke population. white matter hyperintensity volume may therefore be a useful tool with which to identify an enriched sample of stroke patients that is more oxidatively stressed, and this subpopulation would therefore be more susceptible to the potential of antioxidant stroke treatments.

Specific Aim #1: To determine whether, in acute stroke patients, there is an association between white matter hyperintensity volume and biomarkers of oxidative stress measured

at baseline (less than 9 hours) after stroke onset. We will label this relationship the correlation at baseline.

Hypothesis: Levels of biomarkers of oxidative stress measured at baseline will correlate directly with the volume of white matter hyperintensity, independent of known stroke risk factors and stroke characteristics, such as infarct volume.

Specific Aim #2: To determine whether, in acute stroke patients, there is an association between white matter hyperintensity volume and biomarkers of oxidative stress measured at 48 hours after stroke onset. We will label this relationship the correlation at 48 hours.

Hypothesis: Levels of biomarkers of oxidative stress measured at 48 hours after stroke onset will correlate directly with the volume of white matter hyperintensity, independent of known stroke risk factors and stroke characteristics, such as infarct volume.

Specific Aim #3: To compare the correlation at baseline with the correlation at 48 hours.

Hypothesis: We hypothesize that the correlation at baseline will be stronger than the correlation at 48 hours, because of the added contribution of acute oxidative stress.

Methods

Author Contributions

Patients for the study of white matter hyperintensity as a biomarker of oxidative stress in acute ischemic stroke were selected from a larger parent study of biomarkers in ischemic stroke conducted at the Massachusetts General Hospital and Brigham and Women's Hospital. The author (Z.A.C.) spent a year at the Massachusetts General Hospital to conduct the substudy. The following were his overall duties, the specifics of

each are described in detail in the methods below: 1) to complete the research group's protocol for certification as a reader of white matter hyperintensity, 2) to obtain patient information subsequent to enrollment in the parent study and screen them for this substudy, 3) to retrieve and store the MRIs from the medical imaging systems at the hospitals, 4) to quantify the white matter hyperintensity on the MRIs after retrieval, 5) to review the analyzed MRIs with the overreader (N.S.R.), 6) to assist in the quantification of 8-hydroxy-2-deoxyguanosine for a subset of the parent study cohort, specifically measuring output peaks on the mass spectrometer in the collaborating laboratory, 7) to calculate the total white matter hyperintensity volume for each MRI read, and 8) correct, or normalize, those volumes for head size when possible.

Other members of the research group physically recruited the patients, conducted the patient interviews, recorded patient clinical data, and collected biological samples from the patients. Collaborating laboratories quantified the values of clinical indicators, F2-isoprostane, the Oxygen Radical Absorbance Capacity, and completed the measurement of 8-hydroxy-2-deoxyguanosine. The statistical analysis was conducted by a collaborating biostatistician (M.K.P.).

Subject Selection and Screening

As mentioned previously, this project was a substudy of a larger observational study. In the parent study, consecutive patients presenting within 9 hours of onset of acute ischemic stroke symptoms were enrolled at the Massachusetts General Hospital and Brigham and Women's Hospital. If stroke onset time was unknown, onset time was defined as the time midway between when the subject was last seen well and when the symptoms were first noted. Exclusion criteria were as per the protocol of the parent

study: 1) Stroke cause was believed to be vasculitis, endocarditis, venous infarction, or primary hemorrhagic stroke. 2) No baseline CT or MRI was available. 3) Other acute intracerebral pathology was noted on baseline imaging (i.e. subarachnoid hemorrhage, brain tumor, central nervous system infection). 4) Isolated transient monocular blindness was present. 5) Neurological deficits that were rapidly resolving. 6) Temperature was above 101 degrees Fahrenheit or white blood cell count was greater than 15,000 cells per milliliter. 7) Chronic kidney disease existed requiring dialysis or end-stage hepatic dysfunction. 8) Active metastatic malignancy was diagnosed at the time of the stroke. 9) Informed consent was unable to be obtained. 10) Stroke, MI, or a major thrombolytic event had occurred within 30 days. 11) Major surgery had occurred within 30 days.(92)

The author reviewed the recorded stroke onset times for all patients enrolled in the parent study by consulting either the medical record or the larger study's database. He then consulted the medical record to determine if an MRI had been performed for clinical or research purposes within approximately 72 hours of stroke onset. Patients were included as subjects in this substudy if they had an MRI that was available within the time period and analyzable as determined by either the author and/or the overreader. All patients included in this analysis were enrolled at Massachusetts General Hospital.

