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Magnetic resonance imaging and ^{1}H-magnetic resonance spectroscopy in amyotrophic lateral sclerosis

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Abstract We aimed to increase confidence in the combined use of MRI and proton MR spectroscopy (^{1}H-MRS) in diagnosis of amyotrophic lateral sclerosis (ALS). We investigated 12 patients with ALS, seven definite and five probable, taking into account clinical measures of motor neuron function. On T2-weighted images we found high signal in the corticospinal tract in six and low signal in the primary motor cortex in seven of the 12 patients. Atrophy of the precentral gyrus was apparent in all the patients apart from one with probable ALS. Absolute quantification of cerebral metabolites using ^{1}H-MRS demonstrated a significantly lower mean concentration of N-acetylaspartate (NAA) in the precentral gyrus of patients with probable and definite ALS (8.5 ± 0.62) than in control subjects (10.4 ± 0.71; $P < 0.001$). NAA concentration in primary motor cortex correlated with Norris scale scores ($r = 0.30$; $P < 0.0001$) but not with the ALS Functional Rating Scale score or disease duration. Significantly lower levels of NAA were detected in patients with low signal in the motor cortex than in those without ($P < 0.01$). Mean choline (Cho) and creatine (Cr) values did not differ between patients with ALS and controls.

Key words Sclerosis, amyotrophic lateral · Magnetic resonance imaging · Magnetic resonance spectroscopy

Introduction

Amyotrophic lateral sclerosis (ALS) is the most common and well-recognised form of motor neuron disease. It affects adults and has a progressive course leading to death. Its occurrence is mostly sporadic, a familial form being recognised in only 5–10% of patients [1]. At some time during the illness, the patients show a combination of upper (UMN) and lower (LMN) motor neuron signs [2]. The pathological basis of these signs is destruction of motor neurons in the spinal cord and brain stem, with associated peripheral and cranial nerve axonal degeneration, and in the cortex, associated with corticospinal and corticobulbar tract degeneration [3].

The aetiology and pathogenesis of ALS are unknown, although neurotoxic, free radical-mediated, immunological mechanisms and aberrant growth-factor signalling have been suggested [4, 5, 6, 7].

According to the current diagnostic criteria, the El Escorial Criteria of the World Federation of Neurology (WFN), there must be an absence of radiological abnormalities in the skull and spine, no significant abnormalities of the brain or spinal cord on MRI and no compressive lesions of the spinal cord or roots [8]. Although these criteria must be satisfied for a clear diagnosis of ALS, some abnormalities can often be detected on T2-weighted images, such as high signal in the corticospinal and corticobulbar tracts and low signal in the motor cortex, particularly the precentral gyrus [9, 10, 11, 12]. These abnormalities have been considered suggestive of, even if not pathognomonic, for ALS [13].

The usefulness of proton ^{1}H-MRS has been recognised in the study of the natural history, efficacy of treatment and clinical outcome of several disorders of the central nervous system (CNS), including neurodegenerative diseases [14].

Research using ^{1}H-MRS on patients with ALS, shows a reduction of N-acetylaspartate (NAA), a marker of neural integrity, indicative of degeneration and loss of motor neurones [15, 16, 17, 18, 19, 20, 21, 22]. However, few studies have been performed on the relationship between ^{1}H-MRS findings, MRI abnormalities and clinical impairment [23, 24, 25, 26, 27].

This study was aimed at revealing abnormalities in the brain of patients with definite or probable ALS by MRI, and biochemical changes in the motor cortex by ^{1}H-MRS. Absolute quantification of cerebral metabolites was carried out instead of using metabolite ratios, which could give rise to a misinterpretation of the results [28, 29]. ^{1}H-MRS data were correlated with clinical scores of neurological impairment and the imaging findings.

Patients and methods

We examined 12 patients with definite or probable ALS [8]. Other CNS diseases which could mimic ALS were excluded by clinical examination, laboratory findings, evoked potentials and MRI. They included inflammatory myopathies, neuromuscular transmission disorders, radiculopathies, plexopathies, polyneuropathies, spondylosis with myelopathy or radiculopathy, anterior horn cell disorders, syringomyelia, multiple sclerosis, parathyroid and thyroid diseases, Lyme disease, syphilis, HIV infection, toxic neuropathy and the postpolio syndrome [30].

The diagnosis was made using the El Escorial criteria [8]. Definite ALS was diagnosed when UMN and LMN signs coexisted in the bulbar region and at least in two other spinal regions (cervical, thoracic, lumbosacral) or when UMN and LMN signs coexisted in all three spinal regions and worsened over a 12 month period following initial diagnosis. Probable ALS was diagnosed when UMN and LMN signs existed in at least two different regions and there were UMN signs rostral to a region with LMN signs, which worsened over a 12-month period following initial diagnosis.

