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Global *N*-Acetylaspartate in Normal Subjects, Mild Cognitive Impairment and Alzheimer's Disease Patients

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Abstract.
Background: Mild cognitive impairment (MCI) is an intermediary state on the way to Alzheimer's disease (AD). Little is known about whole brain concentration of the neuronal marker, *N*-acetylaspartate (NAA) in MCI patients.
Objective: To test the hypothesis that since MCI and AD are both neurodegenerative, quantification of the NAA in their whole brain (WBNAA) could differentiate them from cognitively-intact matched controls.
Methods: Proton MR spectroscopy to quantify the WBNAA was applied to 197 subjects (86 females) 72.6 ± 8.4 years old (mean $\pm$ standard deviation). Of these, 102 were cognitively intact, 42 diagnosed as MCI, and 53 as probable AD. Their WBNAA amounts were converted into absolute concentration by dividing with the brain volume segmented from the MRI that also yielded the fractional brain volume (fBPV), an atrophy metric.
Results: WBNAA concentration of MCI and AD patients (10.5 ± 3.0 and 10.1 ± 2.9 mM) were not significantly different ($p = 0.85$). They were, however, highly significantly 25–29% lower than the 14.1 ± 2.4 mM of normal matched controls ($p < 10^{-4}$). The fBPV of MCI and AD patients (72.9 ± 4.9 and 69.9 ± 4.7%) differed significantly from each other (4%, $p = 0.02$) and both were significantly lower than the 74.6 ± 4.4% of normal elderly (2%, $p = 0.003$ for MCI; 6%, $p < 10^{-4}$ for AD). ROC curve analysis has shown WBNAA to have 70.5% sensitivity and 84.3% specificity to differentiate MCI or AD patients from normal elderly versus just 68.4 and 65.7% for fBPV.
Conclusion: Low WBNAA in MCI patients compared with cognitively normal contemporaries may indicate early neuronal damage accumulation and supports the notion of MCI as an early stage of AD. It also suggests WBNAA as a potential marker of early AD pathology.

Keywords: Alzheimer's disease, magnetic resonance spectroscopy, mild cognitive impairment, N-acetylaspartate, normal aging

INTRODUCTION

Neuropathological studies have shown Alzheimer's disease (AD) to follow a stagewise progressive course involving the medial temporal lobe and subsequently spread to temporal and parietal cortex [1]. Clinically, the decline into AD is thought to proceed through an intermediate stage of mild cognitive impairment (MCI) characterized by subjective memory complaints accompanied by decline in memory or other cognitive domain performance but preserved general intellectual function and absence of dementia [2–4]. Estimated to

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be tenfold more common than AD, MCI affects anywhere from 12.5% to as high as 21.8% of the elderly [5–7]. Indeed, while the annual incidence rates of AD in normal elderly are under 5% [8–10], they are estimated at 10% in MCI, especially of the amnestic type [3, 8]. These estimates are even higher in clinical settings [11]. Since MCI is associated with greater mortality [12, 13] and risk to develop AD [3], it is clearly of special public health interest.

Despite growing relevance of MCI to public health, its diagnostic criteria and neuroimaging features are still debated. This may be due at least in part to the several identified subtypes (amnestic, non-amnestic, single or multiple domains) that may also help explain the variable courses [4]. For example, 10 to 20% of individuals initially diagnosed as MCI revert to "normal" at follow-up [8–10]. Consequently, early detection is difficult, partly due to the lack of well-established, non-invasive markers not necessitating radiation exposure. This can confound proper selection of patients who could benefit from early interventions.

Postmortem examinations show more than half of the subjects with MCI have a Braak stage of III/IV [14]. While these involve mostly entorhinal and transentorhinal regions, neurofibrillary tangles were already found in the striatum, thalamus, and isocortex [1]. Over half the MCI patients in that study also met the consortium to establish a registry for AD (CERAD) criteria for probable or definite AD [14]. Similarly, although imaging indicates that AD manifests first in medial temporal lobe morphology, abnormalities in MCI are not limited to the hippocampus [15]. In fact, multiple white matter (WM) regions in all lobes exhibited diffusion properties [16–18] and magnetization transfer differences from controls [19]. These reports all suggest widespread cortical and subcortical pathology already in the prodromal stage, warranting examination of multiple regions or global changes.