Demographics, Clinical Measures, and Outcomes

As part of the parent study, baseline demographics, vital signs, basic clinical laboratory data, medical history, medication history, stroke risk factors, and stroke characteristics were collected, including the NIH Stroke Scale, for each patient. After 3 months (or 70 to 110 days), the modified Rankin Scale score and Barthel Index of Activities of Daily Living were determined.

Biological Samples

As part of the parent study, peripheral plasma and urine samples were collected at baseline (less than 9 hours after stroke onset), and peripheral plasma was collected again at 48 hours (between 36 and 60 hours) after stroke onset. These samples were obtained by research coordinators, research fellows, and study investigators affiliated with the larger study. Urinary creatinine was quantified by the clinical laboratory at the Massachusetts General Hospital.

Quantification of White Matter Hyperintensity Volume

MRIs were performed at the Massachusetts General Hospital on 1.5 Tesla scanners (GE Healthcare, Little Chalfont, Buckinghamshire, U.K.). Because the majority of the available MRIs were obtained for clinical purposes, motion artifact was prominent in many of the images.

The author was trained and certified to read white matter hyperintensity per the research group's protocol prior to analyzing patient images. The method of quantifying white matter hyperintensity volume and normalizing the volume to intracranial area has been described previously.(93, 94) In this manner, the author conducted white matter hyperintensity volume analysis using a semi-automated multistep process with MRIcro software (Center for Advanced Brain Imaging, Atlanta, GA), which is recapitulated here: 1) Sagittal T1 images, when available, were reviewed to determine the two most midline slices. For these slices, the intradural space was outlined manually by tracing the inner table. The computer calculated the intradural area, which served as an estimate of intracranial area with which to normalize white matter hyperintensity volume.

2) As these MRIs were performed on patients suffering acute ischemic stroke, areas of restricted diffusion were generally visible on diffusion-weighted imaging. The author reviewed diffusion-weighted imaging sequences to determine the anatomical distribution of the ischemia.

3) The author then reviewed axial T2 fluid-attenuated inversion recovery images to determine a slice with a representative quantity of white matter hyperintensity visible. Using this slice as a guide, a threshold intensity filter was manually adjusted to generate a region of interest for the whole sequence. Because T2 signal is not specific to white matter hyperintensity, this region of interest included components of bone, calcifications in the grey matter, brain infarcted at various stages, and other structures.

4) To limit the region of interest to white matter hyperintensity, axial T2 fluid-attenuated inversion recovery sequences were examined with coregistration of diffusion-weighted image sequences, to ensure regions of acute ischemia were not included as white matter hyperintensity. For individual cerebral hemispheres, the author grossly outlined areas of white matter hyperintensity (including both periventricular hyperintensity as well as subcortical infarcts) on each slice. The computer then determined the region of interest overlap between the intensity filter and white matter hyperintensity outlines, which represented an estimate of white matter hyperintensity volume.

5) As a final step, the author manually refined this estimate by editing the white matter hyperintensity region of interest so that it better reflected the heterogeneity of white matter hyperintensity.

6) As mentioned above, the MRIs with quantified white matter hyperintensity volumes were individually reviewed in person with the overreader (N.S.R.), who would ensure

that the appropriate regions of the image had been classified as white matter hyperintensity.

7) Computation of total white matter hyperintensity volume depended on several additional rules:

a) If the ischemia was infratentorial, the white matter hyperintensity volume of both hemispheres was summed.

b) If the ischemia involved only one cerebral hemisphere or if prominent intracranial pathology interfered with white matter hyperintensity measurements in one cerebral hemisphere, the white matter hyperintensity volume of the contralateral hemisphere was doubled.

c) If ischemia or prominent intracranial pathology interfered with the measurement of white matter hyperintensity volume of both cerebral hemispheres, the white matter hyperintensity volume of both hemispheres was summed.

Quantification of F2-isoprostane

To assay F2-isoprostane, baseline and 48-hour plasma samples were frozen at -80 degrees Celsius prior to processing. F2-isoprostane was quantified using the 8-Isoprostane Enzyme Immunoassay Kit (Cayman Chemical, Ann Arbor, MI) by the Antioxidant Research Laboratory at the Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University.

