Short echo time proton magnetic resonance spectroscopy in Alzheimer’s disease: a longitudinal multiple time point study

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Short echo time localized proton magnetic resonance spectroscopy provides quantification of brain metabolites, including N-acetyl-aspartate, myo-inositol, creatine/phosphocreatine and choline-containing compounds, which may be useful biomarkers for monitoring Alzheimer’s disease. We aimed to quantify the rate of metabolite change in Alzheimer’s disease, to assess factors influencing changes and to investigate the potential for serial magnetic resonance spectroscopy as an Alzheimer’s disease trial biomarker. A total of 42 patients and 22 controls each had up to six magnetic resonance spectroscopy examinations over a 2-year period, using a midline posterior cingulate single-voxel point resolved spectroscopy sequence (1.5 T; time to repetition = 2000 ms; echo time = 30 ms; 192 averages). Metabolite ratios N-acetyl-aspartate:creatine/phosphocreatine, choline-containing compounds:creatine/phosphocreatine, and myo-inositol:creatine/phosphocreatine were measured using online software (PROBE-Q) and the N-acetyl-aspartate:myo-inositol ratio was derived. Baseline ratios were compared between patients and controls. A linear mixed model was used to quantify longitudinal changes and extended to assess the effect of age, disease severity and baseline use of acetylcholinesterase inhibitors. Patients and controls were matched for age (patients: 68.9 ± 7.2 years; controls: 69.1 ± 6.7 years); 71% of the patients were on acetylcholinesterase inhibitors at baseline; mean Mini-Mental State Examination for patients was 19.4 ± 4.1. A total of 307 spectra were acquired. In cross-sectional analyses, patients were significantly different from controls for N-acetyl-aspartate:creatine/phosphocreatine (11% lower, P < 0.001), N-acetyl-aspartate:myo-inositol (24% lower, P < 0.001), and myo-inositol:creatine/phosphocreatine (17% higher, P < 0.001). After adjustment for N-acetyl-aspartate:myo-inositol, none of the other variables differed significantly. In patients there was significant decline in N-acetyl-aspartate:creatine/phosphocreatine (mean: 2.2%/year; 95% confidence interval: 0.9–3.5) and N-acetyl-aspartate:myo-inositol (mean: 3.7%/year; 95% confidence interval: 1.7–5.7), with no evidence for influence by age, disease severity or acetylcholinesterase inhibitor use. There was significant excess decline in patients compared with controls only in N-acetyl-aspartate:myo-inositol (mean: 3.6%/year; 95% confidence interval: 0.8–6.4; P = 0.014). Between-subject standard deviation for N-acetyl-aspartate:myo-inositol was 0% for controls and 3.5%/year for patients; within-subject standard deviation for a 1 year, two-time-point study was 9.2%/year for both patients and controls. These results confirm that magnetic resonance spectroscopy can be used to quantify excess metabolite decline in Alzheimer’s disease, which may provide a useful measure of disease.
progression. We found no evidence that age, disease severity or acetylcholinesterase inhibitor use influenced rate of decline, although numbers were small. The substantial variability in longitudinal measurements that drives sample size requirements is principally within-subject and technique related: technical developments to reduce this variability may make serial magnetic resonance spectroscopy a viable biomarker in clinical trials for Alzheimer's disease.

**Keywords:** Alzheimer's disease; magnetic resonance spectroscopy; clinical trial design

**Abbreviations:** Cho = choline-containing compounds; Cr = creatine/phosphocreatine; MRS = magnetic resonance spectroscopy; NAA = N-acetyl-aspartate

**Introduction**

Intensive efforts are being made to devise novel treatments to slow the progression of Alzheimer's disease, the commonest neurodegenerative form of dementia. Biomarkers, including structural and functional neuroimaging, are increasingly being used in clinical trials in an attempt to distinguish compounds providing symptomatic effect from those that are truly disease modifying. Short echo time localized proton magnetic resonance spectroscopy (MRS) allows for non-invasive and repeated quantification of brain metabolites, including N-acetyl-aspartate (NAA), myo-inositol, creatine/phosphocreatine (Cr) and choline-containing compounds (Cho) (Valenzuela and Sachdev, 2001; Jones and Waldman, 2004; Kantarci, 2007). NAA is thought to be a marker of healthy neuronal density; Cho is thought to reflect mainly the products of membrane phosphotidyl choline breakdown, themselves the precursors of choline and acetylcholine synthesis; and myo-inositol may reflect glial cell proliferation or gliosis (Kantarci, 2007).

