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ABSTRACT

BACKGROUND AND PURPOSE: Oxidative stress has been implicated as an important pathologic mechanism in the development of Alzheimer disease. The purpose of this study was to assess whether glutathione levels, detected noninvasively with proton MR spectroscopy, are associated with brain amyloidosis and memory in a community-dwelling cohort of healthy older adults.

MATERIALS AND METHODS: Fifteen cognitively healthy subjects were prospectively enrolled in this study. All subjects underwent [1]H-MR spectroscopy of glutathione, a positron-emission tomography scan with an amyloid tracer, and neuropsychological testing by using the Repeatable Battery for the Assessment of Neuropsychological Status. Associations among glutathione levels, brain amyloidosis, and memory were assessed by using multivariate regression models.

RESULTS: Lower glutathione levels were associated with greater brain amyloidosis in the temporal ($P = .03$) and parietal ($P = .05$) regions, adjusted for *apolipoprotein E ε4* carrier status. There were no significant associations between glutathione levels and cognitive scores.

CONCLUSIONS: This study found an association between cortical glutathione levels and brain amyloidosis in healthy older adults, suggesting a potential role for [1]H-MR spectroscopy measures of glutathione as a noninvasive biomarker of early Alzheimer disease pathogenesis.

ABBREVIATIONS: AD = Alzheimer disease; ADNI = Alzheimer's Disease Neuroimaging Initiative; *APOE* = *apolipoprotein E*; GSH = glutathione; PiB = Pittsburgh compound-B; RBANS = Repeatable Battery for the Assessment of Neuropsychological Status

Alzheimer disease (AD), a devastating neurodegenerative disorder afflicting >11% of individuals older than 65 years of age,[1] is currently the sixth leading cause of death in the United States. Various forms of therapy have failed to show clinical benefit in individuals with AD.[2] In the absence of disease-modifying pharmacotherapy for AD, identifying and potentially targeting early pathologic processes that may lead to the development of AD are essential in developing prevention strategies.

Oxidative stress, defined as excessive production of free radicals relative to total tissue antioxidant reserves, has emerged from in vitro and preclinical studies as a key pathologic process in the development of AD.[3-8] In transgenic mouse models, depletion of the reduced form of the tripeptide thiol glutathione (GSH)—the most abundant intracellular antioxidant and free radical scavenger and a reliable marker of oxidative stress[9]—has been reported to precede amyloid oligomerization and plaque formation,[10,11] both pathologic hallmarks of AD. A self-propagating cycle of free radical formation, oxidative stress, and amyloid plaque formation has also been shown in vitro.[12] Furthermore, it has been suggested that amyloid may have antioxidant properties, thereby serving as a compensatory mechanism in the presence of oxidative stress.[13] However, the relationship between oxidative stress and amyloidosis in humans remains poorly understood, particularly early in the disease course when oxidative stress may serve as a potential target for disease-modifying interventions.

The primary aim of this study was therefore to assess the relationship between proton MR spectroscopy measures of GSH levels and brain amyloidosis, as assessed by positron-emission tomography with the amyloid tracer Pittsburgh compound-B (PiB),[14] in a prospective cognitively healthy community cohort of elderly subjects. Secondarily, we aimed to assess the relationship between GSH levels and memory. Last, we investigated whether GSH levels were associated with potentially modifiable AD risk factors.

MATERIALS AND METHODS

Subjects

Fifteen cognitively healthy subjects, recruited through flyers posted in the community, newspaper advertisements, and ambulatory care clinics, were prospectively enrolled. All subjects gave written informed consent to participate in this study, which was approved by the institutional review board of our institution.

Inclusion criteria consisted of individuals between 55 and 75 years of age with intact ability to perform all routine activities of daily living, including living independently in the community. None of the subjects met the criteria for mild cognitive impairment or AD. Subjects were also excluded if they had comorbid medical conditions that could impact brain function, including major psychiatric disorders (ie, major depression, bipolar disorder, psychosis), brain tumors, prior strokes, significant traumatic brain injury (defined as requiring a visit to the emergency department or a hospital admission), seizure disorders, recent illicit drug use, alcohol abuse, and other major medical conditions, such as heart failure, recent myocardial infarction, renal failure, liver disease, chronic obstructive pulmonary disease, and malignancy.