Quantification of 8-hydroxy-2-deoxy-guanosine

To measure 8-hydroxy-2-deoxy-guanosine, urine samples were taken at baseline and frozen at -80 degrees Celsius prior to processing. The level of 8-hydroxy-2-deoxy-

guanosine was quantified using a high performance liquid chromatography / mass spectrometry technique developed at the Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University.

8-hydroxy-2-deoxy-guanosine was extracted from urine samples by solid-phase extraction using an Oasis HLB cartridge using a urine cleanup procedure previously described.(95) Chromatographic separation of 8-hydroxy-2-deoxy-guanosine from other eluent constituents was conducted using an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA) fitted with a Phenomenex Synergi Max-RP 80A (4 μm, 150 mm x 4.60 mm i.d.), C18 analytical column. The eluent was directed to an API 3000 triple-quadrupole mass spectrometer (Applied Bioscience, Foster City, CA) equipped with a TurboIon spray source. The recovery and quantification of 8-hydroxy-2-deoxy-guanosine present in samples was determined by comparison to a stable isotope 8-hydroxy-2-deoxy-guanosine kindly provided by Dr. Miral Dizdaroglu, at the National Institute of Standards and Technology, Gaithersburg, MD. The author analyzed output curves for many samples to quantify the amount of 8-hydroxy-2-deoxy-guanosine present in the urine. This resulting 8-hydroxy-2-deoxy-guanosine amount was corrected with the concentration of urine creatinine.

Quantification of the Oxygen Radical Absorbance Capacity

Plasma samples for the Oxygen Radical Absorbance Capacity were drawn at baseline. Plasma samples for the Oxygen Radical Absorbance Capacity (total) and Oxygen Radical Absorbance Capacity (perchloric acid) were drawn at baseline. Samples destined for the Oxygen Radical Absorbance Capacity (perchloric acid) assay received 0.5 M perchloric acid added at a 1:1 ratio and vortexed vigorously for 30 second.

Samples were then centrifuged at 13,000 rpm using microplate centrifuge for 15 minutes and frozen at -80 degrees Celsius prior to processing. The Oxygen Radical Absorbance Capacity was quantified by the Antioxidant Research Laboratory at the Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University according to methods previously described.(96)

Statistical Analysis

Statistical analysis was performed using SAS statistical software (SAS Institute, Cary, NC). White matter hyperintensity volume was normalized with intracranial area by dividing the white matter hyperintensity volume by the ratio of intracranial area over a standard intracranial area value. Normalized white matter hyperintensity volume was log-transformed prior to analysis. Non-parametric Spearman correlations were performed between the log of the normalized white matter hyperintensity volume and age, functional scores, and the biomarkers of oxidative stress, F2-isoprostane, 8-hydroxy-2-deoxy-guanosine, and the Oxygen Radical Absorbance Capacity. Non-parametric tests were used because the age, functional scores, and biomarker values were not normally distributed. Multivariable regression modeling was performed to explore the relationship between the plasma biomarkers of oxidative stress, NIH Stroke Scale (a marker for stroke severity), and the log of the normalized white matter hyperintensity volume.

Results

Table 1 lists demographics and clinical features of the cohort. We studied 158 participants, with a mean age of 71±15 years. The population was 56% male, and mean

body mass index was 28±6. Prevalent stroke risk factors included hypertension (71%), hyperlipidemia (44%), atrial fibrilliation (32%), and diabetes mellitus (20%).

The distribution of the values of the log of the normalized white matter hyperintensity, F2-isoprostane, the Oxygen Radical Absorbance Capacity, and 8-hydroxy-2-deoxy-guanosine are specified in Table 2. Mean log of the normalized white matter hyperintensity volume was 1.38±1.32. Measured at baseline, median value of F2-isoprostane was 54 pg/mL, interquartile range 36-72. The Oxygen Radical Absorbance Capacity at baseline had a median of 1561 μmol Trolox equivalents per liter, interquartile range 1200-1983. Baseline levels of 8-hydroxy-2-deoxy-guanosine had a median of 18 ng 8-hydroxy-2-deoxy-guanosine per mg creatinine, interquartile range 12-31. F2-isoprostane measured at 48 hours after stroke onset had a median value of 43 pg/mL, interquartile range 28-64.