The mean age of the patients was 53.0 ± 5.32 years. They underwent clinical evaluation and assessment of functional impairment using the Norris and ALS Functional Rating Scales [31, 32]. Muscle strength was measured in the arm with a dynamometer.

The patients underwent electrodiagnostic investigation, MRI and ^{1}H-MRS. MRI and MRS were also carried out in 10 healthy age-matched (50.4 ± 4.4 years) control subjects. The study protocol was approved by the Ethics Committee of Perugia Municipality and all patients and control subjects gave written consent.

All patients met the El Escorial electrophysiological criteria [8] for ALS with evidence of LMN degeneration in muscle innervated by different roots and spinal nerves and by different cranial or peripheral nerves, in two or more of four different body regions: bulbar, cervical, thoracic and lumbosacral. The abnormalities consisted of fibrillation potentials, reduced recruitment, increased amplitude and duration of motor-unit potentials. Nerve conduction studies showed that sensory nerve action potentials were preserved and motor conduction velocities were reduced in all patients.

MRI and ^{1}H-MRS were carried out on a clinical 1.5 T system using a standard head coil. Sagittal T1-weighted fast spin-echo (FSE) (TR/TE/excitations 540/18 ms/2, echo-train length [ETL] 2), axial proton-density and T2-weighted spin-echo (SE) images (2500/30/100 ms/1) and coronal T2-weighted FSE images (4000/100 ms/3, ETL 8) were acquired with 5 mm slice thickness, 1 mm gap, acquisition matrix 256 × 256, field of view (FOV) 24 × 24 cm. An axial T1-weighted FSE sequence (400/18 ms/1, ETL 8, 5 mm slice thickness, no gap, FOV 24 × 18, acquisition matrix 256 × 192) was performed to define the volume of interest (VOI) for recording spectra.

Cortical and subcortical signal intensity abnormalities on T2-weighted images and atrophy of the precentral gyri were subjectively assessed independently by two experienced neuroradiologists (GPP, PC). Low signal in the grey matter and high signal in the white matter were considered to be present when seen on two or more images. Discrepancies in interpretation were resolved by consensus.

Spectra were acquired, following imaging, from the precentral gyrus, identified according to anatomical landmarks [33]. Typical voxel size ranged from 4 to 6 cc.

The homogeneity of the magnetic field over the VOI was optimised by observing the ^{1}H-MRS signal of tissue water, measured with the spatially selective stimulated-echo acquisition mode (STEAM) sequence. Typical line widths, full width at half maximum, of 4–5 Hz were achieved for the water peak. The STEAM sequence was also used to acquire spectra, after water peak suppression by the CHESS sequence.

Absolute quantification of signal intensities of NAA, choline (Cho) and creatine (Cr) was performed by a method based on the water compartmentation theory of Ernst et al. [28], using a vial of pure water as the external standard.

Subjects were instructed not to move their heads, which were cushioned with foam padding. MRS acquisition and subsequent examination of the spectra did not show any influence of movement. The total acquisition time for both MRI and MRS was 70–75 min.

Cerebral water content from selected VOI was obtained with 12 acquisitions with TR 10 s and TE 30, 40, 50, 70, 80, 100, 300, 600, 900, 1200 1500 and 1800 ms. The calculation of the water peak was performed for each free induction decay (FID) with fast Fourier transform (FFT) and Gaussian fit (Levenberg-Marquardt method). Data were elaborated with a biexponential fit to separate the contribution of cerebrospinal fluid (S_{csf}, T2 ≅ 1 s) from that of cerebral water (S_{bw} , T2 ≅ 80 ms) and to obtain the value of cerebral water signal intensity for TE 0 ms.

For the water in the external standard, five acquisitions were carried out with TR 10 s and TE 270, 500, 1000, 1500 and 2000 ms. The water peak was calculated for each FID with FFT and Gaussian fit. Data were elaborated with a monoexponential fit to obtain the signal intensity value of pure water (S_h) for TE 0 ms.

For cerebral metabolites, we carried out five acquisitions with TR 4 s and TE 40, 70, 136, 204 and 272 ms, each with 128 averages. The unsuppressed water peak (8 averages) was also acquired for eddy-current correction. Metabolite peaks were calculated for each FID using FFT and Gaussian fit. No correction was made for the baseline. Data for each peak were elaborated with a monoexponential fit to obtain metabolite signal intensity (S_{met}) for TE 0 ms. The effect of the resonance of macromolecules with short T2 was negligible due to the long echo times.

