Relationships among Cortical Glutathione Levels, Brain Amyloidosis, and Memory in Healthy Older Adults Investigated In Vivo with $^1$H-MRS and Pittsburgh Compound-B PET


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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 affecting $>$11% of individuals older than 65 years of age, 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. 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. 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—has been reported to precede amyloid oligomerization and plaque formation, 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. Furthermore, it has been suggested that amyloid may have antioxidant properties, thereby serving as a compensatory mechanism in the presence of oxidative stress. 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), 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.

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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. 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.

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 and the Beck Anxiety Inventory. Immediate and delayed memory were involved recalling a list of words and a short story. Additional assessment of Neuropsychological Status (RBANS) based on tasks that assessed by using subscores of the Repeatable Battery for the Assessment and Analysis of Neuropsychological Status (RBANS) battery. The RBANS has been previously reported to have 90% accuracy for discriminating between cognitively healthy individuals and those with mild cognitive impairment.

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.

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 × 2.5 × 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. 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 and illustrated in Fig 1. Although it has been suggested that a TE of 120 ms is optimal for GSH detection by J-editing, 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-cysteyin resonance at 4.56 ppm while avoiding excitation of oxidized GSH α-cysteiny1 at 3.28 ppm.32 This process resulted in 2 subspectra in which reduced GSH, but not oxidized GSH, was of oxidized GSH

ment of Imaging Neuroscience, London, UK), because the “normal-

of a clear outlier (high parietal amyloidosis and low GSH) on the

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

<table>
<thead>
<tr>
<th>Glutathione levels (± SE)</th>
<th>Frontal Amyloidosis (P value)</th>
<th>Cingulate Amyloidosis</th>
<th>Parietal Amyloidosis</th>
<th>Temporal Amyloidosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>−39 ± 90 (.67)</td>
<td>−27 ± 174 (.88)</td>
<td>−308 ± 143 (.05)</td>
<td>−209 ± 85 (.03)</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Glutathione levels (± SE)</th>
<th>Immediate Memory Subscore</th>
<th>Delayed Memory Subscore</th>
<th>Visuospatial/Construction Subscore</th>
<th>Language Subscore</th>
<th>Attention Subscore</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>317 ± 198 (.14)</td>
<td>232 ± 171 (.20)</td>
<td>113 ± 183 (.55)</td>
<td>−24 ± 242 (.92)</td>
<td>361 ± 247 (.17)</td>
</tr>
</tbody>
</table>

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 e4 carrier status is known to be a risk factor for AD, 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 e3/e3 genotype, 2 (13%) subjects carried the APOE e2/e3 genotype, and 3 (20%) subjects had the APOE e3/e4 genotype. Eight (53%) subjects had co-morbid hypercholesterolemia, and 7 (47%) subjects had co-morbid 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 e4 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 parietal region amyloidosis was −0.51, indicating a large effect size. The correlation coefficient between GSH and temporal 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 e4 status. The partial eta-squared for GSH and APOE e4 was 0.33 and 0.25, respectively, for the temporal region. The partial eta-squared for GSH and APOE e4 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...
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 1H-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 1H-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. An association between oxidative stress and amyloidosis has also been suggested by clinical studies on AD. Mandal et al found that GSH levels measured by 1H-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, depleted GSH levels accompanied the diagnosis of AD. There have also been reports associating GSH depletion with mitochondrial dysfunction and neuronal degeneration. 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. 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 1H-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 1H-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 and predict time-to-progression from cognitively healthy to mild cognitive impairment. 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

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**Table 3: Association between Alzheimer risk factors and glutathione levels**

<table>
<thead>
<tr>
<th>Glutathione levels (± SE)</th>
<th>Body Mass Index</th>
<th>Exercise (hr per wk)</th>
<th>Comorbid Hypertension</th>
<th>Comorbid Hypercholesterolemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regression Coefficients (<em>10⁻⁵</em>)</td>
<td>.22 ± 1.23</td>
<td>−13 ± 14</td>
<td>−5.5 ± 9.9</td>
<td>−8.4 ± 11.0</td>
</tr>
<tr>
<td>P value</td>
<td>.08</td>
<td>.36</td>
<td>.59</td>
<td>.46</td>
</tr>
</tbody>
</table>

Note: −SE indicates standard error.
reported conflicting results, with one reporting GSH deficits in mild cognitive impairment and AD50 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 Yaffe57 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 1H-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 1H-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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