Clinical Data

All subjects completed detailed questionnaires about their medical history and medical records were also examined. Clinical data collected included recent weight and height, cholesterol levels, blood pressure measurements, and the number of hours of exercise per week, because these factors have been reported to be associated with the risk for AD.[15,16] Exercise was defined as physical activity more strenuous than daily routine activity. We also elicited a family history of dementia, because genetics could explain increased brain amyloidosis in otherwise cognitively healthy subjects.[17]

Cognitive Battery

Cognitive testing was performed by a board-certified neuropsychologist (L.D.R.). Patients were first screened for depression and anxiety by using the Beck Depression Inventory-II[18] and the Beck Anxiety Inventory.[19] Immediate and delayed memory were assessed by using subscores of the Repeatable Battery for the Assessment of Neuropsychological Status (RBANS) based on tasks that involved recalling a list of words and a short story.[20] Additional cognitive domains assessed included visuospatial and constructional function, assessed with figure copying and line orientation tasks; attention, assessed with digit span and coding tasks; and language, assessed with picture naming and semantic fluency tasks. The RBANS has been previously reported to have 90% accuracy for discriminating between cognitively healthy individuals and those with mild cognitive impairment.[21]

Apolipoprotein E ε4 Genotyping

Blood samples were obtained from all subjects to isolate DNA for *Apolipoprotein E* (*APOE*) genotyping, which was performed by using polymerase chain reaction amplification, allele-specific primers, and identification of fragments on an agarose gel.[22]
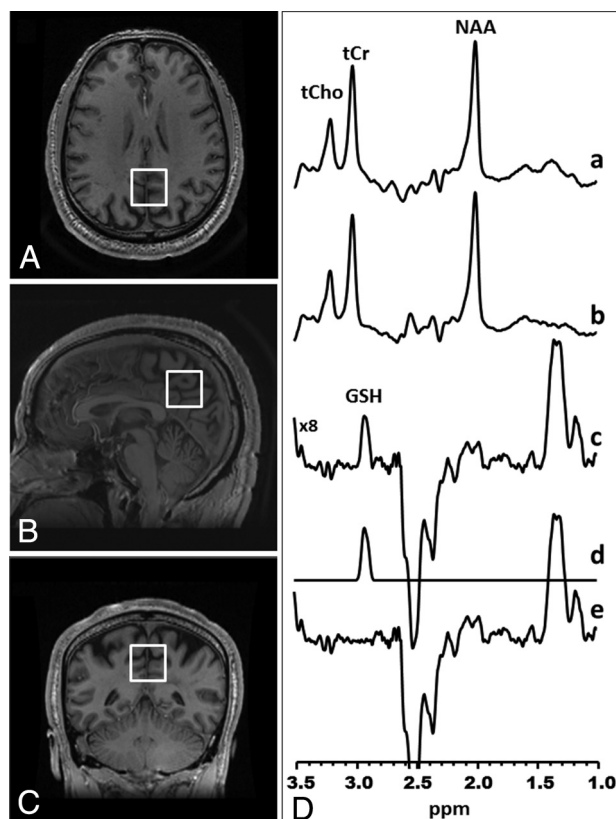

FIG 1. Glutathione detection in the medial parietal lobe with J-edited ¹H-MR spectroscopy. Axial (*A*), sagittal (*B*), and coronal (*C*) MR images of a human brain, with depiction of the size, location, and angulation of the voxel of interest in the medial parietal lobe. *D*, Demonstration of in vivo human brain glutathione detection by ¹H-MR spectroscopy: spectra *a* and *b*, single-voxel subspectra acquired in 15 minutes with the editing pulse on and off and 290 (580 total) interleaved averages; spectrum *c*, difference between spectra *a* and *b*, showing the edited brain GSH resonance at 2.98 ppm; spectrum *d*, model fitting of spectrum *c* to obtain the GSH peak area; spectrum *e*, residual of the difference between spectra *c* and *d*. tCho indicates total choline; tCr, total creatine.

MR Imaging and Spectroscopy Data Acquisition and Analysis

All subjects underwent standardized structural MR imaging of the brain and single-voxel ¹H-MR spectroscopy on a research-dedicated 3T MR imaging system (Excite HD; GE Healthcare, Milwaukee, Wisconsin) with an 8-channel phased array head coil. The MR imaging protocol consisted of a structural T1-weighted spoiled gradient-recalled echo volumetric scan for tissue segmentation and an axial fast fluid-attenuated inversion recovery scan to exclude focal pathology.