Metabolite concentration was obtained by:

$$\frac{n_{met}}{m_b} = \frac{S_{met}}{S_{bw}} R \frac{\varrho_w}{\varrho_b} \frac{10^3}{M} \frac{\nu_{met}}{2} \text{ (moles per kg) wet weight}$$

where n_{met} is number of metabolite moles, m_b is brain mass, $R = S_{bw}/(S_h\text{-}S_{csf})$, ϱ_w and ϱ_b are water and brain density, M is water

Table 1 Clinical details of patients with ALS

Patient	Sex, age (years)	Diagnosis	Events temporally associated with onset	Disease duration (months)	First symptoms and signs
1	M, 53	Probable	Skull trauma	24	Lower motor neurone signs
2	M, 64	Definite	Flu	8	Upper limb signs, dysphagia
3	M, 59	Definite	Not identified	17	Upper limb signs, dysarthria
4	M, 68	Probable	Not identified	28	Dysarthria, dysphagia
5	M, 61	Definite	General anaesthesia	10	Upper limb signs, dysarthria
6	M, 60	Definite	Not identified	12	Upper limb signs, dysphagia, dysarthria
7	M, 67	Definite	Not identified	20	Lower limb signs, dysarthria
8	M, 66	Probable	Not identified	32	Upper limb signs
9	M, 72	Probable	Not identified	36	Dysphagia, dysarthria
10	M, 63	Definite	Dental implant	17	Upper limb signs, dysarthria
11	M, 69	Definite	Not identified	10	Upper limb signs, dysphonia, dysarthria
12	M, 54	Probable	Not identified	38	Upper limb signs

molecular weight and ν_{met} is number of protons for molecule that contribute to the resonance signal.

Our purpose was to obtain absolute quantification of brain metabolites, and this was not possible with accuracy for glutamate, glutamine or inositol with the methods we used: inositol, glutamine and glutamate have a strongly complex coupled spectrum which could give rise to questionable results and both glutamate and glutamine have a signal-to-noise ratio which is too low, even at TE 40 ms.

Statistics included the analysis of variance (ANOVA) to compare the mean values of the metabolites in the control group with those of the patients and subgroups of patients with definite and probable ALS. Fisher's least significant difference was used to compare the main effects in the ANOVA. We chose 5 % for two-tailed tests as the level of statistical significance. Pearson's correlation coefficient was also calculated between the brain metabolite concentrations and the ALS and Norris scale scores of the patients.

Table 2 Individual scores and dynamometric findings

Patient	ALS scores		Strength of arms (kg)	
	Functional Rating	Norris	Right	Left
1	9	25	0	0
2	7	18	0	0
3	14	32	12	5
4	24	48	26	24
5	17	37	8	7
6	20	43	10	12
7	18	38	10	10
8	21	45	5	2
9	13	27	10	15
10	13	28	0	10
11	10	26	4	5
12	27	52	7	8

Results

There were seven patients diagnosed as having definite ALS, as they showed evidence of UMN and LMN signs in four regions. The other five patients were diagnosed as having probable ALS on the basis of evidence of UMN and LMN signs in two regions.

Table 1 shows details of diagnosis, symptoms and signs at onset, factors having occurred before their appearance, duration of disease and scores on ALS Functional Rating and Norris scales. Onset was spinal in four cases, bulbar in two and both bulbar and spinal in the others. Disease duration was longer (13.6 ± 5.7 months) in patients with probable than in those with definite ALS (13.4 ± 4.5 months; $P < 0.0001$), although the mean age of both groups did not differ.

Individual clinical assessment scores and dynamometer measurements of strength in the arms are shown in Table 2. The mean ALS Functional Rating and Norris scores were 16.0 ± 6.17 (range 7–27) and 34.9 ± 18 (range 34–52), respectively. The mean arm strength measurements were right 7.6 ± 7.2 kg (range 0–26 kg) and left 8.3 ± 6.9 kg (range 0–24kg).

MRI findings are given in Table 3. On T2-weighted images, there was more or less evident high signal in the corticospinal tract (Fig. 1) in six patients and low signal in the ascending frontal gyrus in seven (Fig. 1 f). In four there was also low signal in the postcentral gyrus and medial frontal area. More or less significant atrophy of the precentral gyrus was detected in all patients, apart from one with probable ALS.

