Early metabolic alterations in the normal-appearing grey and white matter of patients with clinically isolated syndrome suggestive of multiple sclerosis: A proton MR spectroscopic study

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Received February 12, 2023; Accepted April 18, 2023

DOI: 10.3892/etm.2023.12048

Abstract. Proton magnetic resonance spectroscopy (^{1}H-MRS) is an advanced method of examining metabolic profiles. The present study aimed to assess *in vivo* metabolite levels in areas of normal-appearing grey (thalamus) and white matter (centrum semiovale) using ^{1}H-MRS in patients with clinically isolated syndrome (CIS) suggestive of multiple sclerosis and compare them to healthy controls (HCs). Data from 35 patients with CIS (CIS group), of which 23 were untreated (CIS-untreated group) and 12 were treated (CIS-treated group) with disease-modifying-therapies (DMTs) at the time of ^{1}H-MRS, and from 28 age- and sex-matched HCs were collected using a 3.0 T MRI and single-voxel ^{1}H-MRS (point resolved spectroscopy sequence; repetition time, 2,000 msec; time to echo, 35 msec). Concentrations and ratios of total N-acetyl aspartate (tNAA), total creatine (tCr), total choline (tCho), myoinositol, glutamate (Glu), glutamine (Gln), Glu + Gln (Glx) and glutathione (Glth) were estimated in the thalamic-voxel (th) and centrum semiovale-voxel (cs). For the CIS group, the median duration from the first clinical attack to ^{1}H-MRS was 102 days (interquartile range, 89.5.-131.5). Compared with HCs, significantly lower Glx(cs) (P=0.014) and ratios of tCho/tCr(th) (P=0.026), Glu/tCr(cs) (P=0.040), Glx/tCr(cs) (P=0.004), Glx/tNAA(th) (P=0.043) and Glx/tNAA(cs) (P=0.015) were observed in the CIS group. No differences in tNAA levels were observed between the CIS and the HC groups; however, tNAA(cs) was higher in the CIS-treated than in the CIS-untreated group (P=0.028). Compared with those in HC group, decreased Glu(cs) (P=0.019) and Glx(cs) levels (P=0.014) and lower ratios for tCho/tCr(th) (P=0.015), Gln/tCr(th) (P=0.004), Glu/tCr(cs) (P=0.021), Glx/tCr(th) (P=0.041), Glx/tCr(cs) (P=0.003), Glx/tNAA(th) (P=0.030) and Glx/tNAA(cs) (P=0.015) were found in the CIS-untreated group. The present findings showed alterations in the normal-appearing grey and white matter of patients with CIS; moreover, the present results suggested an early indirect treatment effect of DMTs on the brain metabolic profile of these patients.

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Key words: proton magnetic resonance spectroscopy, clinically isolated syndrome, multiple sclerosis, normal-appearing grey/white matter, treatment effect

Introduction

Conventional magnetic resonance imaging (MRI) protocols in multiple sclerosis (MS) are oriented toward recognizing mainly structural abnormalities since they are focused on morphology and topographical changes of the MRI signal, thus failing to identify subtle and/or diffuse abnormalities in normal-appearing white matter (NAWM) and normal-appearing grey matter (NAGM) that are not visible with the use of conventional MRI (1). However, changes in NAWM and NAGM, including deep GM nuclei like the thalamus, have been described in neuropathological studies and can be present from the early stages of MS (2-4). In particular, the thalamus is frequently affected during the MS pathogenetic

process, being a key site of demyelination and neurodegeneration (such as brain atrophy) (5,6). In studying the early clinical stage of MS, the clinically isolated syndrome (CIS), e.g. the first clinical episode with characteristics of inflammatory demyelination on the brain and/or spinal MRI suggestive of MS) (7), holds interest. Notably, the estimated risk for CIS to progress to MS has been estimated as 42-82% (8) depending on the follow-up duration.

The aforementioned limitations of conventional MRI in the study of normal-appearing (NA) tissue could be partially overcome with the use of other MRI techniques such as proton magnetic resonance spectroscopy (^{1}H-MRS), a non-invasive advanced method that can be considered a 'metabolic biopsy'. Specifically, ^{1}H-MRS is capable of detecting certain metabolite peaks from a region of interest and quantifying the concentrations of these metabolites by measuring regional tissue metabolism (9-12). Particularly in MS, ^{1}H-MRS enables examination of neurometabolic profiles that may be altered during the pathogenesis of the disease in the brain and spine; moreover, depending on the voxel placement it can examine not only regions with visible lesions on conventional MRI but also NA ones, thus assessing indirectly the degree of tissue damage based on the calculated biochemical changes (10,13-15).