Cross-sectional studies in Alzheimer's disease have consistently demonstrated reduction in NAA and elevation of myo-inositol compared with controls, providing a potential ‘signal’ of disease activity (Valenzuela and Sachdev, 2001; Kantarci, 2007). Furthermore, studies in pre-symptomatic familial Alzheimer’s disease (Godbolt et al., 2006) and mild cognitive impairment (Kantarci et al., 2000, 2002a, 2003; Catani et al., 2001; Falini et al., 2005; Wang et al., 2009) have suggested that MRS has the potential to distinguish patients with Alzheimer's disease from controls very early in the course of the disease, at a time when disease-modifying therapies are likely to be most beneficial. Serial MRS studies of NAA levels in Alzheimer’s disease or mild cognitive impairment have been performed at long echo times (Adalsteinsson et al., 2000; Jessen et al., 2001; Dixon et al., 2002; Kantarci et al., 2007; Olson et al., 2008); correlations between decline in NAA levels and clinical symptom progression have been demonstrated (Jessen et al., 2001; Kantarci et al., 2007) and serial MRS has been proposed as a potentially useful outcome measure for therapeutic studies.

The feasibility of using serially acquired MRS as an outcome measure in a clinical trial is dependent on a number of factors, including the variability and reproducibility of the technique, which in turn drive the required sample sizes. In this study, we used multiple serially acquired proton MRS measurements from a single posterior cingulate voxel to quantify the rate and variability of metabolite change in controls and patients with established Alzheimer’s disease and to assess factors influencing these changes. Using these data, we then aimed to critically evaluate the potential for serial MRS as a biomarker of Alzheimer’s disease progression for use in clinical trials.

**Material and methods**

**Subjects**

All subjects were recruited as part of the MIRIAD (Minimal Interval Resonance Imaging in Alzheimer’s Disease) study, the details of which have previously been described (Schott et al., 2005, 2006, 2008). In brief, 46 patients with a diagnosis of probable sporadic Alzheimer’s disease and 23 age-matched controls were recruited. Patients with Alzheimer’s disease fulfilled the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer’s Disease and Related Disorders Association criteria for a diagnosis of probable Alzheimer’s disease, had a Mini-Mental State Examination score between 12/30 and 26/30 and had no contraindication to MRI. Acetylcholinesterase inhibitor use was not a contraindication to recruitment, although all patients were stable on or off treatment for a minimum of 1 month at the start of the study. Controls, usually the spouses of the patients, were matched for age, sex, education and handedness and had no history of cognitive decline, head injury, stroke or psychiatric disease. No subject had diabetes mellitus. The study was approved by the local ethics committee and all participants gave written, informed consent.

All subjects were seen at baseline and underwent a detailed clinical and neuropsychological assessment and structural brain MRI (Schott et al., 2005, 2006). Subjects were requested to attend for proton MRS imaging at the following intervals from baseline: 0 weeks (Visit 1), 3 months (Visit 2), 6 months (Visit 3) and 1 year (Visit 4). Patients completing Visit 4 scanning while the study was still ongoing attended for further scanning at 18 months (Visit 5) and 2 years (Visit 6). At each time point a record was made of any changes in medication.

**Spectroscopy**

Single-voxel proton magnetic resonance spectra were acquired at each time point on the same 1.5T Signa 5× system using an automated point-resolved spectroscopy technique (time to repetition = 2000 ms, echo time = 30 ms, 192 averages; General Electric Medical Systems, Milwaukee, WI, USA) using the standard General Electric birdcage head coil. All spectra were acquired by the same experienced radiographer, at the same time of day. A T1-weighted spin echo sagittal scout was obtained, followed by six axial 5 mm fast inversion recovery images. A 15 mm-thick midline voxel of interest was placed within...
three adjacent slices (Fig. 1), with the other voxel dimensions sized individually to maximize grey matter cingulate content (volume range 3.0–7.4 ml). For serial acquisitions, the same voxel size was used for each patient and the voxel was repositioned as accurately as possible by the study radiographer, using a hard copy image as a guide. Acquired spectra were reviewed visually for quality and significant artefacts that would interfere with metabolite quantification by a neuroradiologist experienced in \textit{in vivo} spectroscopy. For each individual the metabolite ratios NAA/Cr, Cho/Cr and myo-inositol/Cr were measured using online software (PROBE-Q, General Electric Medical Systems, Milwaukee, WI, USA). NAA/myo-inositol ratios were derived from NAA/Cr and myo-inositol/Cr. Typical spectra are shown for a patient with Alzheimer’s disease (Fig. 2A) and normal control (Fig. 2B).