Since MCI and AD target neuronal cells, progression could be monitored via their specific marker, the amino-acid derivative *N*-acetyl-aspartate (NAA), whose prominent peak in proton MR spectroscopy (^{1}H-MRS) of the mammalian brain makes it simple to quantify [20, 21]. Its decline reported for all CNS disorders [22] is most commonly seen in hippocampi and middle temporal lobes of AD and MCI patients [23, 24] but also reported in paratrigonal WM and the posterior cingulate [25, 26]. The diffuse nature and the neuronal substrate of NAA, render its whole-brain (WBNAA) quantification well suited to track the total disease load [27, 28] in order to distinguish normal from MCI and AD, as shown by Falini et al. [29]. To our knowledge, however, no other study has compared WBNAA among these three diagnostic groups. Consequently, in the present study we examine the WBNAA levels in large cohorts of normal elderly, MCI and AD patients to test three hypotheses: (i) That MCI patients' WBNAA is lower than cognitively intact normal contemporaries; (ii) that AD patients' WBNAA is lower than MCI; and (iii) that these WBNAA levels are sufficiently different to enable unique assignment.

MATERIALS AND METHODS

Human subjects

One hundred and ninety seven elderly (111 males, 86 females), recruited from the Memory Clinic, Department of Geriatrics, University Hospital Basel, Switzerland, were enrolled. Of these 102 [64 men and 38 women, 72.4 ± 8.3 (51 to 89) years old) were deemed cognitively normal based on extensive neuropsychological testing (see below). Forty two individuals (24 men and 18 women, 70.8 ± 7.8 (50 to 84) years old) fulfilled criteria for MCI [4] and 53 subjects (23 men and 30 women, 74.9 ± 8.6 (49 to 89) years old) met the NINCDS-ADRDA criteria for probable AD [30]. All participants gave written Institutional Review Board-approved informed consent.

General cognitive abilities in all subjects were assessed with Mini Mental State Examination [31]. Screening for depression was performed with Geriatric Depression Scale [32], where a cutoff score of 5 determined lack of depressive symptoms, a cutoff of 10 separated mild and severe depressive symptoms. Subjects scoring higher than 10 were excluded. A battery of cognitive tests was administered. California Verbal Learning Test [33] or CERAD Word List [34] or Rey-Osterrieth Complex Figure, Immediate and Delayed Recall [35] or CERAD Figures – Delayed Recall [34] were used interchangeably to assess episodic memory. Digit span and Corsi blocks [36] allowed for working memory evaluation. Computerized test of attention [37], Stroop Color-Word Interference Test, Card 1 [38] and Trail Making Test, Part A [39] were used to examine attention and cognitive speed. Language was assessed with Boston Naming Test [40], category [41] and phonemic fluency tests [42]. Visuospatial abilities were evaluated with Rey-Osterrieth Complex Figure, Copy [35] or CERAD Figures, Copy [43] and Clock Drawing Test [44]. Executive functions were assessed with Trail Making Test, Part B [39], Stroop Color-Word Interference Test, Card 3 [38], and Design Fluency [45]. To confirm subject's cognitive status

a knowledgeable informant was questioned using a shortened 16- item version of the Informant Questionnaire on Cognitive Decline in the Elderly [46].

The diagnosis of MCI was based on the criteria proposed by Winblad et al. [4]. According to these criteria among the 42 participants with MCI, 35 were diagnosed as amnestic multiple domain MCI, 2 as amnestic single domain, 3 as non-amnestic multiple domain and 2 as non-amnestic single domain. The diagnosis of AD was based on the NINCDS-ADRDA criteria, where subjects experienced progressive decline in at least two cognitive domains including memory and impaired functioning, not attributed to other causes of cognitive deterioration [30].