A variety of metabolites of the central nervous system (CNS) can be detected and quantified with ^{1}H-MRS including N-acetyl aspartate (NAA), creatine (Cr), choline (Cho), glutamine (Gln) and glutamate (Glu), myoinositol (mIns) and glutathione (Glth) (9,10,16,17). NAA is one of the most common compounds assessed by ^{1}H-MRS; it is produced by mitochondria, localized in neuronal cell bodies and found in high concentrations in oligodendrocytes/myelin (18). It is considered a marker of neuronal viability. Reduced NAA levels are reported specifically in MS, which is usually interpreted as an indirect reflection of neuronal/axonal dysfunction or loss (19). The neurometabolite Cr was shown to be linked with cellular energy metabolism in metabolically active tissue such as the brain; the influence of the demyelinating process in Cr concentration is ambiguous since both diminished and increased levels were previously demonstrated (10). Another key molecule examined using ^{1}H-MRS is Cho. Cho-containing compounds are considered precursors but also products of cellular membrane turnover, having been reported in different studies on patients with relapsing and/or progressive MS either as high (10,19,20) or as low (10) levels and possibly attributed to inflammation, demyelination and/or remyelination. Glu is one of the main excitatory neurotransmitters in the CNS, with a molecular structure similar to Gln, participating in the interplay of the Glu/Gln cycle between neurons and surrounding astrocytes (21). Data on Glu remain unclear in MS spectroscopy; both normal and increased levels have been reported in white matter lesions and NAWM (22), while also decreased levels in mixed grey and white matter (WM) tissue (23,24). The mIns is another peak on the ^{1}H-MRS spectrum and is considered a marker of glia, a key molecule in cellular signaling systems, and also an organic osmolyte (14). The levels of mIns are increased in several ^{1}H-MRS MS studies (10,25,26). Glth is considered a 'protective' metabolite against oxidative stress and is synthesized mainly by glia; Glth levels are reported to be higher in astrocytes than in neurons (27,28) At present, to the best of our knowledge, only a few ^{1}H-MRS studies have estimated Glth in MS and decreased levels are observed in patients with secondary progressive MS (SPMS) (29,30).

To the best of our knowledge, the existing literature on ^{1}H-MRS of patients with CIS is very limited, with only a few studies (31-35) reporting neurometabolic alterations compared with healthy controls (HCs). Therefore, the present study aimed to evaluate the metabolic signatures of brain NAWM and NAGM in patients with CIS, focusing on possible underlying biochemical alterations that could be present in the NA areas on conventional MRI and also to identify possible markers of early cellular changes that may precede severe inflammation and degeneration by comparing metabolite concentrations of participants with CIS with those of HCs.

Materials and methods

Study population. The present study enrolled prospectively 38 consecutive female and male patients with CIS (CIS group), aged 18-50 years, diagnosed and recruited at the First Department of Neurology at Eginition Hospital, Athens, Greece. In addition, 29 age- and sex-matched HCs with no past medical history were also recruited (HC group). Inclusion criteria for the CIS group were as follows: i) History of a single clinical attack within 6 months before ^{1}H-MRS acquisition with objective clinical evidence of at least one lesion due to an acute inflammatory demyelinating event in the CNS with a duration ≥24 h in the absence of fever or infection (36,37); ii) baseline brain and spinal MRI scans at CIS onset demonstrating T2-weighted lesions in ≥1 of the four typical CNS locations for MS (periventricular, infratentorial, juxtacortical or spinal cord) according to the 2010 revisions to the McDonald criteria (37); iii) absence of thalamic lesions on baseline brain MRI and iv) aged 18-50 years. Exclusion criteria were as follows: i) a clinical relapse or administration of oral or intravenous corticosteroids within 4 weeks preceding ^{1}H-MRS acquisition; ii) history of other medical conditions associated with WM brain lesions (e.g. presence of multiple vascular risk factors, substance abuse) and iii) pregnancy. All differential diagnoses of CIS/MS, including other autoimmune inflammatory diseases of the CNS and infectious or vascular diseases were excluded by appropriate blood and cerebrospinal fluid (CSF) laboratory tests.

The MRI/^{1}H-MRS studies were performed from October 2015 to January 2017; moreover, on the date of the scan, all patients with CIS had a history of ≤6 months from the first clinical attack and all participants were evaluated by a neurologist at the Eginition Hospital (DT) including neurological examination. Assessment of the Expanded Disability Status Scale (EDSS) (38) was also performed for the CIS group. All participants provided written informed consent for participation in the study and publication of data. Written approval of the study protocol was obtained from the Ethics Committee of Eginition Hospital (approval no. 518/5.10.2015).

Imaging techniques and data analysis. All participants underwent brain MRI using a 3.0 T MRI Philips manufactured scanner (Achieva 3T TX; Philips Healthcare) equipped with an eight-channel head receive coil. The brain imaging protocol included a T2-weighted fluid-attenuation-inversion-recovery

(FLAIR) sequence in the axial plane [repetition time (TR), 11,000 msec; inversion time (TI), 2,800 msec; echo time (TE), 125 msec; voxel size, 0.45x0.45x4.00 mm; scanning time, 3 min 40 sec] for lesion detection and a high-resolution three dimensional (3D)-T1-weighted turbo field echo (3D-T1w) in the sagittal plane (TR, 9.9 msec; TE, 3.7 msec; voxel size: 1.0x1.00x1.00 mm; scanning time, 6 min) to obtain morphological images and also spectroscopic sequences.