**Statistical analysis**

When analysing ratios it is not advisable to work on an untransformed scale, as the mean of a ratio of two variables is, in expectation, typically greater than the ratio of the means of the two variables, inducing a bias in favour of the numerator. As a simple illustration, consider two variables, \(X\) and \(Y\), measured in two subjects. If \(X\) takes the value 150 and \(Y\) takes the value 50 in the first subject and \(X\) takes the value 50 and \(Y\) takes the value 150 in the second subject, the means of both \(X\) and \(Y\) are 100. However the arithmetic mean of the ratio of \(Y\) to \(X\) (and of the ratio of \(X\) to \(Y\)) is 1.67, substantially >1. This bias is removed by analysing data on logarithmic scales. Accordingly, we report geometric means of the ratios Cho/Cr, NAA/Cr, myo-inositol/Cr and the derived variable NAA/myo-inositol (the ratio of NAA/Cr to myo-inositol/Cr) calculated by logarithmically transforming each measurement, calculating the mean of these log-transformed values and then back-transforming to the original scale in patients and controls. In practice, there are no substantial differences between the geometric and arithmetic means in these data. Changes on the logarithmic scale are expressed as percentage increases. Geometric mean levels were compared between the groups using \(t\)-tests on log-transformed values (allowing different variances in the two groups). Ratios of geometric means with 95\% confidence intervals are also reported. Linear regression models (on log-transformed values and with robust standard errors to allow different variances by group) were used to adjust the analysis of each creatine ratio (e.g. Cho/Cr) for the other two (e.g. NAA/Cr, myo-inositol/Cr). In addition to these mutual adjustment models, the analysis of each creatine ratio was adjusted for NAA/myo-inositol in an analogous fashion.

Linear mixed models, with fixed subject specific intercepts and random slopes, were used to calculate annualized percentage change for each of the three metabolite ratios using all available time points; as data from all available time points are used, this approach gives more statistical power than considering only the first and last points. The models were fitted to log-transformed variables and allowed different between-subject variances in slopes and different within-subject residual variances, in patients and controls. Extensions of these linear mixed models allow rates of change to vary with covariates, including age, disease severity (as assessed using the Mini-Mental State Examination) and baseline acetylcholinesterase inhibitor use.

![Figure 1](image1.png) **Figure 1** Typical voxel placement.

![Figure 2](image2.png) **Figure 2** Typical MRS spectrum for (A) control and (B) Alzheimer’s disease. ml = myo-inositol.
From the estimated components of variance the statistical power of 1- and 2-year clinical trials using MRS as an outcome measure was investigated. Sample sizes were calculated to give 80% statistical power (using a 5% two-sided significance level) to detect a 25% reduction in the rate of decline in excess of the mean rate in controls.

Data were analysed using Stata version 10 (Stata Corporation, College Station, TX, USA) and SAS (SAS Institute Inc., Cary, NC, USA).

Results

Of the 46 patients and 23 controls recruited to the MIRIAD study, 43 and 22, respectively, had MRS imaging at baseline. Seven patients and one control have subsequently come to post-mortem; all had confirmation of diagnosis with the exception of one patient who had dementia with Lewy bodies and was therefore excluded from the analysis. Thus, 42 patients and 22 controls were included in the analysis. Patient demographics are shown in Table 1. Patients and controls were well matched for age, handedness, gender, years of education and smoking history. As expected, the patients had significantly lower Mini-Mental State Examination scores than the controls. Seventy-one percent of patients were on acetylcholinesterase inhibitor treatment at the start of the study. Ten patients started acetylcholinesterase inhibitor treatment during the study due to lack of efficacy. A total of 199 individual spectra were acquired for the patients (i.e. ~4.7 spectra per individual), and 108 for the controls (~4.9 spectra per individual). The number of spectra acquired at each visit (1–6) is shown in Table 2. Due to a poor quality spectrum, myo-inositol was not available for one subject on one occasion.