Table 4 gives data on metabolites. Mean values of NAA in the precentral gyrus of the patients were significantly lower than in the control subjects. Fig. 2 shows spectra from a control subject and a patient with definite ALS, acquired at TE 40 ms; a reduction in the NAA peak is evident in the patient.

No significant difference in NAA was found between patients with definite and probable ALS. NAA concentration in the precentral gyrus correlated weakly with Norris ($r = 0.30$; $P < 0.01$; Fig. 3), but not with ASL Functional Rating scores or disease duration. Significantly lower NAA was detected in the seven patients

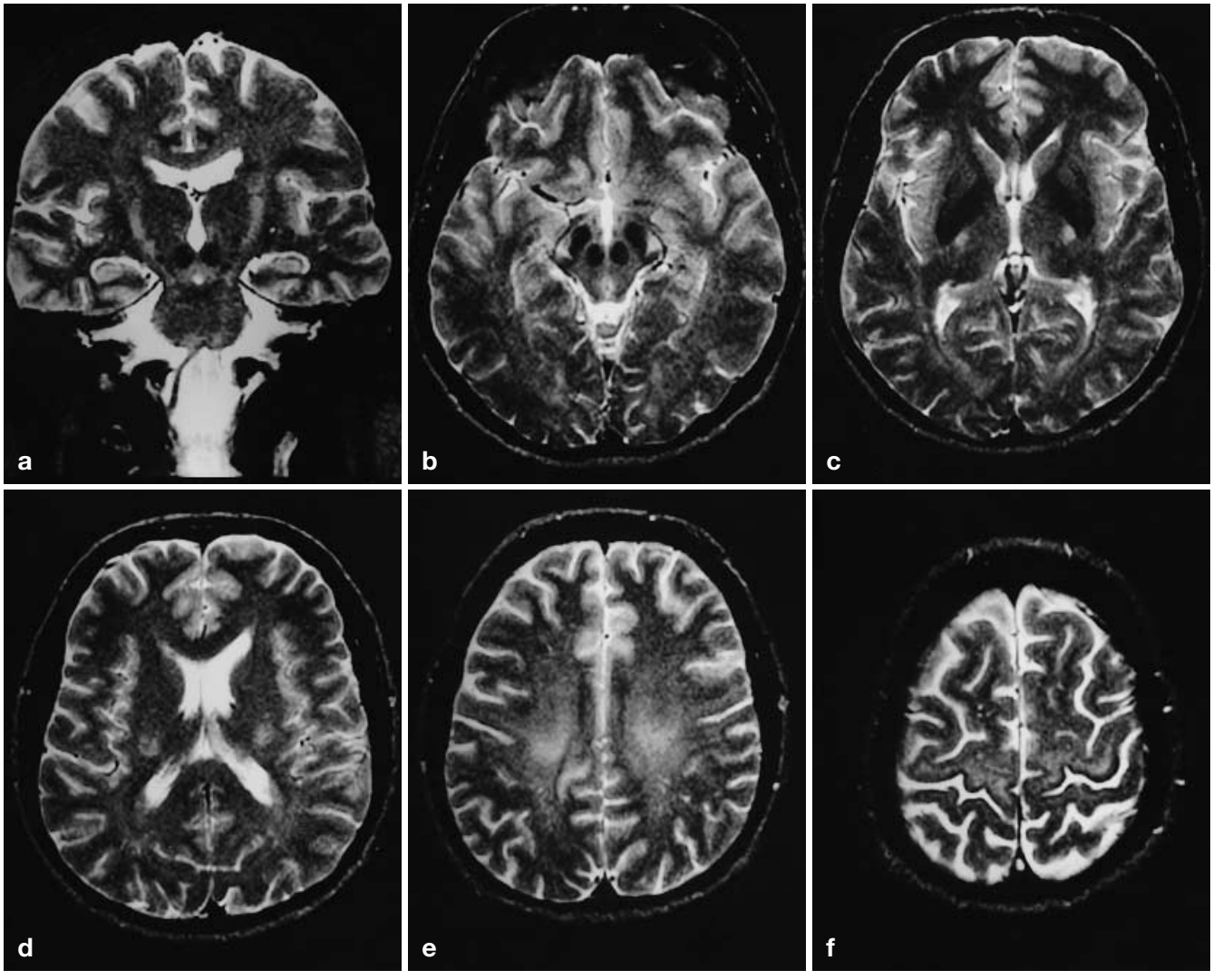

Fig. 1 **a** Coronal fast spin-echo (FSE); **b–f** axial spin-echo (SE) T2-weighted images. Bilateral symmetric high signal of the corticospinal tract extending from the precentral gyrus to the midbrain. In the precentral gyrus, in addition to the high signal of the white matter, it is possible to observe a strip of low signal underlying the primary motor cortex (in **f**) due to the deposition of iron or other metals

with low signal in the motor cortex than in those without (8.0 ± 0.63 vs 8.9 ± 0.10, $P < 0.009$; Fig. 4). Patients with bulbar onset had lower NAA in motor cortex than those with limb onset (7.9 ± 1.2 vs 8.6 ± 0.18, $P < 0.05$; Fig. 5).