MR acquisition

All measurements were done in a 3 Tesla MR head scanner (Magnetom Allega, Siemens AG, Erlangen Germany) using its standard transmit-receive head coil. After placing each subject head-first, supine into the magnet, localizer images in three orthogonal orientations were obtained to verify correct head placement. The magnetic field homogeneity was then adjusted over the whole head, followed by T_1-weighted sagittal Magnetization Prepared RApid Gradient Echo (MP-RAGE) [TE/TR/TI: 3.49/2150/ 1000 ms, 7° tip angle, 144 slices 1.1 mm thick, and 256×224 matrix over a $280 \times 245\,\text{mm}^2$ field-of-view (FOV) for 1.1 mm isotropic spatial resolution] MRI for brain volumetry.

The amount of brain NAA, Q_{NAA}, was obtained with non-localizing $TE/TI/TR = 0/940/10^4$ ms ^{1}H-MRS [28]. The $TR \gg T_1$ and $TE \approx 0$ result in the insensitivity to T_1 and T_2 variations that is desirable in pathologies where neither is likely to be known.

Brain volumetry

Each subject's brain tissue volume, V_B, was segmented from the MP–RAGE images using our in-house FireVoxel software [47]. The automatically detects of a "seed" region in periventricular WM to yield its signal intensity, I_{WM}, as shown in Fig. 1. Following selection of all pixels at or above 55% (but below 135% to exclude fat) of I_{WM}, a tissue-mask is constructed in three steps: (i) morphological erosion; (ii) recursive region growth retaining pixels connected to the "seed;" and (iii) morphological inflation to reverse the effect of erosion. Pixels of intensity under 55% of I_{WM} are defined as CSF. The sum of the pixels in all tissue-masks multiplied by their volume yields V_B. The precision of this process (the agreement between segmentation results for the same head from multiple scans) for T_1-weighted MRI has been recently quantified at 3.4% [47].

The intracranial (brain + CSF) volume, V_{IC}, was estimated using MRIcro, a free downloadable segmentation package: http://www.mricro.com [48]. It subtracted the cranium from the images using a "skull strip" tool leaving behind a mask of just brain and CSF. V_{IC} is the pixels' volume × their number in the mask and the fractional brain parenchyma volume (fBPV) is $V_B/V_{IC} \times 100$.

Whole-brain NAA (WBNAA) quantification

Absolute quantification was done with phantom-replacement against a reference 3 L sphere of 1.5×10^{-2} mole NAA in water. Subject and reference NAA peak areas, S_{S} and S_R, were obtained by manual phasing and selection of the NAA peak limits of integration using our in-house software, as shown in Fig. 1a, by four operators blinded to each other's result. A result more than two average standard deviations (for the four readers' over all the patients, ∼8%) from the mean for that patient, was rejected. If more than one was rejected than that entire set was excluded as "poor quality." The results were then averaged into $\overline{S_S}$ and $\overline{S_R}$ and Q_{NAA} estimated as [28],

$$Q_{\text{NAA}} = 1.5 \times 10^{-2} \cdot \frac{\overline{S_{\text{S}}}}{\overline{S_{\text{R}}}} \cdot \frac{V_{\text{S}}^{180^\circ}}{V_{\text{R}}^{180^\circ}} \text{ moles}, \quad (1)$$

where $V_{\text{R}}^{180^\circ}$ and $V_{\text{S}}^{180^\circ}$ are the transmitter voltages for non-selective 1 ms 180° inversion pulses on the reference and subject. Note that although macromolecules and other *N*-acetyl species also resonate around 2 ppm [49], their contribution to that peak area is estimated at under 10% [50].