Cross-sectional, baseline data are shown in Table 3. Geometric mean levels of NAA/Cr in patients were 11% lower than those in controls (P < 0.001). This difference remained statistically significant after adjustment for Cho/Cr and myo-inositol/Cr. Geometric mean levels of Cho/Cr were 9% higher in patients than in controls (P = 0.012), but this difference was not statistically significant after adjustment for NAA/Cr and myo-inositol/Cr. Geometric mean levels of myo-inositol/Cr were, on average, 17% higher in patients than controls (P < 0.001). Following adjustment for NAA/Cr and Cho/Cr, this effect was reduced in magnitude and only of borderline statistical significance (P = 0.103). NAA/myo-inositol showed the largest differences between the groups. Geometric mean levels were 24% [95% confidence interval (CI): 17–30%] lower in patients than controls. After adjustment for NAA/myo-inositol, none of the other three variables differed significantly between groups. NAA/myo-inositol was able to distinguish patients from controls with 83% sensitivity and 77% specificity (receiver operating characteristic plot shown in Fig. 3).

The estimates for longitudinal change for both patients and controls are shown in Table 4. In controls there were no significant changes over time in any of the four ratios. In the patient group, there was a significant decline in NAA/Cr and the derived NAA/myo-inositol ratios over time and a borderline statistically significant (P = 0.082) increase in myo-inositol/Cr. On average Cho/Cr remained stable. Comparing change over time between patients and controls, only change in the NAA/myo-inositol ratio reached significance, declining in patients by an additional 3.6% /year (P = 0.014). Allowing the rate of decline to vary with age at baseline and adjusting the comparison between groups for this did not materially alter the results. In the patient group there was no significant association between rate of NAA/myo-inositol decline and age, disease severity or use of acetylcholinesterase inhibitors.

The estimated between-subject standard deviation in NAA/myo-inositol decline rates (expressed as a percentage increase) was 3.5%/year. However within-subject variability was large: the estimated within-subject standard deviation of decline rates estimated from two visits 1 year apart was 9.2%/year; and that for two visits 2 years apart was 4.5%/year. For controls, the model-derived estimate of between-subject variability was zero. Within-subject variability was very similar to that seen in patients, with estimated standard deviation of decline rates from two visits one year apart being 9.2%/year.

Using these estimates, a 1-year study of a treatment with 80% statistical power (using a two-sided significance level of 5%) to detect a 25% reduction in the rate of decline in excess of the mean rate in controls (i.e. a reduction from 3.68%/year to 2.76%/year) would require ~1700 subjects in each group. An analogously powered 2-year study would require ~600 subjects in each group. These sample sizes could be reduced slightly if an

### Table 1 Demographics

<table>
<thead>
<tr>
<th></th>
<th>Patients</th>
<th>Controls</th>
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<tbody>
<tr>
<td>n</td>
<td>42</td>
<td>22</td>
</tr>
<tr>
<td>Age at study entry (years)</td>
<td>68.9 ± 7.2</td>
<td>69.1 ± 6.7</td>
</tr>
<tr>
<td>Males (%)</td>
<td>38</td>
<td>50</td>
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<tr>
<td>Mean (±SD) baseline Mini-Mental State Examination</td>
<td>19.4 ± 4.1</td>
<td>29.5 ± 0.7</td>
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<tr>
<td>Right-handed (%)</td>
<td>90</td>
<td>86</td>
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<td>School-leaving age (years)</td>
<td>16.4 ± 1.4</td>
<td>16.5 ± 1.4</td>
</tr>
<tr>
<td>Lifelong non-smokers (%)</td>
<td>55</td>
<td>68</td>
</tr>
<tr>
<td>Years of symptoms</td>
<td>4.5 ± 2.2</td>
<td>–</td>
</tr>
<tr>
<td>Taking acetylcholinesterase inhibitor at baseline</td>
<td>71%</td>
<td>–</td>
</tr>
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</table>

a Significant difference between groups (P < 0.0001, Mann–Whitney U-test).