^{1}H-MRS protocol. Single voxel ^{1}H-MRS spectroscopic data were acquired using point resolved spectroscopy sequence, receiving 1,024 samples with 2,000 Hz spectral bandwidth, 2,000 msec TR, 35 msec TE, 128 averages combined with excitation water suppression technique (scanning time, ~5 min). For each participant, two ^{1}H-MRS-voxels were located, one in the left thalamus and the other in the left centrum semiovale (CS), adjusting their dimensions to maximize the volume while avoiding contamination from neighboring structures or lesions; thereby only NAGM and NAWM were included, respectively. In the CIS group, in case an increased lesion load was detected in the left CS on T2/FLAIR images, the voxel was located on the right CS provided that no lesions were included.

^{1}H-MRS data analysis. The ^{1}H-MRS spectroscopy data were processed with TARQUIN software (version no. 4.3.10; tarquin.sourceforge.net/index.php) following the standard procedure implemented in the toolbox (39,40) Briefly, preprocessing stages included: i) Subtraction of the post-acquisition residual water by applying a signal model that contained a range of frequencies (-fs/2,+45 Hz), where fs is the sampling frequency from the free induction decay nuclear medicine resonance signal; ii) phase adjustment applying a zero and first-order phase correction to the undergoing signal and iii) automatic referencing to optimal signal fitting. Subsequently, the TARQUIN algorithm using the basing simulated set of brain metabolites was applied to solve the non-linear least squares fitting problem providing metabolite concentrations. To gain reliable spectral data the following inclusion criteria were defined: Signal-to-noise ratio >5 (Q), fit quality <2.5 (index provided by TARQUIN) and absence of visually detected baseline abnormalities and artifacts. Specifically, the absolute concentrations of eight metabolites were estimated: i) total (t)NAA; ii) tCr; iii) tCho; iv) mIns; v) Gln; vi) Glu, vii) Gln + Glu (Glx) and viii) Glth.

Statistical analysis. Preliminary testing showed that metabolite concentrations and metabolite concentration ratios within groups were not normally distributed. Therefore, the non-parametric method of quantile regression was used to compare median values between the following groups: i) CIS vs. HC; ii) CIS-treated with disease modifying therapies (DMTs) at ^{1}H-MRS acquisition vs. CIS-untreated and iii) CIS-untreated vs. HC. The STATA statistical software package (version no. 13; StataCorp LP) was used for statistical analysis.

Results

Patient characteristics. Following the initial evaluation of the MRI data and ^{1}H-MRS spectra, four participants were excluded from the analysis, of whom three were patients with CIS and artifacts were affecting spectral quality and one was a participant from the HC group with WM brain lesions detected on FLAIR images. In the CIS group, no thalamic lesions were identified on FLAIR and 3D-T1w images, a finding in accordance with their baseline MRI scan. Accordingly, the analysis included 35 patients with CIS and 28 HCs; the CIS group included 23 females and 12 males with a median age of 32 years and an interquartile range (IQR) of 27.00-36.50 years, whereas the HC group contained 20 females and 8 males with a median age of 32 years and IQR of 28.75-36.25 years.

The clinical presentations at first clinical attack for the participants in the CIS group were typical for CNS demyelination (37): Unilateral optic neuritis (n=8); brainstem syndrome (n=3); myelitis (n=16); ataxic syndrome (n=1); isolated sensory symptoms due to a cerebral lesion (n=3); multifocal/polysymptomatic (n=2) and symptoms of undetermined location (n=2). A total of 30 patients fulfilled the criteria for dissemination in space (DIS), 15 for dissemination in time (DIT), and 14 for DIS and DIT according to 2010 revisions to the McDonald criteria (37). In addition, lesions in the cervical and/or thoracic spine were identified in 27 patients with CIS on their baseline MRI scan before ^{1}H-MRS examination. Regarding CSF findings, of 30 patients with CIS who underwent lumbar puncture, oligoclonal bands were detected in 26 and elevated IgG index (>0.65) in 21. At the ^{1}H-MRS, the median duration from the CIS onset (first clinical attack) was 102 days and the median EDSS score date was 1 for the CIS group. DMTs were started in the majority of the patients with CIS that met the DIS and DIT criteria for MS. Specifically, 12 out of 14 patients fulfilling the DIS and DIT criteria were receiving DMTs at the time of the ^{1}H-MRS acquisition; these included interferon β-1a (n=7); peginterferon β-1a (n=1); glatiramer acetate (n=2); natalizumab (n=1) and dimethyl fumarate (n=1). Treatment duration was short with a median of 23.50 days (IQR, 12.00-35.50). The demographic, clinical and laboratory features of the CIS group are summarized in Table I.

^{1}H-MRS. For the CIS group, the median ^{1}H-MRS voxel size for the thalamus was 1.3 cm^3 (IQR, 0.95-1.55) and for the CS 3.62 cm^3 (IQR, 3.22-4.11). Similar values were noted for the HC group with thalamic-voxel (th) 1.29 cm^3 (IQR, 1.08-1.64) and CS-voxel (cs) 3.88 cm^3 (IQR, 2.99-4.45). Representative ^{1}H-MRS voxels and spectra from two participants in the CIS group are shown in Fig. 1. The estimated concentrations of tNAA, tCr, tCho and mIns in 'th' and 'cs' did not differ significantly between the CIS and HC groups. Concentrations of Gln(th), Gln(cs), Glu(th), Glu(cs), Glx(th) and Glx(cs) were lower in the CIS group than the HC group, however, only Glx(cs) was significantly decreased. Additionally, the concentration of Glth was increased in the CIS group compared with that in HCs in both applied voxels, but the difference did not reach statistical significance. The ^{1}H-MRS metabolite concentrations in the CIS and HC groups are represented in Table II and Fig. 2A. Ratio of tNAA, tCho, mIns, Gln, Glu, Glx and Glth concentrations relative to tCr concentration and also the ratio of Glx and Glth concentrations relative to tNAA concentration were evaluated; significantly lower ratios of tCho/tCr(th), Glu/tCr(cs), Glx/tCr(cs), Glx/tNAA(th) and Glx/tNAA(cs) were observed in patients with CIS compared with those in the HC group (Table II; Fig. 2B).