Mean Cho and Cr values did not significantly differ between patients and controls, nor between patients with definite and probable ALS (Table 4). Pearson analysis showed no significant correlation between Cho and Cr and disability scores or disease duration.

Discussion

Our findings indicate the usefulness of combining MRI and ^{1}H-MRS to increase confidence in diagnosis of ALS and to explore confirm the neuropathological processes underlying it.

High signal in the corticospinal tract on T2-weighted images is a possible MRI correlate of the pathological abnormalities observed in motor neuron disease. This abnormality was detected bilaterally in 6 of the 12 patients we examined. It may be the expression of the primary degenerative process responsible for Wallerian degeneration in the corticospinal tract, which is mainly bilateral and symmetrical [10, 11]. However, it does not seem to be pathognomonic, because it can be observed in other multisystem degenerative diseases involving the corticospinal tract and in a small percentage of control subjects [34, 35, 36, 37, 38, 39].

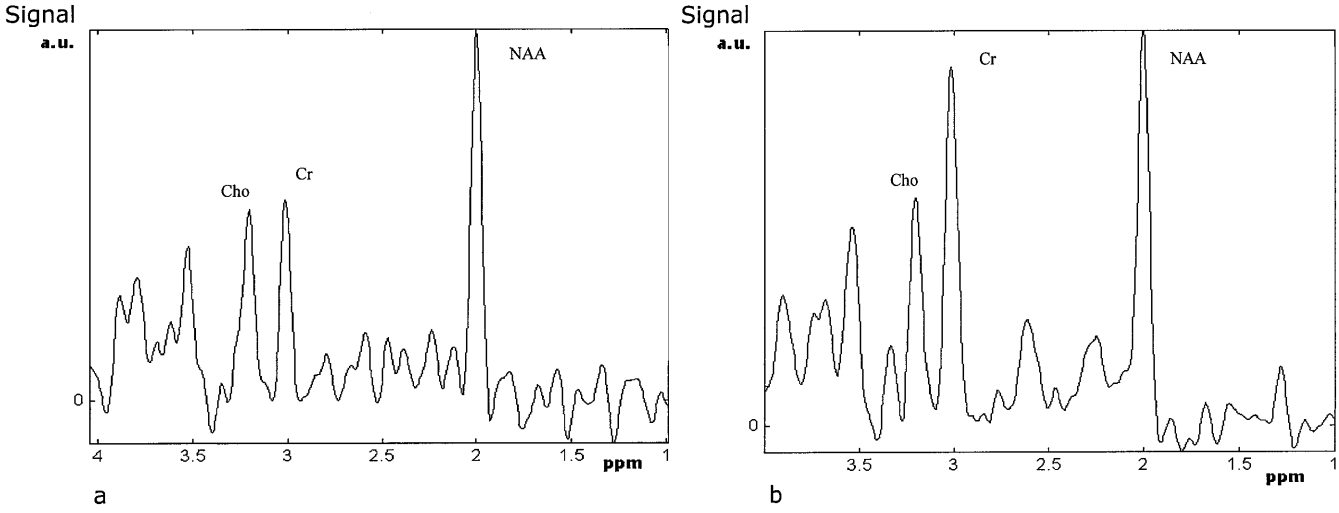

Fig. 2 Spectra obtained from the precentral gyrus of **a** a control subject and **b** a patient with definite amyotrophic lateral sclerosis (ALS)

Table 3 MRI findings

Patient	Diagnosis	High signal in cortico-spinal tract	Low signal in primary motor cortex	Selective atrophy of primary motor cortex
1	Probable	No	No	Yes
2	Definite	Yes	Yes	Yes
3	Definite	No	Yes	Yes
4	Probable	No	Yes	Yes
5	Definite	Yes	No	Yes
6	Definite	Yes	Yes	Yes
7	Definite	Yes	No	Yes
8	Probable	No	No	No
9	Probable	No	Yes	Yes
10	Definite	Yes	Yes	Yes
11	Definite	Yes	Yes	Yes
12	Probable	No	No	Yes

Table 4 Mean brain metabolite concentrations(mmol/kg wet weight ± 2 SD) in patients and control subjects