To normalize for differences in brain size among subjects, Q_{NAA} was divided by the brain parenchyma volume, V_B, to yield the whole-brain NAA concentration:

$$\text{WBNAA} = Q_{NAA}/V_B \text{ mM}. \quad (2)$$

This is a specific (brain size independent) metric and its *inter*- and *intra*-subject variability in younger healthy individuals has been shown to be better than ±8% [51]. The whole head NAA (WHNAA) concentration, a marker of NAA atrophy, was then estimated as,

$$\text{WHNAA} = Q_{NAA}/V_{IC} \text{ mM}, \quad (3)$$

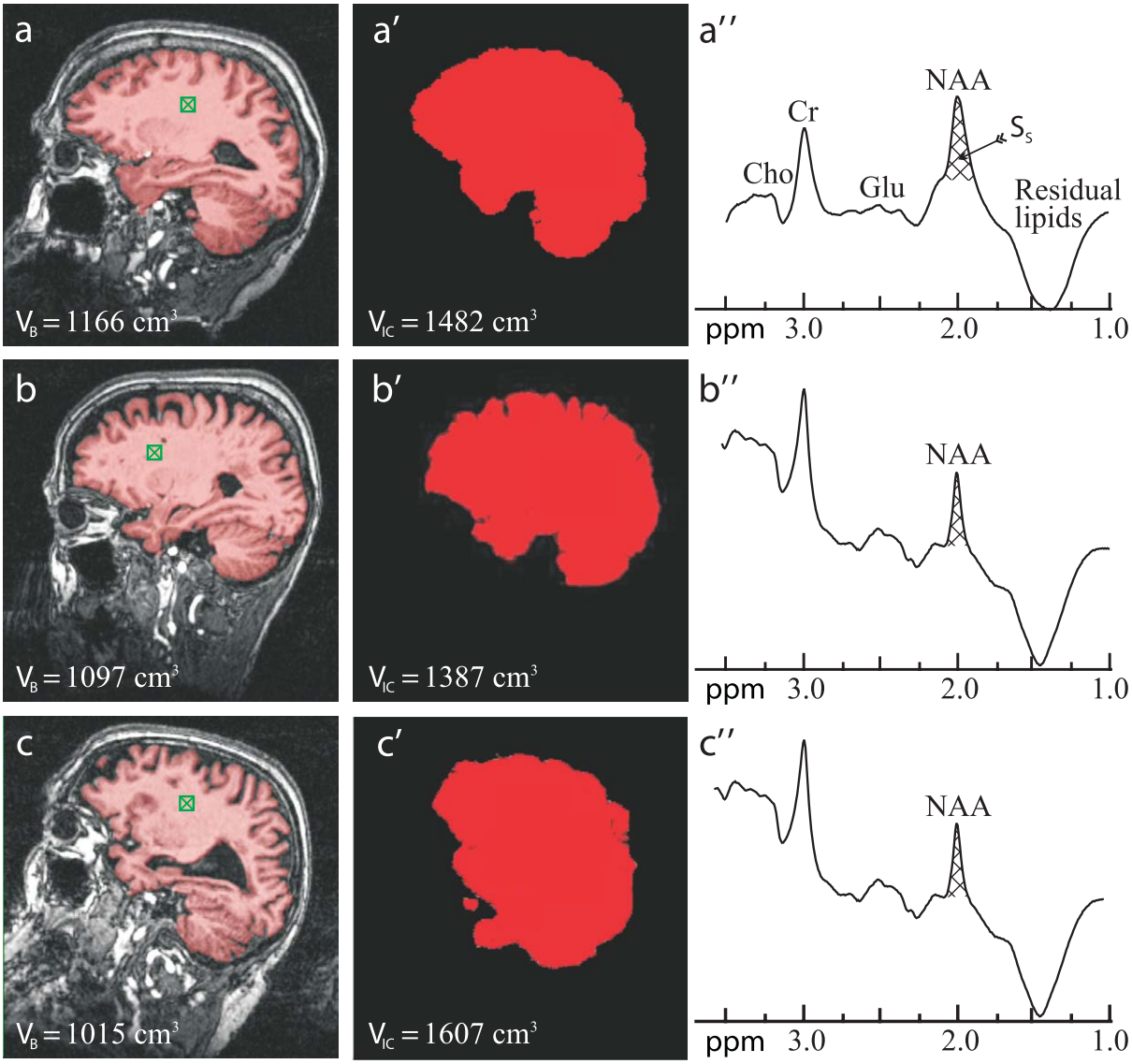

Fig. 1. Sample FireVoxel and MRIcro segmentation used for V_B and V_{IC} (indicated on the images) and whole-head NAA spectra (not normalized for V_B) from one subject from each cohort: 73 year old normal woman (a, a', a"), 73 year old female MCI (b, b', b"), and 80 year old male AD patient (c, c', c"). Note the "seed" in the white matter (green hatched box in a–c), brain-capture performance of FireVoxel (a–c), intracranial volume capture of MRIcro (a'–c'), and well defined whole-head ^{1}H spectra (a"–c") for straightforward integration of S_s for Equation [1]. Note the NAA peak at 2 ppm, lipids suppression performance and that of all the other peaks in the spectrum, e.g., glutamate (Glu), total creatine (Cr) and total choline (Cho), only NAA is implicitly localized by its biochemistry to just the brain.

that is sensitive to both atrophy *as well as* the NAA concentration in the remaining tissue.

Statistical analysis

Analysis of variance based on ranks was used to compare diagnostic groups in terms of the three study endpoints: fBPV, WBNAA, and WHNAA (separate analysis for each). In each case, the observed values of the endpoint were converted to ranks used as the dependent variable in order to satisfy underlying distributional assumptions. p values are reported for the comparisons with adjustment for potential confounding effects of age, gender and multiple comparisons. Receiver operating characteristic (ROC) curve analyses were used to assess and compare study endpoints utility to discriminate cognitively impaired (groups 2 and 3) from normal (group 1). A statistical jackknife

procedure was used to compare endpoints with respect to area under the ROC curve (AUC) while a McNemar test was used to compare endpoints in terms of the diagnostic accuracy when selected threshold values were used as test criterion for diagnosis of cognitive impairment. Results were declared significant if associated with a two-sided p value under 0.05. SAS version 9.0 (SAS Institute, Cary, NC) was used for all statistical analyses.