Table I. Demographic, clinical, and laboratory features of the CIS group.

Characteristic	Patients (n=35)
Female/male ratio, (%)	23/35 (65.70)
Caucasian (%)	35 (100)
Median (IQR) age at MRI/^{1}H-MRS acquisition, years	32 (27.00-36.50)
Median (IQR) duration from the CIS onset (first clinical attack) to ^{1}H-MRS acquisition, days	102 (89.50-131.50)
Median (IQR) EDSS score at ^{1}H-MRS acquisition	1 (1.00-1.50)
Number of patients with CIS and spinal cord lesions	27
Number of patients with CSF-OCBs	26
Number of patients fulfilling the criteria for DIS	30
Number of patients fulfilling the criteria for DIT	15
Number of patients fulfilling the criteria for DIS and DIT	14
Number of patients treated with DMTs at the time of ^{1}H-MRS acquisition	12
Median (IQR) DMT duration, days	23.50 (12.00-35.50)

CIS, clinically isolated syndrome; ^{1}H-MRS, proton magnetic resonance spectroscopy; IQR, interquartile range; EDSS, expanded disability status scale; CSF-OCBs, cerebrospinal fluid-oligoclonal bands; DIS, dissemination in space; DIT, dissemination in time; DMT, disease-modifying therapy.

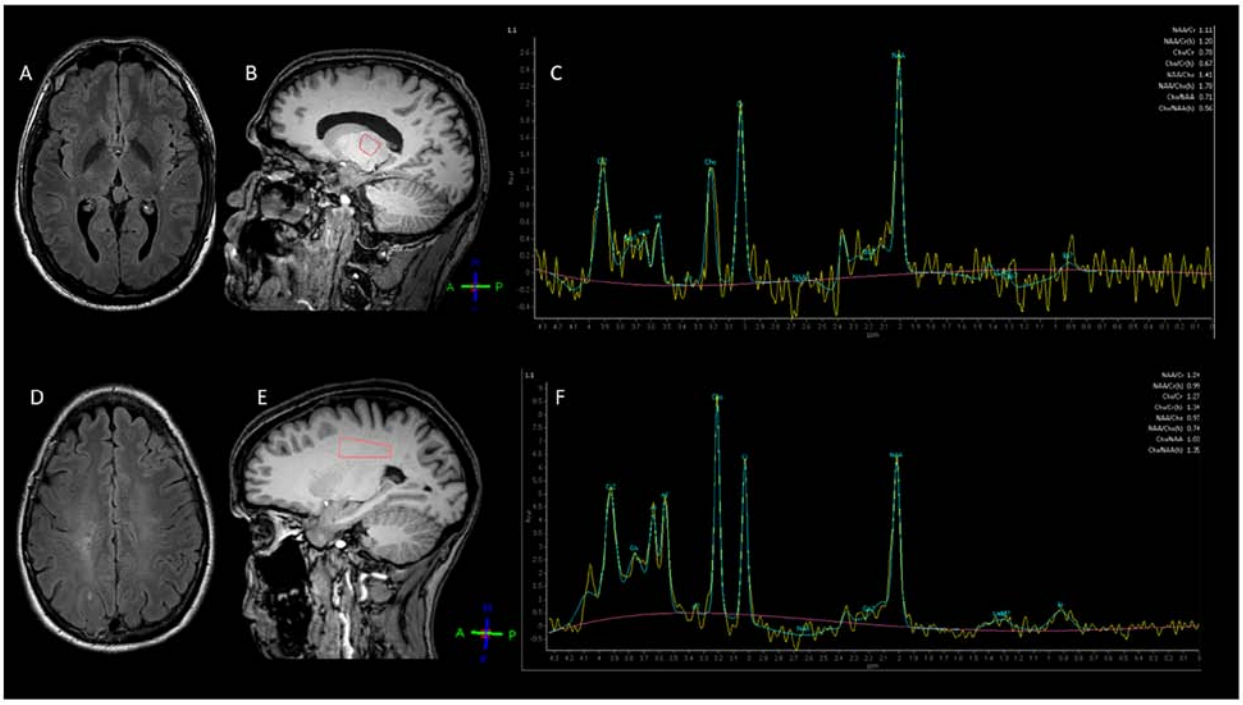

Figure 1. Representative 3.0 T ^{1}H-MRS images in two patients with CIS. (A) Brain axial FLAIR MRI image of a 35-year-old male patient with CIS. (B) Positioning of the single voxel in the left thalamus (orange) and its (C) ^{1}H-MRS spectrum. (D) MRI scan of a 32-year-old female patient with CIS (axial FLAIR). (E) Single voxel was placed in the left centrum semiovale (orange) including only normal-appearing white matter and (F) the derived spectrum. ^{1}H-MRS, proton magnetic resonance spectroscopy; CIS, clinically isolated syndrome; FLAIR, fluid-attenuated inversion recovery; tNAA, total N-acetyl aspartate; tCr, total creatine; tCho, total choline; mIns, myoinositol; Gln, glutamine; Glu, glutamate; Glx, glutamate + glutamine; Glth, glutathione.