The log of the normalized white matter hyperintensity volume was strongly correlated with age ($\rho=0.62$, $p<0.0001$), modified Rankin Scale ($\rho=0.20$, $p=0.04$), and Barthel Index ($\rho=-0.21$, $p=0.04$), as shown in Table 3. Table 4 demonstrates that the log of the normalized white matter hyperintensity volume did not correlate significantly with levels of, F2-isoprostane, 8-hydroxy-2-deoxy-guanosine, or the Oxygen Radical Absorbance Capacity. Multivariable regression modeling did not reveal any significant predictive value for log of the normalized white matter hyperintensity volume from the biomarkers, taken together and in combination with stroke severity, represented by NIH Stroke Scale.

Table 1. Demographics and Comorbidities of the Patient Cohort

Age (years), mean±SD	71±15
Body Mass Index, mean±SD	28±6
Race, n (%)	
White	149 (94)
Black	4 (3)
Other	5 (3)
Ethnicity, n (%)	
Hispanic	16 (10)
Gender, n (%)	
Male	88 (56)
Female	70 (44)
Comorbidities / Risk Factors, n (%)	
Hypertension	112 (71)
Diabetes Mellitus	31 (20)
Hyperlipidemia	69 (44)
Atrial Fibrillation	50 (32)
Prior Stroke	28 (18)
Prior Transient Ischemic Attack	11 (7)
Coronary Artery Disease	24 (15)
Body Mass Index ≥ 30	44 (33)
Current Smoking	32 (20)

Table 2. Distribution of White Matter Hyperintensity Volume and Oxidative Stress Biomarkers Measured at Both Timepoints after Stroke Onset

				n	Mean±SD	
Log of the normalized white matter hyperintensity volume				158	1.38±1.32	
	Baseline (within 9 hours)			48 hours		
	n	Median	25-75 IQR[A]	n	Median	25-75 IQR[A]
F2-isoprostane (pg/mL)	144	54	36-72	107	43	28-64
The Oxygen Radical Absorbance Capacity (μmol TE/L)[B]	142	1561	1200-1983	N/A		
8-hydroxy-2-deoxy-guanosine (ng/mg)[C]	27	18	12-31	N/A		

[A]25 - 75 IQR = the 25th to 75th percentile interquartile range

[B]μmol TE/L = micromoles of Trolox equivalents per liter

[C]8-hydroxy-2-deoxy-guanosine is measured as a ratio of its quantity divided by that of urine creatinine

Table 3. Correlation between White Matter Hyperintensity Volume and Age and Functional Scores

	n	Correlation (ρ)	p value
Age	158	0.62	< 0.0001
Modified Rankin Scale	109	0.20	0.04
Barthel Index of Activities of Daily Living	98	-0.21	0.04

Table 4. Correlation between White Matter Hyperintensity Volume and Oxidative Stress Biomarkers Measured at Both Timepoints after Stroke Onset

	Baseline (within 9 hours)			48 hours		
	n	Correlation (ρ)	p value	n	Correlation (ρ)	p value
F2-isoprostane	144	-0.01	0.91	107	0.15	0.11
The Oxygen Radical Absorbance Capacity	142	-0.07	0.39	N/A		
8-hydroxy-2-deoxy-guanosine	27	-0.01	0.95	N/A		

Discussion

White matter hyperintensity volume does not correlate with levels of established molecular biomarkers of systemic oxidative stress in our cohort of patients with acute ischemic stroke. Potential explanations for our negative findings include: 1) our systemic measures of oxidative stress may not be suitably accurate or sensitive as measures of oxidative stress in the brain; 2) we may not have a large enough sample; 3) we may not have measured oxidative stress at an appropriate time point to observe chronic oxidative state; or 4) white matter hyperintensity may not be related to oxidative stress, but other neuroimaging findings may be.