RESULTS

The original data set consisted of 225 subjects. 28 of them (16 cognitively normal (NL), 5 MCI, and 7 AD) failed the WBNAA data quality criterion described in section "Whole-brain NAA (WBNAA) quantification," above and were, consequently, excluded from the analyses. Thus the final set included 197 subjects.

The WBNAA concentrations (mean ± standard deviation) were 14.1 ± 2.4, 10.5 ± 3.0, and 10.1 ± 2.9 mM for normal elderly, MCI, and AD patients, as shown in Fig. 2a. While the MCI and AD patients means were not statistically different ($p = 0.85$), they were highly significantly *lower* than normal contemporaries' (25%, $p < 10^{-4}$ for MCI; 29% $p < 10^{-4}$ for AD).

The WHNAA concentrations were 10.5 ± 2.0, 7.6 ± 2.2, and 7.0 ± 2.1 mM for normal elderly, MCI, and AD patients, as shown in Fig. 2b. Again, no significant difference was observed between the means of the MCI and AD patients ($p = 0.37$) but each was highly significantly *lower* than the controls' (27%, $p < 10^{-4}$ for MCI group; 31% $p < 10^{-4}$ for AD group).

The fBPVs were 74.6 ± 4.4, 72.9 ± 4.9, and 69.9 ± 4.7% for the normal, MCI, and AD patients, as shown in Fig. 2c. As expected, compared to normal controls fBPVs were significantly lower in MCI (2%, $p = 0.003$) and AD patients (6%, $p < 10^{-4}$). Moreover, the MCI and AD groups also differed significantly from each other (4%, $p = 0.02$). All p values are adjusted for age and gender which, therefore, played no role in the differences observed among the cohorts.

All relationships remained virtually unchanged when analyses were repeated after exclusion of non-amnestic MCI patients.

We re-analyzed our data with ANCOVA using actual and log transformed values for WBNAA, WHNAA, and fractional brain parenchymal volume. The overall results and between group differences (adjusted for multiple comparisons) remained the same:

- WBNAA actual values, ANCOVA adjusted for age and gender $F_{4,192} = 48.9$, $p < 10^{-4}$. There was no difference between MCI and AD ($p = 1.00$); however, both MCI and AD differed from the NL group at $p < 10^{-4}$.
- WBNAA log transformed values, ANCOVA adjusted for age and gender $F_{4,192} = 45.9$, $p < 10^{-4}$. There was no difference between MCI and AD ($p = 1.00$); however, both MCI and AD differed from the NL group at $p < 10^{-4}$.