The present study also investigated if the use of DMTs could have an early indirect impact on metabolic ^{1}H-MRS profiles. Accordingly, the estimated metabolite concentrations and ratios in patients with CIS who received DMTs (CIS-treated group, n=12) were compared with patients with CIS who were DMT-naïve at the ^{1}H-MRS acquisition (CIS-untreated group, n=23). In the CIS-treated-group tNAA(cs) was significantly higher and tCr(cs) was also elevated showing a trend toward significance (Table III, Fig. 3A). The comparisons between metabolic ratios indicated a non-significant trend for increased Gln/tCr(th) ratio in the CIS-treated group (Table III, Fig. 3B).

Compared between CIS-untreated and HC group, there were significant differences (Table IV). CIS-untreated-group showed significantly decreased Glu(cs) and Glx(cs) and lower Gln(th), however this was not significant (Fig. 4A). Comparisons between metabolite ratios revealed significantly decreased tCho/tCr(th),

Table II. ^{1}H-MRS metabolite concentrations and ratios in the CIS and HC groups.

A, Median concentration (IQR), mM

Metabolite	CIS group (n=35)	HC group (n=28)	P-value
tNAA(th)	4.55 (2.60-5.36)	5.23 (2.90-5.95)	0.114
tNAA(cs)	6.33 (5.46-7.11)	6.08 (4.88-6.93)	0.557
tCr(th)	5.39 (4.02-6.52)	5.49 (4.67-6.18)	0.952
tCr(cs)	5.03 (4.55-5.56)	4.96 (4.67-5.38)	0.724
tCho(th)	1.53 (1.06-1.86)	1.66 (1.29-1.88)	0.535
tCho(cs)	1.92 (1.80-2.09)	1.91 (1.78-2.12)	0.908
mIns(th)	3.40 (1.79-4.93)	2.56 (1.86-3.40)	0.252
mIns(cs)	2.90 (2.46-3.56)	2.93 (2.57-3.48)	0.853
Gln(th)	2.47 (0.42-3.42)	3.64 (2.31-4.67)	0.156
Gln(cs)	1.99 (0.42-3.48)	2.74 (2.36-4.49)	0.335
Glu(th)	4.37 (1.13-6.10)	4.69 (1.10-6.42)	0.736
Glu(cs)	3.41 (2.59-4.81)	4.07 (3.59-5.64)	0.129
Glx(th)	6.58 (3.17-9.55)	8.53 (4.55-11.10)	0.292
Glx(cs)	5.26 (3.17-8.73	7.59 (6.06-8.71)	0.014[a]
Glth(th)	0.68 (0.27-0.97)	0.46 (0.00-1.14)	0.100
Glth(cs)	0.55 (0.36-0.72)	0.44 (0.29-0.66)	0.741

B, Median ratio (IQR)

Metabolite	CIS group (n=35)	HC group (n=28)	P-value
tNAA/tCr(th)	0.79 (0.53-0.96)	0.876 (0.52-1.08)	0.283
tNAA/tCr(cs)	1.29 (1.08-1.40)	1.26 (1.03-1.46)	0.675
tCho/tCr(th)	0.26 (0.22-0.31)	0.29 (0.27-0.33)	0.026[a]
tCho/tCr(cs)	0.38 (0.35-0.42)	0.38 (0.36-0.40)	0.431
mIns/tCr(th)	0.57 (0.37-0.72)	0.48 (0.35-0.73)	0.505
mIns/tCr(cs)	0.57 (0.46-0.69)	0.58 (0.51-0.68)	0.678
Gln/tCr(th)	0.46 (0.09-0.65)	0.62 (0.45-0.90)	0.166
Gln/tCr(cs)	0.41 (0.05-0.69)	0.55 (0.47-0.91)	0.387
Glu/tCr(th)	0.59 (0.32-1.04)	0.90 (0.10-1.36)	0.399
Glu/tCr(cs)	0.66 (0.55-0.86)	0.87 (0.69-1.11)	0.040[a]
Glx/tCr(th)	1.14 (0.56-1.78)	1.40 (0.89-2.01)	0.573
Glx/tCr(cs)	0.99 (0.67-1.54)	1.52 (1.25-1.78)	0.004[a]
Glth/tCr(th)	0.12 (0.06-0.20)	0.07 (0.00-0.20)	0.197
Glth/tCr(cs)	0.10 (0.07-0.14)	0.09 (0.06-0.13)	0.514
Glx/tNAA(th)	1.23 (0.92-2.23)	1.95 (1.28-2.74)	0.043[a]
Glx/tNAA(cs)	0.95 (0.65-1.23)	1.35 (0.93-1.59)	0.015[a]
Glth/tNAA(th)	0.17 (0.06-0.25)	0.07 (0.00-0.20)	0.106
Glth/tNAA(cs)	0.08 (0.06-0.12)	0.07 (0.04-0.12)	0.748

[a]P<0.05. CIS, clinically isolated syndrome; HC, healthy control; ^{1}H-MRS, proton magnetic resonance spectroscopy; IQR, interquartile range; (th), thalamic-voxel; (cs), centrum semiovale-voxel; tNAA, total N-acetyl aspartate; tCr, total creatine; tCho, total choline; mIns; myoinositol; Gln, glutamine; Glu, glutamate; Glx, glutamate + glutamine; Glth, glutathione.