In vivo ¹H-MR spectroscopy data were obtained from a $2.5 \times 2.5 \times 2.5$ cm³ voxel prescribed in the medial parietal lobe to include the posterior cingulate gyrus and precuneus—a region chosen because multiple prior studies reported early involvement of these regions by AD due to their inclusion in the memory network.[23-26] The standard J-edited spin-echo difference method with TE/TR = 68/1500 ms was used to measure the levels of reduced GSH, as previously described[27-30] and illustrated in Fig 1. Although it has been suggested that a TE of 120 ms is optimal for GSH detection by J-editing,[31] we opted to use a TE of 68 ms because it yields a difference spectrum in which the coedited as-

partyl (CH2) resonances of NAA around 2.5 ppm are inverted and clearly separated from the noninverted GSH resonance, facilitating spectral fitting (Fig 1).[27,28]

Briefly, a pair of frequency-selective inversion pulses was inserted into the standard point-resolved spectroscopy sequence method and was applied on alternate scans at the frequency of the GSH α-cysteinyl resonance at 4.56 ppm while avoiding excitation of oxidized GSH α-cysteinyl at 3.28 ppm.[32] This process resulted in 2 subspectra in which reduced GSH, but not oxidized GSH, was alternately inverted or not inverted. Subtracting these 2 subspectra yielded a [1]H-MR spectrum consisting of only the edited GSH β-cysteinyl resonance at 2.98 ppm. A high test-retest reliability has been reported for detection of γ-aminobutyric acid with this MR spectroscopy technique on the same 3T GE Healthcare Excite HD instrument.[33] Spectral data for this study were acquired in 15 minutes by using 290 interleaved excitations (580 total) with the editing pulses on or off. The area under the GSH resonance, which is proportional to the concentration of GSH in the voxel of interest, was obtained by frequency-domain spectral fitting as previously described.[28] The derived GSH peak areas were then expressed semiquantitatively as ratios relative to the unsuppressed intravoxel water signal for normalization across subjects before being used in group analyses. To estimate the proportions of gray matter, white matter, and CSF contained in the voxel of interest, the volumetric spoiled gradient-recalled echo MR imaging data were segmented by using Statistical Parametric Mapping software (SPM8; http://www.fil.ion.ucl.ac.uk/spm/software/spm8).

Pittsburgh Compound-B PET Image Acquisition and Analysis

All subjects underwent an amyloid PET scanning on a Biograph PET-CT scanner (Siemens, Erlangen, Germany; 1-mm full width at half maximum, 25-cm FOV) by using a standardized research protocol.[17] All subjects received an intravenous catheter for injection of 15 mCi of PiB. Sixty minutes after injection, subjects were scanned for 30 minutes with their eyes open in a quiet, dimly lit room. A low-dose CT scan was acquired for attenuation correction, and all images were reconstructed into a 512 × 512 matrix.

Summed PET images corresponding to the 60–90 minutes of PiB data were generated and nonlinearly normalized to a PiB template. The PiB template was generated by averaging the summed images of 48 cognitively healthy individuals in the same age range, which were downloaded from the Alzheimer's Disease Neuroimaging Initiative (ADNI) on-line data repository (adni.loni.usc.edu). The ADNI was launched in 2003 as a public-private partnership, led by Principal Investigator Michael W. Weiner, MD, with the primary goal of testing whether serial MR imaging, positron-emission tomography, other biologic markers, and clinical and neuropsychological assessment may be combined to measure the progression of mild cognitive impairment and early Alzheimer disease. More information may be obtained at www.adni-info.org.

Orientation and origin for all the PiB PET images were automatically fixed to the anterior commissure to match the templates used in Statistical Parametric Mapping (SPM; Wellcome Department of Imaging Neuroscience, London, UK), because the "normal-ize" function of SPM uses the origin as a starting estimate. These reoriented PiB PET images and the mean PiB template were skull-stripped with the FSL Brain Extraction Tool (http://fsl.fmrib.ox.ac.uk/fsl/fslwiki/BET)[34] to avoid any bias induced by skull staining. All the skull-stripped PiB PET images were then nonlinearly warped to the skull-stripped mean PiB template. Gray matter regions were parcellated by using the Automated Anatomical Labeling atlas of SPM to obtain 116 automated ROIs.[35] Regional PiB uptake values were then normalized by the subject's cerebellar reference uptake.

