Lower Levels of N-Acetylaspartate in Multiple Sclerosis Patients With the Apolipoprotein E ε4 Allele

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Background: In multiple sclerosis (MS), the ε4 allele of apolipoprotein E (APOE ε4) has been associated with more rapid clinical worsening and more severe tissue damage on magnetic resonance imaging.

Objective: To use proton magnetic resonance spectroscopy (1H-MRS) to further explore the biochemical changes in the brains of patients with MS associated with APOE ε4.

Design: A 2-year clinical and 1H-MRS follow-up cohort study.

Setting: The MS outpatient clinic, Department of Neurology, and Magnetic Resonance Center of Karl-Franzens University.

Patients: We performed 1H-MRS of the central portion of both hemispheres and APOE genotyping in 72 patients (52 women and 20 men; mean±SD age, 34.8±8.8 years) with clinically definite relapsing-remitting MS. Repeated studies were performed in 44 patients after a mean±SD interval of 34±9 months.

Main Outcome Measure: Levels of N-acetylaspartate as measured by 1H-MRS.

Results: Patients with MS and an ε4 allele (n=19) had a significantly lower mean±SD N-acetylaspartate–creatine ratio than those without an ε4 allele (n=53) (1.73±0.26 vs 1.89±0.24; P=.04) despite the absence of significant differences in age at onset, disease duration, Expanded Disability Status Scale score, and number of previous relapses between subgroups. During follow-up, the drop in the N-acetylaspartate–creatine ratio of ε4 carriers was also significantly larger (−0.31 vs −0.10; P=.01). This was paralleled by a higher number of relapses (mean±SD, 4.1±2.7 vs 1.7±1.6; P=.02) and a faster although nonsignificant progression of disability (mean±SD ΔExpanded Disability Status Scale score, 0.9±1.8 vs 0.3±1.1; P=.19).

Conclusions: The APOE ε4 allele has a negative effect on the course of MS, and increasing axonal damage may be an important mechanism.

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In particular, measurements of disability were obtained by the EDSS. The disease duration was 85 ± 71 months. All patients were women and 20 men with a mean ± SD age of 34.8 ± 8.8 years. Written informed consent was obtained from all patients. Blood samples were taken from the 28 individuals who could not be reexamined at or beyond 24 months, 13 refused further participation in the study owing to significant clinical deterioration and 11 denied multiple sclerosis lesions. Four patients were lost to follow-up because they had moved to another town. Dropouts were more frequent in carriers of the ε4 allele (10/19; 53%) than in the remainder of the group (18/53; 34%).

The clinical data collected were age at onset of MS, disease duration, disease severity according to the EDSS, the number of previous relapses, and the number of relapses during follow-up. Patients underwent clinical evaluation at 6-month intervals or more frequently in case of an exacerbation. A relapse was defined as the appearance of a new symptom or the worsening of an old symptom attributable to MS, accompanied by an appropriate new neurologic abnormality or focal neurologic dysfunction lasting at least 24 hours in the absence of fever and preceded by stability or improvement for at least 30 days. All clinical data were obtained by a single neurologist (S.S.-F.) masked to the patients’ genotypes and spectroscopic data. Long-term immunomodulatory and immunosuppressive medication use was recorded. At baseline, there were no significant differences concerning the frequency of interferon beta therapy between patients with (10/19; 53%) and without (34/53; 64%) the ε4 allele or in the proportion of individuals obtaining other disease-modifying and immunosuppressive drugs (intravenous immunoglobulins: ε4 group, n=1; non-ε4 group, n=1; methotrexate: non-ε4 group, n=2; and mitoxantrone hydrochloride [Novantrone; Wyeth-Lederle, Catania, Italy]: ε4 group, n=1) (P = .34). However, change in therapy during follow-up occurred more often in patients with the ε4 allele (4/9; 44%) than in the remainder of the group (6/35; 17%; P = .17). In the ε4 group, therapies were switched from interferon beta-1b (Betaferon; Schering, Berlin, Germany) to interferon beta-1a (Avonex; Biogen, Paris, France) and from interferon beta-1a (Rebif; Serono, Geneva, Switzerland) to glatiramer acetate (Copaxone; TEVA Pharmaceutical Industries Ltd, Petach Tikva, Israel) in 1 patient each. New therapies were started with Betaferon in 1 patient and with intravenous immunoglobulins in another. In the non-ε4 group, changes were as follows: Betaferon to Avonex (n=2) and intravenous immunoglobulins to Betaferon (n=1). New therapies with Betaferon were initialized in 3 patients.