### Table 2 Numbers and timing of spectra acquired

<table>
<thead>
<tr>
<th>Visit</th>
<th>Time from baseline (days)</th>
<th>Number of Alzheimer's disease spectra</th>
<th>Number of control spectra</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>42</td>
<td>22</td>
</tr>
<tr>
<td>2</td>
<td>83 ± 10</td>
<td>42</td>
<td>22</td>
</tr>
<tr>
<td>3</td>
<td>170 ± 27</td>
<td>40</td>
<td>22</td>
</tr>
<tr>
<td>4</td>
<td>349 ± 14</td>
<td>36</td>
<td>21</td>
</tr>
<tr>
<td>5</td>
<td>542 ± 33</td>
<td>26</td>
<td>13</td>
</tr>
<tr>
<td>6</td>
<td>715 ± 11</td>
<td>13</td>
<td>8</td>
</tr>
</tbody>
</table>
We describe a prospective multiple scan serial MRS study in Alzheimer’s disease and healthy elderly control subjects. This study is, to our knowledge, the first that has involved the systematic acquisition of multiple serial spectra from individual patients. This approach not only allows for quantification of baseline differences between patients and controls and assessment of longitudinal change over time, but also permits an assessment of factors affecting the variability of such measures. At baseline, we found a decrease in NAA/Cr and elevation in Cho/Cr and myo-inositol/Cr ratios in patients with Alzheimer’s disease compared with controls. While these ratios were individually significantly different between patients and controls, none were significant once the derived NAA/myo-inositol ratio was accounted for. NAA/myo-inositol also proved the best means of discriminating between the groups, being ~24% lower in patients than controls. These findings are consistent with previous studies; the values for individual metabolite ratios are concordant with some (Kantarci et al., 2007), probably due to similarities in spectroscopic technique and analysis methods. The reduction of NAA, a marker of neuronal integrity/viability, is likely to reflect neuronal loss or dysfunction in Alzheimer’s disease, and elevation of myo-inositol to reflect gliosis. Alterations in these metabolites in the posterior cingulate region appear to follow the known pathological progression of Alzheimer’s disease (Kantarci et al., 2008), and while myo-inositol increase may predate decline in NAA early in the disease course, both are abnormal in established Alzheimer’s disease. Our finding that NAA/myo-inositol was the best means of discriminating patients from controls also accords with a previous study demonstrating that this ratio was the best predictor of the pathological likelihood of Alzheimer’s disease at subsequent post-mortem, and was strongly associated with histopathological Braak stage (Kantarci et al., 2008).

Imaging biomarkers that can accurately quantify the progression of disease for clinical trials are the subject of intense interest. While numerous longitudinal studies using structural MRI have been reported, relatively few serial MRS studies have been published. We found no evidence for significant longitudinal changes in NAA/Cr, Cho/Cr, myo-inositol/Cr or NAA/myo-inositol in controls. In patients with Alzheimer’s disease, there was significant decline in both NAA/Cr and NAA/myo-inositol over time, but compared with controls only NAA/myo-inositol showed significant excess decline, at a rate of 3.6%/year. Kantarci et al. (2007) have also reported longitudinal results from a two-time-point proton MRS study in controls and patients with Alzheimer’s disease over ~13 months using a single posterior cingulate voxel with similar imaging parameters. They found similar and significant excess decline in NAA/Cr (~1.8%/year in patients with Alzheimer’s disease, 0.5%/year in controls) and non-significant but somewhat different rates of change of Cho/Cr (1.2% in patients with Alzheimer’s disease, 2.6% in controls) and myo-inositol/Cr (0.8% in patients with Alzheimer’s disease, 1.9% in controls). These non-significant differences probably reflect the inherent variability of the technique, which is likely to be greater with fewer study time points.