Group	N-Acetyl-aspartate	Choline	Creatine
Probable ALS	8.5 ± 0.55	1.44 ± 0.08	6.5 ± 0.4
Definite ALS	8.3 ± 0.75	1.42 ± 0.12	6.3 ± 0.6
All ALS	8.4 ± 0.62[a]	1.43 ± 0.09	6.4 ± 0.5
Controls	10.4 ± 0.71	1.48 ± 0.03	6.7 ± 0.7

[a] $P < 0.001$ vs controls

In nearly 60% of our patients, low signal was evident in the primary motor cortex on T2-weighted images. This may to be related to deposition of paramagnetic metals, causing local changes in the magnetic field and subsequent signal loss, which is considered characteristic of ALS [12, 40, 41, 42]. The most probable candidate is iron, although it is difficult to discriminate if its presence is pathological or age-related "paraphysiological" accumulation [43]. It might expresses free radical accumulation, which has been associated to neuronal degeneration and death in several neurodegenerative diseases, including ALS [44].

In several diseases of the CNS, including ALS, ^{1}H-MRS enables in vivo study of cerebral metabolites [15]. NAA is predominantly in neurons and therefore considered indicative of neuronal and axonal integrity [15]. Even if clinical and experimental MRS studies have indicated that decrease in NAA is a marker of neuronal viability, serial observations in conditions such as multiple sclerosis, mitochondrial encephalopathy and stroke-like episodes (MELAS) have shown reversibility in NAA levels, possibly reflecting recovery of neuronal function [45, 46]. Knowledge of NAA localisation is crucial in interpreting MRS findings. The assumption that NAA is specific to neurons is based on immunohistochemical studies on whole brain using NAA-specific antibodies [47, 48]. Other studies show NAA outside neurones, particularly in oligodendrocyte-type 2 astrocyte precursors and immature oligodendrocytes [49]. A recent report also demonstrates NAA in mature oligodendrocyte, supporting the hypothesis that these cells may express NAA in vivo and contribute to the NAA signal on ^{1}H-MRS [50].

Cr is believed to be a marker of the brain energy metabolism, and is evenly distributed in the brain [51]. The Cho signal in ^{1}H-MRS is an expression of a variety of compounds including phosphorylcholine, glycerophosphorylcholine and choline itself. Its increase seems to reflect an alteration in metabolic pathways involved in membrane turnover or increased breakdown and remodelling of myelin, as in multiple sclerosis.

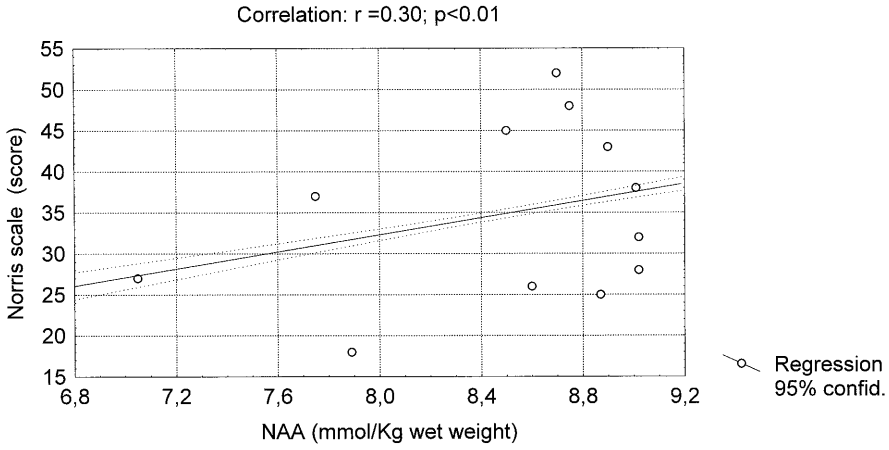

Fig. 3 Correlation between N-acetylaspartate (NAA) values and Norris scale scores of patients