Gln/tCr(th), Glu/tCr(cs), Glx/tCr(th), Glx/tCr(cs), Glx/tNAA(th) and Glx/tNAA(cs) in the CIS-untreated group compared with those in the HC group. Additionally, increased Glth/tCr(th) and Glth/tNAA(th) were observed in the CIS-untreated group, however this was not significant (Table IV; Fig. 4B).

Discussion

The present ^{1}H-MRS study quantified and investigated brain metabolites in patients with CIS and HCs. The present results showed metabolic alterations in the thalamus and

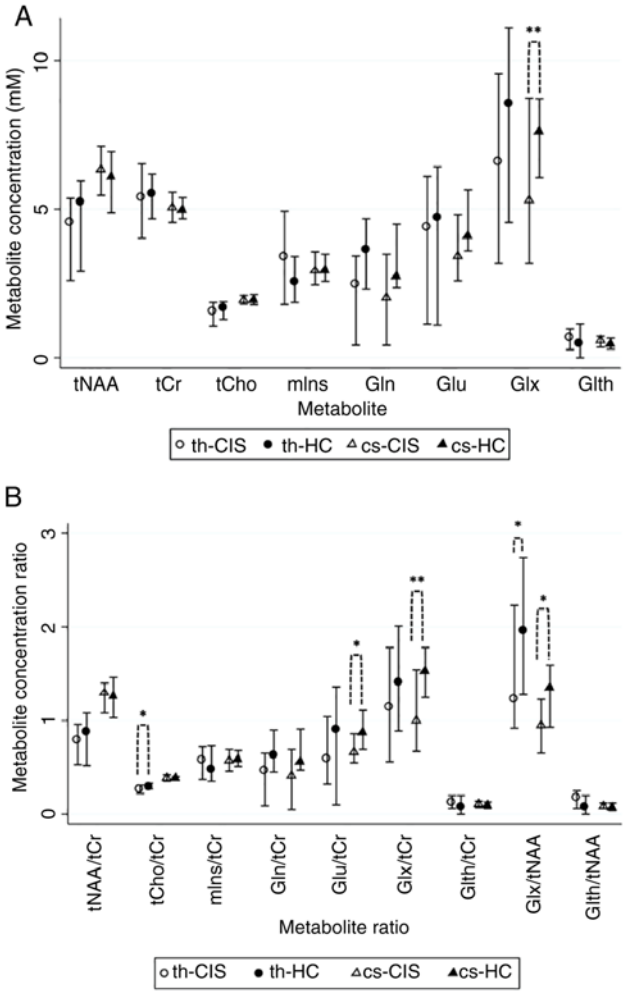

Figure 2. ^{1}H-MRS metabolite concentrations and ratios in the CIS and HC groups. (A) Concentration for each metabolite (median ± IQR) obtained from th and cs. (B) Estimated metabolite concentration ratios (median ± IQR). *P<0.05 and **P<0.01. ^{1}H-MRS, proton magnetic resonance spectroscopy; CIS, clinically isolated syndrome; HC, healthy control; tNAA, total N-acetyl aspartate; tCr, total creatine; tCho, total choline; mIns, myoinositol; Gln, glutamine; Glu, glutamate; Glx, glutamate + glutamine; Glth, glutathione; th, thalamic-voxel; cs, centrum semiovale-voxel; IQR, interquartile range.