### METHODS

**PATIENTS**

Patients from the outpatient clinic for MS at Karl-Franzens University with clinically definite MS according to the criteria of Poser et al.22 who participated in an ongoing prospective serial 1H-MRS study were asked to undergo APOE genotyping. Only patients with a relapsing-remitting course23 were chosen to obtain a more homogeneous cohort. Written informed consent was obtained from all patients. Blood samples were taken from 52 women and 20 men with a mean ± SD age of 34.8 ± 8.8 years and a mean ± SD disease duration of 85 ± 71 months. All patients were white. Their mean ± SD disability score as measured by the EDSS was 1.9 ± 1.5 (score range, 0-6), and the mean ± SD progression index, calculated as EDSS score at baseline divided by disease duration in years, was 0.44 ± 0.46. Immunomodulatory treatment was not considered a reason for exclusion given the high frequency of treated patients with MS in Austria, particularly in outpatient MS clinics. For inclusion in the follow-up cohort of this study we required 24 months as the minimum interval to limit the variation in study intervals and to increase the likelihood of statistically significant changes over time even with relatively small patient numbers. No other specific reasons led to an exclusion from being considered to take part in the follow-up examinations. This criterion was met by 44 patients (33 women and 11 men) with a mean ± SD age of 34.1 ± 8.9 years and a mean ± SD disease duration of 93 ± 81 months. They had a mean ± SD EDSS score of 1.9 ± 1.3 and a mean ± SD progression index of 0.48 ± 0.53. Of the 28 individuals who could not be reexamined at or beyond 24 months, 13 refused further participation in the study owing to significant clinical deterioration and 11 denied multiple examinations for other reasons, including 3 because of claustrophobia. Four patients were lost to follow-up because they had moved to another town. Dropouts were more frequent in carriers of the ε4 allele (10/19; 53%) than in the remainder of the group (18/53; 34%).

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**PROTON MAGNETIC RESONANCE SPECTROSCOPY**

Proton magnetic resonance spectroscopy was performed in a large single voxel to obtain global biochemical information on lesions and normal-appearing white matter as suggested by Arnold et al.20 As illustrated in Figure 1, we obtained metabolite ratios of NAA and choline relative to creatine (Cr) within a large volume of interest including a few multiple sclerosis lesions. C, A representative spectrum showing a significant decrease in the N-acetylaspartate (NAA)–creatine ratio.
uppermost portion of the corpus callosum parallel to a line that connects the inferior border of the genu and the splenium of the corpus callosum. A copy of this survey was used to achieve exact repositioning at follow-up. Axial and coronal images served to ascertain a symmetrical position perpendicular to the midline. We used a PRESS (point resolve spectroscopy) technique (repetition time [TR]/echo time [TE]=1500/136 ms) with automated shimming; water suppression was accomplished using a frequency-selective adiabatic pulse. Spectra were analyzed by means of the user-independent fitting routine Linear Combination of Model Spectra described in detail elsewhere.25 Individual anatomical landmarks were used for voxel repositioning in the follow-up series.