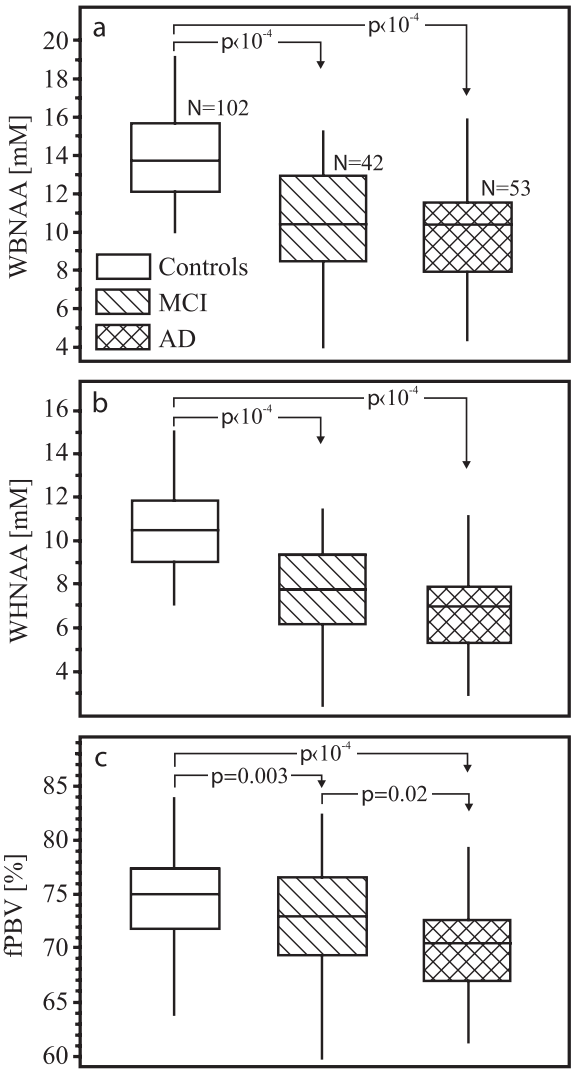

Fig. 2. Top, a) Box plots showing the first, second (median) and third quartiles (box) and ±95% (whiskers) of the WBNAA distribution in the three groups. Note highly significant 25% lower WBNAA for MCI and AD versus their normal contemporaries (arrows). Center, b) Same for the WHNAA distribution. Note the highly significantly 30% lower WHNAA for MCI and AD patients versus their normal controls (but not each other). Bottom, c) Same for the fBPV. Note the significant difference between MCI and AD patients both of whom are highly significantly lower ~5%, than normal contemporaries.

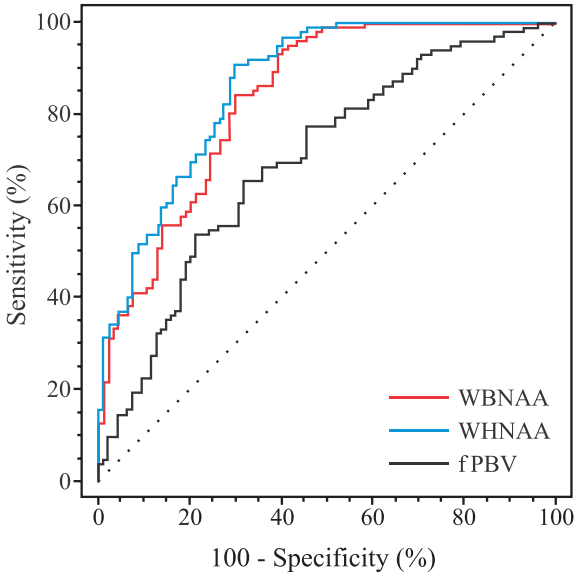

Fig. 3. ROC curves for the WBNAA, WHNAA, and fBPV (red, blue, and black solid lines). The dotted diagonal line represents the probability of a random event. The further a curve is from this diagonal, the greater its AUC, consequently its sensitivity. Note the greater area (i.e., predictive value) under the WBNAA and WHHAA curves compared with the fBPV.