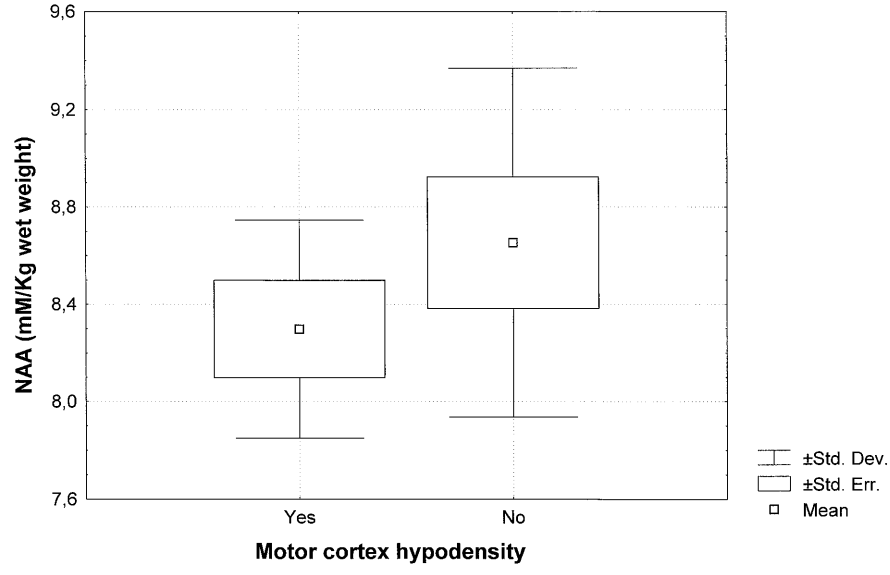

Fig. 4 Mean metabolite values in seven patients with and five without high signal in the motor cortex

The first studies carried out with proton magnetic resonance spectroscopic imaging in patients with ALS concerned the assessment of NAA/Cr and NAA/Cho ratios. Significantly lower ratios were found in patients with ALS than in controls, confirming motor neurone damage and loss [16].

A more accurate evaluation of changes in cerebral metabolites in the motor cortex of patients with ALS was obtained with ^{1}H-MRS using localised VOI [17, 19, 21]. A single-voxel ^{1}H-MRS study confirmed a reduced NAA/Cr in the precentral gyrus of patients with ALS and primary lateral sclerosis (PLS) patients compared with controls ($P < 0.05$) [26]. In the same study, assuming 2.5 standard deviations to be the cut-off point, this ratio was abnormal in 15 (79%) of 19 patients with ALS and 12 (67%) of 18 with PLS, whereas corticospinal tract high signal and atrophy of the primary motor cortex were found in 43 and 24%, respectively. These data suggest that the NAA/Cr ratio, measured with a single-voxel technique, is more sensitive than standard MRI to changes in motor neuron disease.

Significant improvement of ^{1}H-MRS has been achieved thanks to the development of absolute quantification techniques for brain metabolites, which avoid the misinterpretation due to the assumption that Cr values are relatively constant in ^{1}H-MRS studies.

Absolute quantification of metabolites was carried out with a single voxel in the precentral gyrus. As in previous studies, we our research showed a reduction in NAA, interpreted as a correlate of motor neuron loss. This finding concurs with the study by Gredal et al. [20]on seven patients with probable and definite ALS, showing a decrease in NAA, detected with absolute quantification, limited to the motor cortex and not evident in the cerebellum and not seen in patients with progressive muscular atrophy, as already shown by Pioro et al. [16].

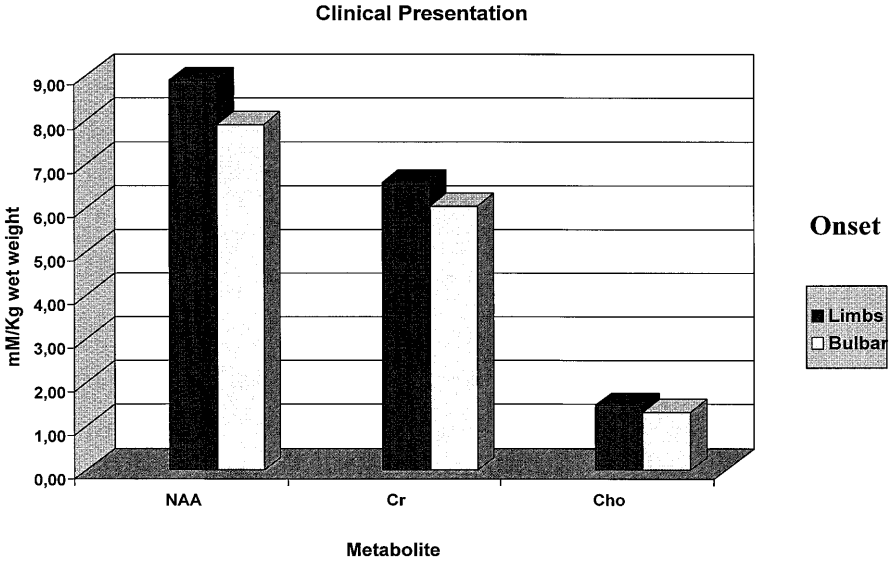

Fig. 5 Mean NAA values in four patients with bulbar and eight with limb onset

Moreover, in a recent study by Bowen et al. [27], water-suppressed MRS from the precentral gyrus, analysed by an LCModel, showed no change in NAA absolute values, but a decrease in NAA and glutamate after adjustment for Cr covariance. With our technique, we could not detect either glutamate or inositol, due to the short T2 and J-coupled resonance of both substances. We could not. therefore, extrapolate information about variations in these two metabolites, which may be relevant for ALS pathology.