Prior ADNI publications determined that the 4 large regions of the brain that are most useful in measuring the degree of brain amyloidosis are the frontal, anterior/posterior cingulate, lateral parietal, and lateral temporal regions, with the cerebellum as a reference region.[36-39] Using ROIs from the Automated Anatomical Labeling atlas, we determined amyloid deposition in the frontal region by averaging the uptake values from the bilateral superior frontal, bilateral superior orbital frontal, bilateral middle frontal, bilateral inferior frontal opercular, bilateral inferior frontal triangularis, bilateral supplemental motor, bilateral medial superior frontal, and bilateral middle orbital frontal regions of the brain. The cingulate region included the bilateral anterior, middle, and posterior cingulum regions. The lateral parietal region included the bilateral superior and inferior parietal regions, as well as the precuneus. The lateral temporal region included the bilateral and superior middle temporal regions.

Statistical Analysis

All statistical analyses were performed in STATA, Version 13 (StataCorp, College Station, Texas).

The potential influence of voxel tissue heterogeneity and brain matter content in the analyses was examined by testing for associations between brain matter proportions in the voxel of interest and both MR spectroscopy measures of GSH levels and PiB PET measures of amyloid levels. We also examined the distribution of brain tissue proportions within our subject cohort to identify outliers.

To assess whether there was an association between GSH and brain amyloidosis, based on uptake on PiB PET, we used ordinary least-squares regression analysis with amyloid levels in each of the 4 brain regions as the outcome variable and GSH as the predictor variable. Because *APOE ε4* carrier status has been shown to be associated with increased brain amyloidosis in the literature,[40-43] carrier status was included as a covariate to adjust for this confounding factor.

The robustness of any association between GSH and amyloidosis was examined by bootstrapping the original cohort of subjects 1000 times to obtain 95% confidence intervals.[44] The effect of a clear outlier (high parietal amyloidosis and low GSH) on the association was examined by performing the analyses both with and without this data point. To assess the effect sizes of our associations, we estimated the correlation coefficients between GSH and amyloidosis, with <0.1 indicating a small effect, $0.1–0.5$ indicating a medium effect, and >0.5 indicating a large effect.[45] We also calculated the partial eta-squared for GSH on the basis of the regression models,[46] with <0.06 indicating a small effect, $0.06–0.14$ indicating a medium effect, and >0.14 indicating a large effect.[47]

Table 1: Results of the regression analyses showing associations between glutathione and regional brain amyloidosis

	Regression Coefficients			
	Frontal Amyloidosis	Cingulate Amyloidosis	Parietal Amyloidosis	Temporal Amyloidosis
Glutathione levels (±SE)	−39 ± 90	−27 ± 174	−308 ± 143	−209 ± 85
P value	.67	.88	.05	.03

Note:—SE indicates standard error.
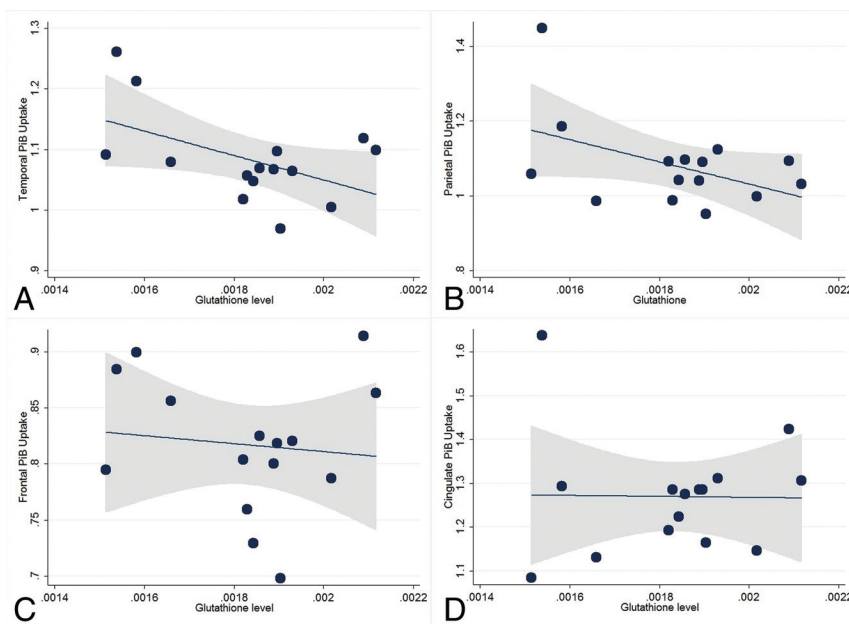
FIG 2. Scatterplots showing the relationship between glutathione levels and brain amyloidosis by region. After we adjusted for *APOE4* carrier status, lower glutathione levels were associated with higher levels of amyloidosis in the temporal (A) (P = .03) and in the parietal (B) (P = .05) regions, but not in the frontal (C) (P = .67) or cingulate (D) (P = .88) regions. Fitted lines and 95% confidence intervals (*shaded area*) are also shown.