APOE GENOTYPING

High-molecular-weight DNA was extracted from peripheral whole blood samples using genomic tips (Quiagen, Hilden, Germany). Genotyping of APOE was performed according to the method of Hixon and Vernier.26 In short, a 244–base pair fragment coding for that part of the protein containing the variant amino acid residues 112 (cysteine-arginine=e4) and 158 (arginine-cysteine=e2) was amplified by polymerase chain reaction. These polymerase chain reaction fragments were digested with 20 U of CfoI over 3 hours at 37°C. Thereafter, samples were electrophoresed on a 20% nondenaturating polyacrylamide gel, stained with ethidium bromide (1.0 µg/mL), and photographed under UV transillumination. The 6 different genotypes then were determined from the banding pattern of the allele-specific fragments. The APOE genotype frequencies in our study group were as follows: e2/e2, n=2 (3%); e2/e3, n=6 (8%); e2/e4, n=2 (3%); e3/e3, n=45 (63%); e3/e4, n=16 (22%); and e4/e4, n=1 (1%). This genotype distribution is comparable to that recently reported in a cohort of 374 patients with MS (P=.66) and a general population of the same ethnic background.19

STATISTICAL ANALYSIS

Normal distribution was first assessed using Kolmogorov-Smirnov statistics. For continuous variables, differences between patient subgroups with a specific APOE allele were then tested using the Mann-Whitney test or the unpaired t test where appropriate. Differences in categorical variables between subgroups were tested using contingency tables (Fisher exact test). Possession of the APOE e4 allele, sex, disease duration, age at onset, and interval between baseline and follow-up 1H-MRS were then entered into a stepwise-forward binary logistic regression model to examine the adjusted contributions of these factors to reaching a low NAA/Cr ratio (a ratio at follow-up less than the median). All statistical tests were performed using a software package (SPSS/PC+, version 10.0.7; SPSS Inc, Chicago, Ill). All data are expressed as mean±SD.

RESULTS

BASELINE COHORT

The demographic, clinical, and spectroscopic variables for all patients with MS investigated at baseline regarding the presence (n=19) or absence (n=53) of an e4 allele are compared in Table 1. Patients carrying the e4 allele had a higher degree of disability as measured by the EDSS, although they were younger and had a shorter disease duration compared with non-e4 counterparts. However, none of these differences reached statistical significance. The proportion of patients receiving any immunomodulatory or immunosuppressive interval treatment was similar in both groups (see the “Patients” subsection). Proton magnetic resonance spectroscopy showed a significantly lower mean ratio of NAA/Cr in patients with than without the APOE e4 allele (1.73±0.26 vs 1.89±0.24; P=.04). No such differences were seen regarding the choline/Cr ratio.

FOLLOW-UP COHORT

Follow-up data after more than 2 years were available in 44 patients (mean follow-up, 34±9 months [e4 group: n=9, 39±13 months; non-e4 group: n=35, 31±6 months; P=.16]). In this subgroup, e4 carriers were also younger and had a shorter disease duration compared with non-e4 carriers (Table 2). Their baseline disease severity as assessed by the EDSS was similar, however. During follow-up, patients with an e4 allele experienced relapses more than twice as often than those without (4.1±2.7 vs 1.7±1.6; P=.02). This resulted in a higher percentage of individuals changing immunomodulatory therapy during follow-up in the e4 subgroup (see the “Patients” subsection). A larger accumulation of disability over time in e4 carriers was not statistically significant (P=.19). Conversion to a secondary progressive course of MS during follow-up was observed in 1 patient in the e4 group and 3 in the non-e4 group.

In the follow-up cohort, the mean NAA/Cr ratio at baseline was similar in APOE e4 carriers and noncarriers (Table 3). During follow-up, however, the NAA/Cr ratio became significantly lower in patients with the e4 al-
lele (1.57 ± 0.14 vs 1.77 ± 0.28; P = .02) because of a significantly greater decrease in the NAA/Cr ratio (−0.31 ± 0.16 vs −0.10 ± 0.23; P = .01) (Figure 2). No such differences were observed concerning the choline/Cr ratio.