Perhaps we were unable to detect a true relationship between white matter hyperintensity and systemic biomarkers of oxidative stress because of theoretical or practical limitations inherent to the study. Oxidative stress biomarkers in acute ischemic stroke are limited by the difficulty of signal-to-noise ratio, which is to say that the measurement of oxidative stress has variability due to chance and that true differences may be within the same scale as chance differences. One study of intraindividual variability predicted that studies with one measurement of F2-isoprostanes would generate observed correlation coefficients 85% of their true value.(97) While this is quite good – indeed, considered the "gold standard" – it shows that, even in a perfect world, measurement of the true changes in oxidative stress are reflected less well in the biomarkers.(32) Other factors inherent to oxidative stress biomarkers contribute to this noise, such as sample oxidation between when it has left the patient and when it is quantified, an issue that has been of particular concern with 8-hydroxy-2-deoxy-guanosine.(26, 91, 98)

Also, the dynamic nature of levels of oxidative stresses and antioxidant defenses combined with the variety of species and mechanisms for both adds a layer of complexity to any measure of oxidative stress, especially when using systemic markers. Different oxidative species effect different types of oxidative damage, which are best measured by different molecular biomarkers.(91) Thus, measurement of overall oxidative damage by any one biomarker is limited, and it is better to work with techniques that combine more than one biomarker together. However, using multiple different biomarkers that represent different oxidant systems will not necessarily reduce complexity of the analysis.

For example, in our scenario, it is possible that the Oxygen Radical Absorbance Capacity and 8-hydroxy-2-deoxy-guanosine will change paradoxically because of processes mediating protection and recovery from injury.(31) Specifically, the Oxygen Radical Absorbance Capacity measures antioxidant capacity, so it is possible that protective mechanisms within the body will increase the concentration of antioxidant species in response to evolving oxidative injury. If the Oxygen Radical Absorbance Capacity detects only the increase of antioxidant species, our study would incorrectly conclude that the oxidative stress within the system has decreased. Though the antioxidant capacity has increased in this system, perhaps the total systemic oxidative stress has either not changed or actually increased and caused the increase in antioxidant capacity. Assaying 8-hydroxy-2-deoxy-guanosine may result in a similar scenario. Evidence has shown that these markers of DNA oxidation are repaired by cellular machinery, and potentially these processes become detrimental to the cell if they divert energy from other more important systems.(43) One could imagine that detectable levels of 8-hydroxy-2-deoxy-guanosine actually increase after the cell has undergone the worst of its damage, when the cells enzymatically remove the damaged bases from the DNA

during the repair process. Thus, in this situation the concentration of these markers paradoxically increases after the tissue has begun recovery from the stress.

The above considerations contribute to the 'noise' in the signal-to-noise ratio, but the 'signal' is another limiting factor in this study. When compared to the size the human body, even the largest brain infarct is miniscule. The brain is approximately 2% of the total body weight, and the largest common vascular distribution for stroke is the middle cerebral artery.(1) Thus, common strokes are significantly less than 1% of the volume of the body. Even with factors such as the high concentration of arachidonic acid and high oxygen metabolism contributing to potentially significant oxidative injury, it is difficult to detect any changes in such a small part of the body given the practical limitations to physically accessing the tissue.(26)

These limitations underscore the paradigm of monitoring processes in the central nervous system with markers in the body's periphery. In the case of persistent ischemia, one would expect any bloodborne markers of tissue damage will need to be brought to detection from tissue that has a severely impaired vascular supply and therefore equally impaired venous return. In this way, more affected tissues may be less likely to convey the impaired state of their function in peripheral biomarker studies. The 8-hydroxy-2-deoxy-guanosine literature hints at this effect in one excellent study where temporary middle cerebral artery occlusion was induced in rats and monitored with peripheral 8-hydroxy-2-deoxy-guanosine measurements.(44) This group found that, when comparing their models of permanent versus temporary ischemia, plasma 8-hydroxy-2-deoxy-guanosine levels were similar. The group implies that this means the level of oxidative damage is similar for the two types of ischemia.(44) However, presumably, there is actually less ischemia in the area reperfused by the temporary versus permanent

occlusion model. If this is the case, the reperfused tissue is evolving as much 8-hydroxy-2-deoxy-guanosine as the completely ischemic tissue, which is actually in a more dire state. Thus, the level of molecular biomarkers is not accurately reflecting the condition of the tissue from which it evolves. One of the reasons for this effect may be the lack of venous return from the most affected areas of tissue in the ischemic region of the brain.