Table 2: Results of the regression analyses showing associations between glutathione and age-adjusted cognitive scores on the Repeatable Battery for the Assessment of Neuropsychological Status

	Regression Coefficients (×10²)				
	Immediate Memory Subscore	Delayed Memory Subscore	Visuospatial/ Construction Subscore	Language Subscore	Attention Subscore
Glutathione levels (±SE)	317 ± 198	232 ± 171	113 ± 183	−24 ± 242	361 ± 247
P value	.14	.20	.55	.92	.17

Note:—SE indicates standard error.

To assess whether there was an association between GSH levels and memory, we again used ordinary least-squares regression analysis with GSH as the predictor and the age-adjusted subscores from the RBANS as the outcome variable. Because *APOE ε4* carrier status is known to be a risk factor for AD,[48,49] it was again included as a covariate.

Finally, we explored the associations between GSH and potential mediators of oxidative stress, including obesity, hypercholesterolemia, hypertension, and exercise, again by using ordinary least-squares regression analyses.

RESULTS

Subjects ranged in age from 55 to 72 years (mean, 63 ± 5 years), and 5 (33%) of the subjects were women. All subjects completed at least a year of college, with a mean of 16 ± 3 years of education.

Five (33%) subjects had a family history of dementia. Ten (67%) subjects had the *APOE ε3/ε3* genotype, 2 (13%) subjects carried the *APOE ε2/ε3* genotype, and 3 (20%) subjects had the *APOE ε3/ε4* genotype. Eight (53%) subjects had comorbid hypercholesterolemia, and 7 (47%) subjects had comorbid hypertension. Body mass index ranged from 22 to 37, with a mean of 29 ± 4. Subjects reported exercising 0–14 hours per week, with a mean of 3 ± 4 hours.

The results of the regression analyses evaluating the association between ¹H-MR spectroscopy GSH and amyloidosis as assessed by PiB PET are provided in Table 1 and shown in Fig 2. There were no significant associations between tissue proportions and GSH levels or amyloidosis. After we adjusted for *APOE ε4* status, GSH levels were inversely associated with levels of amyloidosis in both the temporal region (P = .03, coefficient = −209; 95% confidence interval, −395 to −23) and parietal region (P = .05, coefficient = −308; 95% confidence interval, −621 to 3). Post hoc bootstrapping yielded a P value of .08 (95% confidence interval, −441 to 23) for the temporal region and 0.1 (95% confidence interval, −705 to 88) for the parietal region. In addition, the association between parietal region amyloidosis and GSH appears to have been primarily driven by 1 subject with high amyloidosis and low GSH. The association was no longer significant when this outlier was excluded (coefficient = −62, P = .60). There was no significant association between GSH levels and either frontal (P = .67) or cingulate (P = .88) region amyloidosis.

The correlation coefficient between GSH and temporal region amyloidosis was −0.51, indicating a large effect size. The correlation coefficient between GSH and parietal region amyloidosis was −0.47, indicating a medium effect size. In the regression models, the effect sizes for GSH were large, explaining a greater proportion of the variance in amyloidosis than in *APOE ε4* status. The partial eta-squared for GSH and *APOE ε4* was 0.33 and 0.25, respectively, for the temporal region. The partial eta-squared for GSH and *APOE ε4* was 0.28 and 0.23, respectively, for the parietal region.

The results of the regression analyses evaluating the association between GSH and cognition are provided in Table 2 and shown in Fig 3. None of the associations were statistically significant.