To test whether the effect of the APOE ε4 allele was independent of possible confounders such as age at onset, disease duration, sex, and interval between 1H-MRS examinations, these variables were entered into a logistic regression model, with the NAA/Cr ratio at follow-up below the median (ie, NAA/Cr ratio < 1.67) as the dependent variable. In this analysis, the initial model (−2LL [−2 log likelihood]: 52.46) was significantly improved by stepwise inclusion of possession of the ε4 allele, age at onset, sex (−2LL: 38.90; P < .001), and APOE ε4 remained a significant and independent predictor of a low NAA/Cr ratio at the end of follow-up (P = .02; regression coefficient B = 3.1).

No differences in any of the assessed variables were found between patient groups with and without the APOE ε2 or APOE ε3 alleles (data not presented).

**Table 3. Spectroscopic Follow-up Data for 44 Patients With Multiple Sclerosis**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Non-ε4 Group (n = 35)</th>
<th>ε4 Group (n = 9)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAA/Cr ratio at baseline</td>
<td>1.88 ± 0.26</td>
<td>1.87 ± 0.20</td>
<td>.91</td>
</tr>
<tr>
<td>NAA/Cr ratio at follow-up</td>
<td>1.77 ± 0.28</td>
<td>1.57 ± 0.14</td>
<td>.02</td>
</tr>
<tr>
<td>ΔNAA/Cr ratio</td>
<td>−0.10 ± 0.23</td>
<td>−0.31 ± 0.16</td>
<td>.01</td>
</tr>
<tr>
<td>Cho/Cr ratio at baseline</td>
<td>0.41 ± 0.05</td>
<td>0.43 ± 0.07</td>
<td>.42</td>
</tr>
<tr>
<td>Cho/Cr ratio at follow-up</td>
<td>0.39 ± 0.06</td>
<td>0.39 ± 0.06</td>
<td>.84</td>
</tr>
<tr>
<td>ΔCho/Cr ratio</td>
<td>−0.01 ± 0.06</td>
<td>−0.01 ± 0.06</td>
<td>.84</td>
</tr>
</tbody>
</table>

Abbreviations: Cho, choline; Cr, creatine; NAA, N-acetylaspartate.
*Data are expressed as mean ± SD.

The results of this longitudinal 1H-MRS study substantiate and extend previous findings linking faster progression of MS to the APOE ε4 allele. Moreover, we provide the first in vivo evidence of more extensive axonal damage associated with the ε4 allele.

At the end of almost 3 years of follow-up, carriers of the ε4 allele had deteriorated clinically more rapidly, as measured by the EDSS, which is in accordance with the results of a 2-year follow-up study by Chapman et al., although this difference did not reach statistical significance in our study. Furthermore, during follow-up, patients with APOE ε4 had experienced relapses more than twice as often than those without, although the proportion of patients receiving immunomodulatory interval treatment was similar in both groups and treatment, in general, was intensified the more patients deteriorated. These differences in disease course would have remained undetected in a merely cross-sectional study design because both groups happened to be similar at baseline in terms of EDSS score, annual relapse rate, and number of previous relapses. Presumably, such weaknesses of a cross-sectional approach may account at least in part for the negative results of some previous investigations.

As an important novel finding, ratios of NAA assessed by 1H-MRS in a large central brain volume were significantly lower in individuals possessing the ε4 allele. This difference was already seen in the baseline examination of the entire group and, in view of the non-significant clinical differences, confirms a high sensitivity of this biochemical marker for MS-related brain damage. The amino acid NAA has been shown experimentally to be contained almost exclusively in mature neurons. A loss of neurons, therefore, would be predicted to cause a persistent reduction in the concentration of NAA. In fact, in MS, a reduction in NAA concentration has been demonstrated conclusively in chronic lesions and normal-appearing white matter. Moreover, the degree of NAA reduction seems to be correlated with clinical disability. Axonal pathologic involvement in MS occurs early and frequently and seems to be widespread. On the other hand, NAA concentration was shown to be unaltered in so-called benign MS, indicating relative preservation of axons. Previous findings of a greater proportion of more severe tissue destruction on MRI as indicated by “black holes” in patients with MS and the ε4 allele also seem to be in line with our observations of lower NAA levels in this patient subgroup.