We discussed previously theories of endothelial dysfunction surrounding the development of white matter hyperintensity.(87) In this theory, we relate systemic oxidative stress to endothelial dysfunction, which causes white matter hyperintensities in the brain. By definition, the source of systemic oxidative stress would be from a distant site in the body. The volume in which these reactive oxygen species would be diluted, the time necessary to transport them between their site of formation, and site of action would be quite a bit larger than if the source of oxidative species were local. Were the oxidative species formed in the hypothetical endothelial cells that are experiencing dysfunction, the scale of oxidative stress necessary to produce physiologically significant effects would be much smaller. In this experiment, because we are observing the evolution of events occurring on a molecular level in the brain in the distant peripheral plasma or – even more physiologically removed – urine, we are likely unable to witness local sources of oxidative stress in the brain unless they are on a catastrophic scale. Indeed, the types of endogenous oxidants that keep reactive oxygen species in check in normal biological systems are probably buffering most of the signs of oxidative stress we might detect were white matter hyperintensities being caused by oxidative stress in situ.

When planning for the study, we had hoped to analyze some 250 patients in our cohort. This projection was made from an estimated sample of 259, based on an α set at 0.05 and an assumed lower limit of 0.2 for a correlation coefficient, given a power of

0.90.(99) For this cohort, 158 subjects were included, which was well below our projections. The limitations were largely manpower. Potentially, we were not powered to detect a signal we might have expected. On the other hand, the correlations between white matter hyperintensity volume and functional scores were in the range of 0.2 and were well seen in our analysis.

Interestingly, we may see a trend in the data that points to a true correlation between white matter hyperintensity volume and F2-isoprostane. Observe that Spearman's ρ=-0.01 (p=0.91) at baseline versus ρ=0.15 (p=0.11) at 48 hours after stroke onset. Though neither of these correlations is statistically significant, it is notable that for the correlation at 48 hours, the proposed directionality is correct. We would expect increasing F2-isoprostanes to trend with increasing white matter hyperintensity volume. In addition, the value of the proposed ρ is within the range of the correlations between white matter hyperintensity and the functional scores, which we can conceptualize as positive controls in this study.

Our goals of detecting a correlation at baseline or at 48 hours were ambitious because of the interplay of acute versus chronic oxidative stress. Recall the theory that everyone lives at a level of chronic oxidative stress, which forms a kind of baseline oxidative state from which oxidative stress levels rise during an acute oxidative injury, such as that which evolves in acute ischemic stroke. All of our measurements of oxidative stress biomarkers fall well within the timeframe in which we might expect to see acute oxidative injury evolve. Of course, another factor at play in this situation concerns the idea of white matter hyperintensity producing 'sick brain.' This damaged brain tissue may be without the natural oxidative defense mechanisms that can help it deal with an acute stroke, as evidence has shown that infarct growth is larger in patients

with more white matter hyperintensity.(64) Potentially, the same sized acute ischemic injury in a brain with a lot of white matter hyperintensity would lead to a greater evolution of acute oxidative stress.

If one can visualize again the curve of acute oxidative stress overlaying chronic oxidative state, multiple factors influence oxidative state in the acute phase, including a somewhat delayed inflammatory response that occurs over the scale of days. Thus, it is difficult to put exact timeframes on when one will return to the chronic oxidative state. This dynamic underscores some weaknesses mentioned earlier regarding the current literature of oxidative stress in stroke, given studies that group together patients 5 days after stroke with patients 10 days after stroke.(55) Studies such as the parent study to this analysis endeavor to obtain significantly improved resolution regarding the switch from acute injury and acute oxidative stress back to chronic oxidative state.

In any case, this potential trend may point to a relationship between white matter hyperintensity volume and oxidative stress that is emerging as acute oxidative stress dissipates to the chronic oxidative state. Another potential trend in the values of F2-isoprostane shown in Table 2 may represent this change. When measured at baseline, the F2-isoprostane levels have a median of 54 pg/mL, with an interquartile range of 36-72. At 48 hours, the levels show a median of 43 pg/mL, with an interquartile range of 28-64. Though the difference between these values was not statistically verified, the trend is that values of F2-isoprostane are decreasing over this time period. Given that this is only at 48 hours, an improved measure of oxidative state might have been achieved with levels of the molecular biomarkers between 30 to 90 days after stroke. However, in this timeframe, some variability in chronic oxidative state may become evident. Patients might adopt lifestyle changes after a serious health event such as a stroke, which is of

scientific concern. These lifestyle changes would likely improve their future health but will also certainly impair the ability to retrospectively observe the chronic oxidative state prior to their stroke.