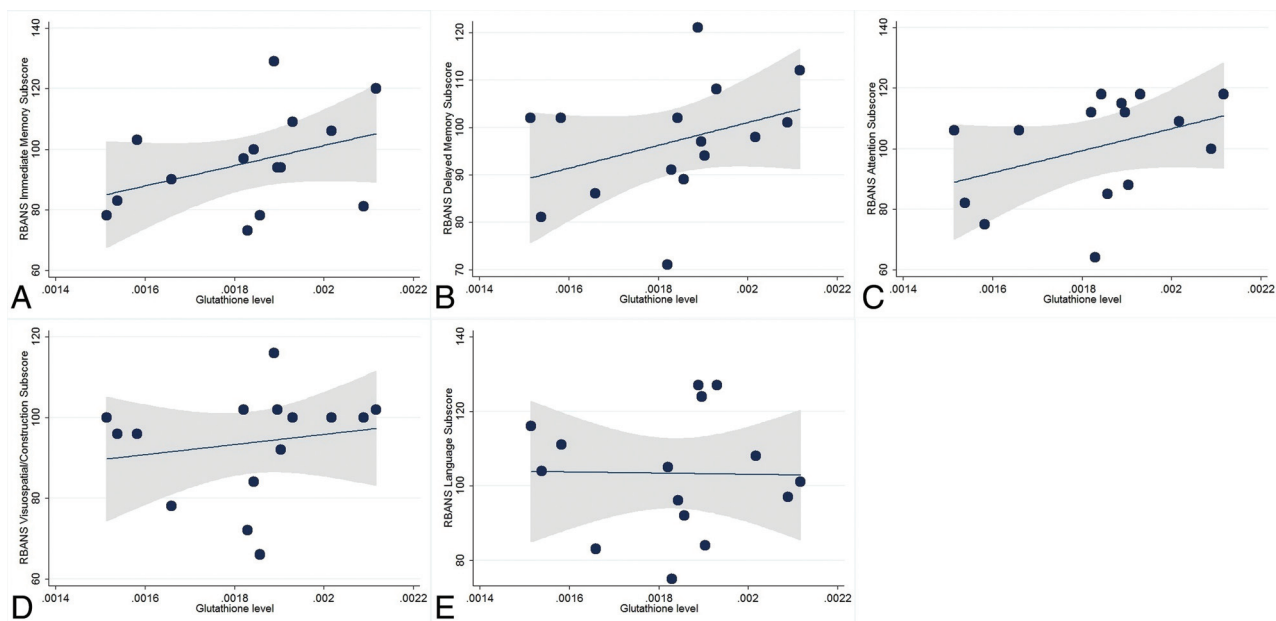The results of the exploratory regression analyses evaluating

FIG 3. Scatterplots showing the relationship between glutathione levels and scores on the Repeatable Battery for the Assessment of Neuropsychological Status. There were no significant associations between higher glutathione levels and higher age-adjusted immediate (*A*) ($P = .14$) and delayed (*B*) ($P = .20$) memory subscores. Higher glutathione levels were not associated with attention (*C*) ($P = .17$), visuospatial/construction (*D*) ($P = .55$), or language (*E*) ($P = .92$) subscores. Fitted lines and 95% confidence intervals (*shaded area*) are also shown.

Table 3: Association between Alzheimer risk factors and glutathione levels

	Regression Coefficients ($\times 10^{-5}$)			
	Body Mass Index	**Exercise (hr per wk)**	**Comorbid Hypertension**	**Comorbid Hypercholesterolemia**
Glutathione levels ($\pm$SE)	-2.2 ± 1.23	-1.3 ± 1.4	-5.5 ± 9.9	-8.4 ± 11.0
P value	.08	.36	.59	.46

Note:—SE indicates standard error.

the association between GSH levels and risk factors for AD are shown in Table 3. There was a trend-level inverse association between body mass index and GSH levels ($P = .08$). Exercise, hypercholesterolemia, and hypertension were not significantly associated with GSH levels ($P > .05$).

DISCUSSION

The value of noninvasive measurement of GSH by [1]H-MR spectroscopy lies in its potential to directly implicate and support a role for oxidative stress in the early stages of AD development. Using this technique, the present study sought to identify a role for oxidative stress in a prospective cohort of healthy older subjects, assessing potential associations between cortical GSH levels and brain amyloidosis and between GSH and memory. The major finding was that GSH levels, measured with [1]H-MR spectroscopy, are negatively associated with brain amyloidosis, as assessed with PiB PET, in the temporal and parietal regions. In this cognitively healthy cohort, there were no associations between GSH levels and immediate and delayed memory.