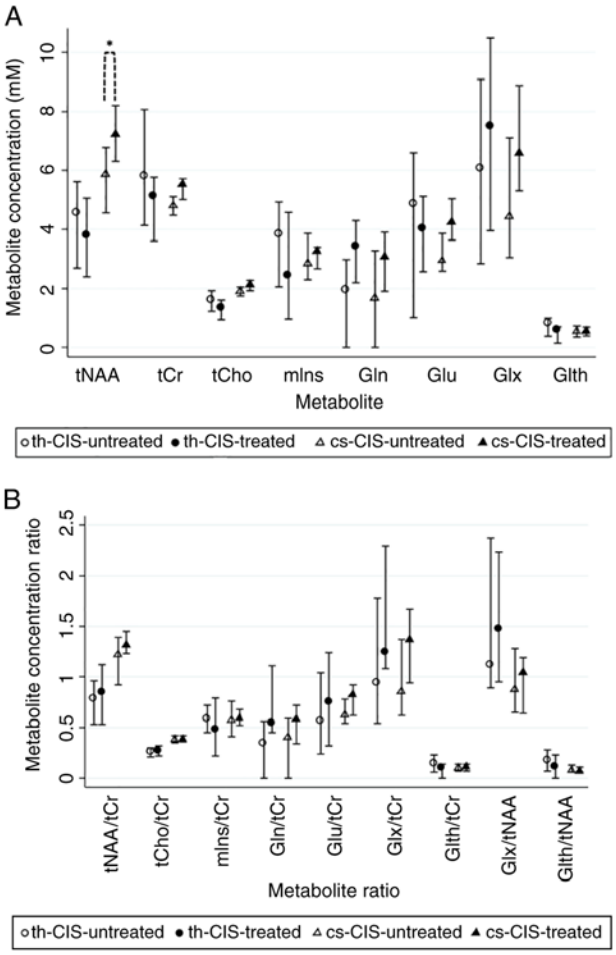

Figure 3. ^{1}H-MRS metabolite concentrations and ratios in the CIS-untreated and the CIS-treated groups. (A) Metabolite concentrations (median ± IQR) in th and cs. (B) Estimated metabolite concentration ratios (median ± IQR). *P<0.05. ^{1}H-MRS, proton magnetic resonance spectroscopy; CIS, clinically isolated syndrome; tNAA, total N-acetyl aspartate; tCr, total creatine; tCho, total choline; mIns, myoinositol; Gln, glutamine; Glu, glutamate; Glx, glutamate + glutamine; Glth, glutathione; th, thalamic-voxel; cs, centrum semiovale-voxel; IQR, interquartile range.

CS in otherwise NA brain tissue on conventional MRI. ^{1}H-MRS protocol was designed to evaluate brain regions that did not show lesions on classic FLAIR/T2 MRI sequences. Accordingly, the voxels were placed strictly in the thalamus and CS areas without including any lesions. Additionally, the present results showed similar dimensions of the 'th' and 'cs' between the patients with CIS and the HCs. Use of short TE ^{1}H-MRS was optimal for Glu and Gln peak detection (41,42).

The present analysis compared the ^{1}H-MRS results of the CIS and HC group and concluded that Glu, its metabolic precursor Gln (43) and Glx were reduced in the thalamus and the CS, indicating possibly diminished glutaminergic activity; however, only the difference of Glx(cs) reached statistical significance. The mixture of Glu/Gln is involved in both excitatory and inhibitory neuronal pathways (9) and concentrations of Glu, Gln and Glx are affected by the interaction between Glu formation/degradation and neurotransmission in neurons and astrocytes (21,44). Zhang and Shen (44) observed higher Glu levels in GM than in WM on ^{1}H-MRS of brain cortices in healthy individuals. Furthermore, a previous 3.0 T ^{1}H-MRS study in young adults showed Glx concentration to be increased in GM (thalamus included) compared with that in WM (CS included). This may be because Glu/Gln is located close to the synapses (45); this observation was in line with the present results demonstrating absolute Glx(th) higher than Glx(cs) in both the CIS and HC groups. ^{1}H-MRS studies in patients with MS also reported decreased Glu, Gln and/or Glx concentrations: Nantes *et al* (23) reported decreased Glu concentrations in the sensorimotor and parietal regions of the left cerebral hemisphere; Chard *et al* (46) reported lower levels of Glx in the cortical GM of clinically early relapsing-remitting (RR)MS compared with those in HCs and Muhlert *et al* (24) reported lower Glu and Glx levels in GM regions in patients with RRMS compared with those in controls at 3.0 T. Notably, another ^{1}H-MRS study involving patients with primary progressive MS (47) demonstrated decreased Glu and Gln concentrations in cortical GM compared with those in healthy individuals; these correlated with increased EDSS scores. Additionally, the ^{1}H-MRS study performed at 7.0 T by Swanberg *et al* (48)

Table III. Metabolite concentrations and ratios in the CIS-untreated and CIS-treated groups at ^{1}H-MRS acquisition.

A, Median concentration (IQR), mM

Metabolite	CIS-untreated (n=23)	CIS-treated (n=12)	P-value
tNAA(th)	4.57 (2.69-5.62)	3.80 (2.40-5.06)	0.558
tNAA (cs)	5.87 (4.56-6.78)	7.21 (6.31-8.20)	0.028[a]
tCr(th)	5.79 (4.14-8.07)	5.11 (3.61-5.77)	0.642
tCr(cs)	4.78 (4.48-5.11)	5.51 (5.01-5.72)	0.073
tCho (th)	1.61 (1.23-1.93)	1.34 (0.94-1.61)	0.432
tCho(cs)	1.90 (1.75-2.05)	2.12 (1.93-2.28)	0.308
mIns(th)	3.83 (2.06-4.93)	2.43 (0.96-4.58)	0.462
mIns(cs)	2.84 (2.30-3.87)	3.25 (2.67-3.40)	0.817
Gln(th)	1.94 (0.00-2.98)	3.42 (2.20-4.30)	0.383
Gln(cs)	1.68 (0.00-3.28)	3.05 (1.91-3.91)	0.109
Glu(th)	4.85 (1.01-6.60)	4.03(2.57-5.12)	0.501
Glu(cs)	2.93 (2.59-3.87)	4.22 (3.64-5.04)	0.113
Glx(th)	6.06 (2.84-9.09)	7.49 (3.96-10.49)	0.406
Glx(cs)	4.44 (3.05-7.11)	6.59 (5.31-8.88)	0.125
Glth(th)	0.82 (0.38-0.99)	0.60 (0.14-0.70)	0.429
Glth(cs)	0.54 (0.35-0.73)	0.55 (0.39-0.69)	0.850