During follow-up, carriers of the ε4 allele showed a more pronounced loss in NAA concentration, which can be viewed as an indicator of more rapid progression of neuronal damage. The effect of APOE ε4 in predicting a greater decrease in the NAA level remained significant after correction for possible confounders such as duration of disease, sex, age at onset of disease, and interval between 1H-MRS examinations. Clinically, there was a parallel trend toward faster accumulation of disability in patients with MS and the ε4 allele, although the differences in EDSS score changes over time did not reach sta-
istical significance in the present study. Also, a higher proportion of patients in the e4 group changed therapy during follow-up. More precise conclusions concerning a possible association between different forms of treatment and the temporal evolution of NAA levels are precluded by the overall small sample size.

Concerning the baseline differences of the entire and follow-up cohorts, some selection toward less severely disabled individuals may have happened because of the requested minimum follow-up time and the necessity to endure further straining 1H-MRS examinations. Therefore, the follow-up cohort must be considered representative of neither the entire group of studied patients nor the MS population as a whole. Nevertheless, findings concerning a close coupling between disability and NAA levels are consistent throughout the study. Thus, the subsample started with similar values for EDSS and NAA in both patient groups, although these had been reached more quickly in the e4 group, whereas in the entire population, e4 carriers were more disabled and had lower NAA levels. The lack of a difference at baseline in the follow-up cohort, therefore, probably represents a chance finding, with the same level of NAA in the e4 carriers reached more quickly. The observed higher dropout rate of e4 carriers should have counteracted any allele-specific differences rather than accentuating them.

Further limitations of the present study include a lack of absolute quantification of the biochemical markers assessed and the examination of a mixture of cerebral tissues, which, however, should have been partly compensated by the large voxel size. Thus, a loss of cell bodies in the basal ganglia may also have contributed to the observed decrease in the NAA level, although only small portions of deep gray matter were included in our voxel. Future 1H-MRS studies may be able to assess biochemical changes in the entire brain, and a separate evaluation of gray and white matter structures regarding the impact of the e4 allele seems desirable. Short-term 1H-MRS follow-up of individual lesions has shown a certain reversibility of the NAA peak after its initial reduction in the acute phase. Such recovery may as well have contributed some variation in the magnitude of observed metabolic changes but would altogether not be expected to have had a significant impact on the overall findings of our study. Similarly, progressing ventricular enlargement cannot serve to explain the relatively large reductions in NAA levels that we observed in patients with MS and the e4 allele, especially as the lateral ventricles only involved a minor portion of the spectroscopic volume of interest.

Absolute quantification of metabolites was technically not feasible for this investigation. However, we used Linear Combination of Model Spectra, which analyzes the in vivo spectrum as a linear combination of a basis set comprising complete model spectra of metabolite solutions. This method is almost fully automatic and requires only the input of acquisition variables, which minimizes user variability. Nevertheless, calculation of the NAA/Cr ratio for relative quantification of NAA levels relies on a rather stable absolute concentration of Cr. In this context, concentrations of Cr have recently been reported to increase with age. However, this should have caused underestimation rather than overestimation of the observed NAA/Cr ratio reduction in e4 carriers because of their younger age. Also, follow-up was similar in those with and without the e4 allele.

Using 1H-MRS, we demonstrated a more rapid decrease in the concentration of the neuronal marker NAA in patients with MS and APOE e4. However, it remains unclear whether increased inflammatory disease per se or an increased predisposition to axonal loss per attack contributes to the observed effect. Supported by a range of experimental data, it seems tempting to speculate on more aggressive disease mechanisms and/or impaired capacity for neuronal repair as the cause for this finding. Although our observations indicate that accumulating axonal damage is an important factor in the previously reported faster progression of MS in association with the e4 allele, they cannot serve to extract any specific etiologic clues.

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