The percentage decline in NAA/myo-inositol over 1 year is comparable to commonly used structural magnetic resonance measures of progression, such as rates of whole brain atrophy (~2%/year) (Schott et al., 2006) and hippocampal atrophy (~4.7%/year) (Barnes et al., 2009). However, for use in a clinical trial, sample sizes are critically dependent on the variance of the measure under consideration. In this study, the use of a mixed

### Table 3 Cross-sectional results

<table>
<thead>
<tr>
<th>Metabolite Ratio</th>
<th>Patients (n = 42) Geometric mean (95% CI)</th>
<th>Controls (n = 22) Geometric mean (95% CI)</th>
<th>Ratio of geometric means (95% CI), P-value</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Cr/Cr</td>
<td>Cr/Cr</td>
<td>Cr/Cr</td>
</tr>
<tr>
<td></td>
<td>NAA/Cr</td>
<td>NAA/Cr</td>
<td>NAA/Cr</td>
</tr>
<tr>
<td></td>
<td>Cho/Cr</td>
<td>Cho/Cr</td>
<td>Cho/Cr</td>
</tr>
<tr>
<td></td>
<td>myo-inositol/Cr</td>
<td>myo-inositol/Cr</td>
<td>myo-inositol/Cr</td>
</tr>
<tr>
<td></td>
<td>NAA/myo-inositol</td>
<td>NAA/myo-inositol</td>
<td>NAA/myo-inositol</td>
</tr>
<tr>
<td>NAA/Cr</td>
<td>1.36 (1.32–1.40)</td>
<td>1.53 (1.48–1.58)</td>
<td>0.89 (0.85–0.93) P &lt; 0.001</td>
</tr>
<tr>
<td>Cho/Cr</td>
<td>0.64 (0.62–0.67)</td>
<td>0.59 (0.55–0.62)</td>
<td>1.09 (1.02–1.17) P = 0.012</td>
</tr>
<tr>
<td>myo-inositol/Cr</td>
<td>0.74 (0.71–0.78)</td>
<td>0.63 (0.60–0.67)</td>
<td>1.17 (1.10–1.25) P &lt; 0.001</td>
</tr>
<tr>
<td>NAA/myo-inositol</td>
<td>1.83 (1.72–1.95)</td>
<td>2.41 (2.26–2.57)</td>
<td>0.76 (0.70–0.83) P &lt; 0.001</td>
</tr>
</tbody>
</table>

Cross-sectional, baseline results are shown for patients and controls. The crude ratio of patients and controls is shown, alongside ‘mutually adjusted ratios’, whereby the crude measure is adjusted for all other metabolite ratios, e.g. results for NAA/Cr adjusted for Cho/Cr and myo-inositol/Cr; results for Cho/Cr adjusted for NAA/Cr and myo-inositol/Cr; and results for myo-inositol/Cr adjusted for Cho/Cr and NAA/Cr.
Effects model allows not only total variance to be estimated, but the contributing factors to be calculated. In the case of NAA/myo-inositol, over 1 year the estimated between-subject standard deviation was 3.5%/year and within-subject standard deviation was 9.2%/year, equating to a total standard deviation of 10% over 1 year. Interestingly, in controls, between-subject variability for longitudinal change approximated zero but within-subject variability was remarkably similar to that seen in patients with Alzheimer’s disease, at 9.2%/year. These findings imply that there is negligible change in metabolite profile in normal ageing over the timescale of the study, and any observed change is likely to reflect measurement error. By contrast, the significant between-subject variability in rate of change in the Alzheimer’s disease group, which was not accounted for by correct for age or disease severity (as measured using the Mini-Mental State Examination), suggests that other, perhaps as yet unidentified, factors must account for inter-individual variability in rate of progression in Alzheimer’s disease.

While other studies have reported an effect of acetylcholinesterase inhibitor on MRS metabolite concentrations (Krishnan et al., 2003; Jessen et al., 2006; Modrego et al., 2006), we found no evidence that patients on acetylcholinesterase inhibitor treatment differed in their rate of decline compared to those not on such treatment, although it should be noted that our study was not designed as a treatment trial and the number of patients not on treatment was small. Moreover, previously reported metabolite changes related to acetylcholinesterase inhibitor treatment have often been transient.

These data confirm that there is a metabolic ‘signal’ which reflects Alzheimer’s disease pathology and may be useful in tracking disease progression; how useful this will be in practice depends very much on the magnitude of any treatment effect relative to within-subject variance of the technique. The effects of successful disease-modifying therapies on metabolite abnormalities seen in Alzheimer’s disease are not yet known. If it is assumed that such treatments simply halt or slow the progression of metabolite abnormality, as a result of the large variance attributable mainly to within-subject variability, the sample sizes necessary to power a clinical study are estimated to be an order of magnitude greater than those using structural MRI measures. In this instance, a substantial reduction in variance would be essential for such a technique to become useful in practice. If, however, NAA depletion or Cho or myo-inositol increase are reversed as a result of the action of agents on brain pathology, current MRS methodology may provide a viable response biomarker.