• WHNAA actual values, ANCOVA adjusted for age and gender $F_{4,192} = 62.4$, $p < 10^{-4}$. There was no difference between MCI and AD ($p = 0.57$), however both MCI and AD differed from the NL group at $p < 10^{-4}$.

• WHNAA log transformed values, ANCOVA adjusted for age and gender $F_{4,192} = 57.5$, $p < 10^{-4}$. There was no difference between MCI and AD ($p = 0.48$); however both MCI and AD differed from the NL group at $p < 10^{-4}$.

• fBPV actual values, ANCOVA adjusted for age and gender $F_{4,192} = 24.2$, $p < 10^{-4}$. MCI had smaller fBPV than the NL group ($p = 0.006$); AD had smaller fBPV than both NL ($p < 10^{-4}$) and MCI group ($p = 0.01$).

• fBPV log transformed values, ANCOVA adjusted for age and gender $F_{4,192} = 23.8$, $p < 10^{-4}$. MCI had smaller fBPV than the NL group ($p = 0.007$), AD had smaller fBPV than both NL ($p < 10^{-4}$) and MCI group ($p = 0.01$).

The AUC of the ROC curves in Fig. 3 were: 0.84, 0.87, and 0.70 for the WBNAA, WHNAA, and fBPV. The maximum sensitivity and specificity for these metrics were: 84.3% and 70.5% for WBNAA, 91.2% and 70.5% for WHNAA, and 65.7% and 68.4% for fBPV. Based on the McNemar tests their maximum accuracy are 77.7% ($p = 0.033$), 81.2% ($p = 0.001$) versus 67.0%. No significant difference was noted between WBNAA and WHNAA for the highest accuracy.

The AUC of the ROC curves for separation between NL and MCI groups were all significant: 0.81, 0.83, and 0.61 for the WBNAA, WHNAA, and fBPV, respectively. The maximum sensitivity and specificity for these metrics were: 80.4% and 64.3% for WBNAA, 88.2% and 64.3% for WHNAA, and 65.7% and 57.1% for fBPV.

Similarly, as expected the AUC of the ROC curves for separation between NL and AD groups were all significant: 0.86, 0.89, and 0.77 for the WBNAA, WHNAA, and fBPV, respectively. The maximum sensitivity and specificity for these metrics were: 86.3% and 71.1% for WBNAA, 92.2% and 77.4% for WHNAA, and 69.6% and 71.7% for fBPV.

Finally, neither the WBNAA nor the WHNAA provided separation between MCI and AD groups. The AUC for fBPV was 0.66 with 61.9% sensitivity and 60.4% specificity.

DISCUSSION

The neurodegenerative nature of AD has been consistently demonstrated by (local) NAA decrease in various gray and white matter brain regions [23–26]. It is interesting, therefore, that (i) significant WBNAA decline (reflecting brain issue "quality") is already present at MCI; which (ii) is not statistically different from AD. Since our MCI group was predominantly amnestic, our findings support the view that this type of MCI constitutes a prodromal stage of AD. This finding is corroborated by the WHNAA, a metric of "NAA atrophy" that accounts for both the reduction in parenchyma volume (quantity) and NAA concentration decrease in the remaining tissue (quality). The WHNAA followed the pattern of the WBNAA among the three cohorts but was consistently lower by about 2%, i.e., by the corresponding fBPV differences.

Together with neuropathological similarities between MCI and AD patients [52] and the fact that 10–15% of MCI patients convert to AD annually [3], our results support the notion that MCI is not just a transitory stage but rather an early manifestation of AD pathology [2].