It is possible to statistically account for the acute stroke in our analysis by using multivariable regression modeling with a marker for stroke severity. The infarct size in our acute stroke population is a potential confounder, as larger acute strokes may be related to larger volumes of white matter hyperintensity as well as larger amounts of acute oxidative stress. In this study, we were able to use the NIH Stroke Scale score to adjust for the acute stroke. As we have MR imaging for all of the subjects, a better marker for the acute stroke would be the acute stroke size on diffusion-weighted imaging. However, infarct size measurements were unavailable for this analysis.

Oxidative stress, both systemic and local, may simply not cause white matter hyperintensity. The "operational" nature of the pathophysiologic explanations for white matter hyperintensity are important to remember.(64) Most of the theories put forth, including endothelial dysfunction, center around chronic ischemia as the root cause.(60) Vascular conditions relatively unrelated to oxidative stress may be chiefly responsible for this ischemia. One of those conditions, mentioned before, is periventricular venous collagenosis.(65) Thickening of the walls of veins in the brain parenchyma near the ventricles eventually leads to venous occlusion. The theory continues that the impaired drainage causes edema and eventually death of surrounding cells.(65) Potentially the result of this process, as it unfolds on a large scale, is seen as white matter hyperintensity.

Finally, even if white matter hyperintensity may not be related to oxidative stress, perhaps periventricular hyperintensity is. As discussed previously, previous authors that found a relationship between periventricular hyperintensity and F2-isoprostane did not

include the subcortical components of white matter hyperintensity in their analysis.(85) Interestingly, they conducted a secondary analysis by grading total white matter hyperintensity and were unable to find any correlations with F2-isoprostane. (Recall that they also did not find any direct correlations with F2-isoprostane and periventricular hyperintensity.) It has been articulated that one should consider "smooth periventricular signal abnormalities" as separate entities from the other components of white matter hyperintensity.(100) Causes of periventricular hyperintensity may be related to arteriosclerosis, seepage of CSF into the parenchyma near the ventricles, or perhaps interstitial fluid accumulating in tissues atrophied due to degenerative disease.(85, 100, 101) This theoretical segmentation is in contradistinction to the inclusive technique conducted in our study, given that we have considered all white matter hyperintensity together. That said, potentially the origins of periventricular hyperintensity differ from white matter hyperintensity in such a way that oxidative stress is related more directly to the formation of the former than the latter.

In this study of ischemic stroke patients, we have likely selected for patients who will have a cardiovascular or cardioembolic pathophysiology for their white matter hyperintensity, as they probably also have these factors contributing to their stroke risk. Thus, all locations of white matter hyperintensity seen in our cohort, periventricular or subcortical, will likely have a similar etiology, and we would not expect to find a distinct cause for the periventricular hyperintensities. In any case, the literature technically demonstrated a relationship between periventricular hyperintensity and biomarkers of oxidative stress, as opposed to white matter hyperintensity.(85) Reanalyzing the MRIs for the patients in this cohort following the established periventricular hyperintensity criteria may be informative in our cohort. Given that a grading scale is a significantly

smaller time-investment than the semi-automated quantification method for measuring white matter hyperintensity volume, periventricular hyperintensity grade may be a reasonable variable to examine in the larger study of the biomarkers of oxidative stress, especially if analyses demonstrated substantially different findings for periventricular hyperintensity.

In conclusion, though white matter hyperintensity volume was correlated with age and functional outcome scores in our study, we did not find a correlation between white matter hyperintensity volume and systemic biomarkers of oxidative stress. Our analysis has several important caveats. To be powered to detect these correlations, we projected a need for a larger cohort than was available at the time of this analysis. Additionally, we did not have an ideal marker for acute stroke severity, thought to be a likely confounder in this study. Finally, periventricular hyperintensity remains to be explored as a potential biomarker for oxidative stress in our cohort. These issues suggest that fertile ground remains in the search for the relationship between white matter hyperintensity and oxidative stress. Indeed, future advances toward a tool for improving trials in emerging stroke therapies will only come through the careful understanding of complex relationships such as these. With such great rewards in sight, great challenges should be expected along the way!

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