While within-subject variability was a much larger source of variance than between-subject variability, the former was almost identical in patients and controls, suggesting that this is a technique- rather than a disease-mediated phenomenon. Both physiological metabolite variations within subjects over time and a number of technical factors potentially contribute to observed within-subject variability in serial in vivo metabolite measures from spatially localized MRS. Differences in voxel placement result in different proportions of grey and white matter and CSF being included within the region of interest. Variations in patient positioning within the head coil, and magnetic resonance system radiofrequency and gradient instability can lead to changes in signal intensity. Moreover, variable efficacy of water suppression and shim sequences result in changes in baseline and spectral line-widths, respectively, and both can affect metabolite estimation (Soher et al., 2000). Due to the relatively weak signal from metabolite protons, which are in the millimolar concentration range, signal to noise ratios of spectra are limited, which introduces further random variation in metabolite measurement. Techniques for fitting and hence quantifying assigned metabolite resonances are also imperfect and introduce further error. The relative contributions of these factors to observed variability cannot be evaluated in our current study; however, there is potential for some of these sources of error to be reduced substantially through technological advances in MRS acquisition and processing.

Improved magnetic resonance radiofrequency and gradient stability and improved algorithms for automated water suppression and shim may improve voxel localization and consistency of spectra. Computerized registration techniques could further improve accuracy of voxel placement. Other approaches to spectroscopic sampling, such as whole brain spectroscopy, avoid effects of variability in voxel location but have been performed at only longer echo times, which precludes myo-inositol determination (Falini et al., 2005). Multiple voxel spectroscopic imaging allows multi-region sampling (Zhu et al., 2006b) and tissue segmentation (MacKay et al., 1996); although signal to noise ratio limits reproducibility of multiple voxel spectroscopic imaging as a quantitative technique, novel approaches to signal processing can improve reliability of metabolite measurement (Zhu et al., 2006a). Acquisition of spectra using a multi-channel head coil or higher

### Table 4 Longitudinal results

<table>
<thead>
<tr>
<th></th>
<th>Patients (n = 42)</th>
<th>Controls (n = 22)</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAA/Cr</td>
<td>–2.2 (–3.5 to –0.9)*</td>
<td>–0.8 (–2.2 to 0.5)</td>
<td>–1.4 (–3.2 to 0.5)</td>
</tr>
<tr>
<td>Cho/Cr</td>
<td>0.0 (–1.5 to 1.6)</td>
<td>0.8 (–1.6 to 3.1)</td>
<td>–0.7 (–3.5 to 2.1)</td>
</tr>
<tr>
<td>ml/Cr</td>
<td>1.6 (–0.2 to 3.5)</td>
<td>–0.9 (–3.2 to 1.5)</td>
<td>2.5 (–0.4 to 5.6)</td>
</tr>
<tr>
<td>NAA/ml</td>
<td>–3.7 (–5.7 to –1.7)*</td>
<td>–0.1 (–2.2 to 2.0)</td>
<td>–3.6 (–6.4 to –0.8)**</td>
</tr>
</tbody>
</table>

*Statistically significant decline over time (P<0.05).
**Statistically significant decline in patients compared with controls (P = 0.014).
ml = myo-inositol.
field strengths may offer potential advantages in metabolite resolution and signal to noise ratio. It is worth noting, however, that available evidence suggests no significant diagnostic benefit of spectroscopy at 3 T over 1.5 T (Kantarci et al., 2003), and potential advantages of higher field may in part be offset by increased susceptibility to field inhomogeneity and shorter metabolite transverse ($T_2$) relaxation times (Barker et al., 2001; Tkac et al., 2001). There is also potential for more accurate and stable automated methods for metabolite quantification. Such advances will require investment and development by the major magnetic resonance system manufacturers.