Most interestingly changes in NAA were not fully reflected in fBPV measurements. First, it is noteworthy that MCI subjects were situated in between NL and AD group. Second, while the fBPV differences were of the order of 5%, WBNAA and WHNAA were fivefold larger, 25–30%. The tissue loss quan-

tified by the fBPV represents an end stage of many pathological processes. Consequently, it is less specific than NAA decline which is a unique marker to neuronal dysfunction and loss. This may help explain the higher sensitivity and specificity of both WBNAA and WHNAA (Fig. 3).

This higher sensitivity may also be attributed to NAA physiology. Specifically, since its production is coupled to ATP synthesis [20], the energy metabolism decline that is known to occur early in the course of AD [53] may also depress the NAA concentration, indicating that functional decline precedes structural damage. Second, the reactive gliosis that accompanies atrophy [54] may mitigate the true extent of neuronal loss biasing the fBPV. Since glial cells do not contain NAA, WBNAA and WHNAA are both insensitive to this bias. Consequently, WBNAA along with other biological markers and clinical tests may present an earlier, more complete picture of the severity and evolution of the disease in any given patient.

The performance of both WBNAA and WHNAA as diagnostic markers merits further discussion. First, although the highest accuracy of either did not differ significantly, the WHNAA AUC was slightly higher, probably reflecting the combined contributions of tissue-quality and volume decline. Second, the diagnostic accuracy for either metric was commensurate with the 85% reported by most volumetric studies focusing on medial temporal structures to discriminate between normal and AD. It is actually higher than most reports comparing these structures between normal and MCI (see review in [15]). In this regard, information on WBNAA and WHNAA is desirable in conjunction with standard diagnostic imaging to exclude alternative pathologies and providing indications for neuronal damage beyond normal aging process.

When discrimination accuracy for the three metrics was examined for individual diagnostic groups, the separation with WBNAA and WHNAA was significant between AD and NL, and MCI and NL groups, but not between MCI and AD subjects. However, this is expected given the comparable levels of WBNAA and WHNAA in both impaired groups.

Furthermore, diagnosis of MCI is currently based on clinical algorithms, e.g., history, mental status exams and neuropsychiatric tests [4] and is accompanied by common uncertainties related among others to the contribution of depression, anxiety, and concurrent medications. Therefore, WBNAA augment the diagnostic MRI (used to exclude other pathologies) with additional level of confidence for presence of pathologic but MRI-occult tissue changes.

Admittedly, this study is subject to several limitations due to a methodology designed to maximize the intrinsically low sensitivity of ^{1}H-MRS and speed up its acquisition. Specifically, since WBNAA, WHNAA, and fBPV are all global averages, they are insensitive to (multi) focal or regional pathologies. Changes, therefore, must be spatially extensive enough to affect the average by the 8–10% intrinsic sensitivity [51]. Disorders that preferentially (or initially) target small regions, e.g., temporal structures in MCI or AD [55], therefore, may go undetected until that threshold is crossed. Moreover, although the technique provides quick and reliable estimate of WBNAA it comes at the cost of missing all other metabolites, e.g., Cho, Cr and Glu, as shown in Fig. 1c. This is because these metabolites, unlike NAA, have signal contributions from other tissue types of the head and the brain's contribution to their signal cannot be separated with this non-localizing sequence). Thus we could not compare the discriminatory values of these other metabolites to that of WBNAA. Finally, since vascular co-morbidities were not quantified, possible differences in their loads are unaccounted for in any of the cohorts. A larger (for more statistical power) study of MCI that compares these subtypes may indicate whether the WBNAA can detect or differentiate these subgroups.

CONCLUSION

We investigated three relatively large cohorts in order to obtain statistically robust data and to compensate for inherent methodological measurement noise. Global ^{1}H-MRS reveals significant diffuse neuroaxonal damage in both AD and MCI patients that is markedly different from normal elderly. We demonstrate that most of the neuronal damage occurred prior to the onset of clinical AD symptoms, indicating that WBNAA may be an early, more sensitive non-invasive marker than MRI-derived global brain volume metrics. Together with the simplicity of the method and the fact that only one measurement is required, further integration of WBNAA into MRI protocols especially in large cohort epidemiological studies appears attractive.

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Authors' disclosures available online (http://www.j-alz.com/disclosures/view.php?id=2437).

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