The inverse association between levels of GSH and temporal and parietal amyloid levels supports a role for oxidative stress in amyloid plaque formation—a finding that is consistent with prior laboratory and preclinical studies.[5,6-8,46-49] An association between oxidative stress and amyloidosis has also been suggested by clinical studies on AD. Mandal et al[50] found that GSH levels measured by [1]H-MR spectroscopy could accurately discriminate among

healthy subjects, individuals with mild cognitive impairment, and patients with AD, with decreased GSH levels being associated with increased levels of cognitive impairment. In postmortem AD brains,[51] depleted GSH levels accompanied the diagnosis of AD. There have also been reports associating GSH depletion with mitochondrial dysfunction[52,53] and neuronal degeneration.[54,55] On the other hand, increased GSH levels have been reported in those with mild cognitive impairment compared with healthy subjects, suggesting that there may be a compensatory up-regulation of GSH in the early stages of AD.[56] However, no direct relationship between oxidative stress and amyloidosis was established in any of the prior clinical studies because subject groups were defined clinically without quantifying the degree of underlying amyloidosis. In the present study, with an advanced [1]H-MR spectroscopy editing technique that enables reliable in vivo measurements of GSH, we have obtained strong preliminary evidence of an inverse relationship between GSH levels and amyloidosis in older adults, even before the onset of mild cognitive impairment. Replication in larger cohorts would both solidify this result and support measurement of brain GSH levels with [1]H-MR spectroscopy as a noninvasive biomarker of AD risk early in disease development.

This study also investigated whether GSH levels are associated with memory because memory deficits are known to be the earliest clinical manifestation of AD[57] and predict time-to-progression from cognitively healthy to mild cognitive impairment.[58] Because oxidative stress can exert deleterious effects on mitochondrial function and neuronal integrity, we surmised that GSH depletion could also lead to memory dysfunction. Two prior studies that included subjects with mild cognitive impairment and AD

reported conflicting results, with one reporting GSH deficits in mild cognitive impairment and AD[50] and the other reporting a potential compensatory increase of GSH in mild cognitive impairment.[56] Our study found no associations between GSH levels and cognitive scores in our cognitively healthy cohort, necessitating further studies in larger cohorts.

In exploring associations between GSH and AD risk factors, we found a trend-level inverse association between GSH levels and body mass index. Barnes and Yaffe[15] previously reported that up to 54% of AD cases may be attributable to modifiable risk factors, with 21% attributable to physical inactivity and 7% attributable to obesity. In the present cohort, we explored the association between these risk factors and GSH levels and found a trend-level negative association with body mass index, which could be consistent with a prior large cohort study of >2000 subjects, which found increased markers of oxidative stress, which would deplete GSH, with increased body mass index.[59] If this finding is validated, monitoring GSH levels by [1]H-MR spectroscopy could also serve as a biomarker of the potential benefits of various lifestyle-modification regimens, without the radiation risk and cost of PET imaging.

Finally, this study has a number of limitations. First, the sample size was relatively small, potentially limiting both statistical power and generalizability of the findings. Replication of these findings in larger cohorts will be necessary. Second, our cohort consisted of cognitively healthy individuals. As a result, subjects did not have significant memory deficits, possibly limiting our ability to detect statistically significant associations between GSH and memory, particularly in a small cohort. Third, we targeted the precuneus for GSH measurement with MR spectroscopy because this region is affected early in AD pathology. However, there may be abnormalities in other brain regions, which would need to be investigated to obtain a more complete understanding of oxidative stress–associated brain damage in AD and its prodromal stages. Furthermore, although we found associations between GSH and amyloidosis, longitudinal studies are necessary to determine whether decreased GSH levels increase subsequent risk of developing AD. Finally, we did not enroll a control group for comparison with our cognitively healthy cohort. As a result, it is not known whether the GSH levels detected in our cohort are significantly abnormal.

CONCLUSIONS
This is the first study, to our knowledge, to explore in vivo associations between GSH and brain amyloidosis, as well as GSH and memory in a cognitively healthy cohort. This supports a role for [1]H-MR spectroscopy measures of cortical glutathione as a potential early biomarker of AD pathology and therapeutic response monitoring of existing or future disease-modifying interventions targeting oxidative stress.

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