At baseline, the NAA/myo-inositol ratio had 83% sensitivity and 77% specificity for the diagnosis of Alzheimer’s disease against normal ageing, similar to previous studies using a single-voxel posterior cingulate voxel, which have ascribed a sensitivity of 82% with a specificity of 73–80% (Kantarci et al., 2002b; Martinez-Bisbal et al., 2004). The 24% mean difference in baseline NAA/myo-inositol ratio between patients and controls was associated with a 3.6%/year rate of subsequent decline in patients with no evidence to suggest influence from baseline age or disease severity. Assuming similar linear rates of decline prior to the start of the study and that patients at some stage had NAA/myo-inositol ratios similar to controls suggests that excess decline in NAA/myo-inositol in patients with Alzheimer’s disease started several years prior to the start of the study and is in keeping with studies demonstrating differences in metabolite ratios between controls and patients with mild cognitive impairment (Kantarci et al., 2000, 2002a; Catani et al., 2001; Chantal et al., 2002). As the average duration of symptoms was 4.5 years at baseline, it is probable that excess decline in the NAA/myo-inositol ratio occurred prior to the onset of symptoms, as has been demonstrated in familial Alzheimer’s disease (Godbolt et al., 2006). Proton spectroscopy may therefore have a role in the early diagnosis of Alzheimer’s disease and be useful in tracking disease progression at this stage.

This study has a number of strengths. We used a posterior cingulate voxel, which samples a well-established, disease-appropriate, midline region of interest containing a substantial proportion of grey matter from the mesial aspects of both parietal cortices. The midline location allows relatively easy shim and hence high quality spectra and reproducible voxel placement. We used voxel position and acquisition parameters similar to those used in earlier Alzheimer’s disease studies, allowing for comparisons with published data. Sources of acquisition-related variance were minimized by acquiring serial spectra on a single MRI system, which was subject to regular spectroscopy quality assurance and meticulous voxel placement by one experienced radiographer. Our use of a mixed model statistical approach allowed rates of change of metabolite parameters to be measured accurately and contributions to variance to be estimated.

A number of different analysis methods are used for MRS data. In this study we chose to use the automated PROBE-Q analysis software, which provides a ratio-based algorithm for metabolite quantification, rather than attempting to calculate absolute metabolite concentrations, for a number of reasons: (i) PROBE-Q has been used in a large number of previous studies in Alzheimer’s disease, thus allowing for a direct comparison with the previous literature; (ii) being a fully automated online technique, it is well suited to the analysis of large data sets such as these and to the potential analysis of longitudinal clinical trial data; and (iii) using a ratio-based technique rather than absolute metabolite quantification minimizes error and hence variability due to radiofrequency inhomogeneity and signal baseline variation, circumvents the need for segmentation of CSF within the acquisition voxel (a process that has the potential to introduce further error) and allows the use of shorter times to repetition, which optimizes MRS acquisition time and hence minimizes protocol length in this vulnerable clinical group.

Other studies using proton MRS in Alzheimer’s disease have acquired spectra from other brain regions, including the frontal lobes and hippocampus (Schuff et al., 1997; Dixon et al., 2002; Zhu et al., 2006a; Bartha et al., 2008; Wang et al., 2009). While the hippocampus is a prominent and early site for Alzheimer’s disease pathology, it is a difficult part of the brain from which to obtain reliable or reproducible spectra. This may have contributed to the failure to determine a significant longitudinal decline in hippocampal metabolites in one longitudinal MRS study (Dixon et al., 2002). More recently, the feasibility of comparing NAA from medial temporal lobe spectra across multiple centres has been reported (Jessen et al., 2009).

**Conclusion**

We have shown that ongoing metabolic changes can be demonstrated and quantified using proton MRS in patients with established Alzheimer’s disease. We found no evidence that age, disease severity or acetylcholinesterase inhibitor use influenced rate of decline, although numbers were small. Our data suggest that in patients with Alzheimer’s disease, MRS measures of metabolites may diverge from controls early in the disease process and perhaps prior to the onset of symptoms, suggesting that MRS may have a role in diagnosis and progression monitoring in early disease. While NAA/myo-inositol declined significantly faster in patients with Alzheimer’s disease than in controls, variability in rate of change, due mainly to technique-based within-subject variance, currently limits utility in clinical trials. Future technical advances are likely to improve the stability of acquisition, and serial MRS may yet prove to be a useful biomarker for therapeutic studies.

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