We also demonstrated a correlation between reduced NAA and the Norris score, which gives a more objective assessment of neurological function than the ALS score, which is based on more subjective evaluation of the patient.

Another ^{1}H-MRS study showed a correlation of NAA/(Cr + PCr) in the motor cortex with the Norris score ($P = 0.01$) and E1 Escorial category ($P = 0.03$) but, as in our study, no significant correlation with disease duration [25]. Both studies revealed lower NAA in patients with bulbar onset than in those with limb onset. In agreement with Bowen et al. [27], we also found lower NAA in patients with low signal in the motor cortex on T2-weighted images.

We did not find significant variations in Cr and Cho. This is not in agreement with previous findings of increased Cho/(Cr + PCr) ratio or absolute Cho in recent studies which suggested a release of these compounds related to membrane degradation [23, 27].

Our data and those from previous studies do not concur with those obtained in vitro. A high performance liquid chromatography study of post-mortem brain and spinal cord samples revealed a reduction in NAA in the spinal cord but not the motor cortex [52, 53]. The discrepancy between these data and those obtained in vivo in motor cortex may be explained by changes in post-mortem brain tissue and different sensitivities of the methods. In particular, release from cells of N-acetylaspartylglutamate (NAAG), which is rapidly metabolised into NAA, can occur post-mortem. On the other hand, using ^{1}H-MRS, the signal of NAA at 2.0 ppm obtained from brain could correspond not only to NAA but also to other N-acetyl-containing moieties such as NAAG [54].

A recent stereological study, providing precise, reliable estimates of the number of neurones in specific regions of the brain, showed no change in the average number of neurons in neocortex and motor cortex of patients with ALS and controls. This finding suggests that results of in vivo ^{1}H-MRS, especially the NAA reduction, attributed to a neuronal loss, may be due to neuronal metabolic dysfunction and/or alterations in the size or volume fraction of the neurons [55].

Further sources of NAA reduction are be improbable, because the primary lesion of ALS is destruction of motor neurones in the cortex, brain stem and spinal cord; other cells, such as oligodendrocytes, do not characteristically appear involved in neuropathological studies of the disease. On the other hand, anabolic products of NAA and NAAG, which are specifically targeted to oligodendrocytes and astrocytes respectively, as glial cell specific signalling, may contribute to the NAA signal due to reactive astrogliosis which is believed to play a role in the pathophysiological events underlying neurodegeneration in ALS [56, 57]. N-acetyl moieties in these cells would appear as part of NAA levels, rather than contributing to its decrease.

Changes in NAA concentration can occur after treatment. The role of ^{1}H-MRS in detecting early changes in motor-cortex metabolites due to the glutamate antagonist riluzole was examined. Patients with ALS were

found to show a significant increase in NAA/Cr within 3 weeks of beginning treatment [58]. As glutamate can trigger the generation of reactive oxygen species in neurones, it has been hypothesised that early changes in NAA may reflect oxidative injury to mitochondria where NAA is synthesised. However, these data do not concur with those of Bowen et al. [27] who found no significant variation in the this metabolite after 2 weeks of treatment with riluzole and gabapentin. Long-term studies are needed to verify the effects of the glutamate antagonist and GABA agonist, as well as those of other innovative drugs in reversing upper motor-neurone injury in ALS [59].

We tried to relate MRI and ^{1}H-MRS findings to the severity of clinical signs. An association between low signal of precentral gyrus, clinical deterioration and reduced NAA levels in the motor cortex emerged, as well as the greater sensitivity of ^{1}H-MRS in detecting brain abnormalities in ALS.

Even if absolute quantification of cerebral metabolites by ^{1}H-MRS may not be considered a routine tool for identifying neuronal loss or dysfunction in ALS, it can be used in selected patients to detect an association between abnormal metabolite levels and clinical impairment [60]. In particular, ^{1}H-MRS of the brain stem might show early, subclinical involvement of motor nuclei of cranial nerves [22]. ^{1}H-MRS may also be used for monitoring the effect of new therapeutic approaches [59, 61].

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