B, Median ratio (IQR)

Metabolite	CIS-untreated (n=23)	CIS-treated (n=12)	P-value
tNAA/tCr(th)	0.78 (0.53-0.96)	0.84 (0.53-1.12)	0.497
tNAA/tCr(cs)	1.22 (0.92-1.39)	1.31 (1.23-1.45)	0.292
tCho/tCr(th)	0.26 (0.21-0.30)	0.27 (0.22-0.32)	0.390
tCho/tCr(cs)	0.38 (0.35-0.42)	0.38 (0.36-0.42)	0.819
mIns/tCr(th)	0.58 (0.45-0.72)	0.48 (0.22-0.79)	0.609
mIns/tCr(cs)	0.57 (0.41-0.76)	0.59 (0.52-0.68)	0.680
Gln/tCr(th)	0.34 (0.00-0.56)	0.54 (0.45-1.11)	0.088
Gln/tCr(cs)	0.40 (0.00-0.59)	0.58 (0.34-0.72)	0.122
Glu/tCr(th)	0.56 (0.24-1.04)	0.75 (0.32-1.24)	0.986
Glu/tCr(cs)	0.62 (0.54-0.78)	0.82 (0.62-0.92)	0.352
Glx/tCr(th)	0.94 (0.54-1.78)	1.24 (1.08-2.29)	0.191
Glx/tCr(cs)	0.85 (0.62-1.37)	1.36 (0.94-1.67)	0.770
Glth/tCr(th)	0.14 (0.06-0.23)	0.10 (0.00-0.14)	0.542
Glth/tCr(cs)	0.10 (0.07-0.14)	0.11 (0.07-0.14)	0.984
Glx/tNAA(th)	1.12 (0.89-2.37)	1.47 (0.95-2.23)	0.566
Glx/tNAA(cs)	0.87 (0.65-1.28)	1.04 (0.64-1.19)	0.497
Glth/tNAA(th)	0.18 (0.07-0.28)	0.11 (0.00-0.23)	0.795
Glth/tNAA(cs)	0.08 (0.06-0.13)	0.07 (0.06-0.11)	0.994

[a]P<0.05. CIS, clinically isolated syndrome; ^{1}H-MRS, proton magnetic resonance spectroscopy; IQR, interquartile range; (th), thalamic-voxel; (cs), centrum semiovale-voxel; tNAA, total N-acetyl aspartate; tCr, total creatine; tCho, total choline; mIns; myoinositol; Gln, glutamine; Glu, glutamate; Glx, glutamate + glutamine; Glth, glutathione.

involving patients with progressive MS, RRMS and healthy controls demonstrated that only patients with progressive MS had lower frontal cortical Glu levels but not reduced Gln compared to healthy individuals; moreover, a negative correlation of Glu levels with MS duration was found, suggesting that these findings may reflect neuronal cell death (48). Even though the present study applied the voxel in deep GM (thalamus) and not in the cortex, it found no significant differences in Glu(th) levels between the CIS and the HC group. Conversely, other ^{1}H-MRS studies estimating Glu or Glx levels in WM reported

Table IV. Metabolite concentrations and ratios in the CIS-untreated and HC groups.

A, Median concentration (IQR), mM

Metabolite	CIS-untreated (n=23)	HC (n=28)	P-value
tNAA(th)	4.57 (2.63-5.71)	5.23 (2.56-5.96)	0.543
tNAA(cs)	5.87 (4.55-6.87)	6.07 (4.86-6.99)	0.639
tCr(th)	5.79 (4.02-8.51)	5.49 (4.59-6.20)	0.611
tCr(cs)	4.77 (4.46-5.11)	4.96 (4.66-5.38)	0.247
tCho(th)	1.61 (1.22-1.95)	1.66 (1.28-1.89)	0.983
tCho(cs)	1.90 (1.73-2.06)	1.90 (1.77-2.12)	0.797
mIns(th)	3.83 (1.95-4.93)	2.56 (1.85-3.40)	0.244
mIns(cs)	2.84 (2.30-4.03)	2.92 (2.55-3.60)	0.949
Gln(th)	1.94 (0.00-3.06)	3.64 (2.15-4.72)	0.068
Gln(cs)	1.68 (0.00-3.40)	2.74 (2.35-4.62)	0.404
Glu(th)	4.85 (0.97-6.76)	4.69 (0.95-6.46)	0.561
Glu(cs)	2.93 (2.59-4.01)	4.06 (3.55-5.67)	0.019[a]
Glx(th)	6.05 (2.73-9.14)	8.53 (4.52-11.10)	0.199
Glx(cs)	4.44 (3.04-7.40)	7.59 (6.04-8.76)	0.014[a]
Glth(th)	0.82 (0.35-0.99)	0.46 (0-1.20)	0.113
Glth(cs)	0.54 (0.34-0.76)	0.44 (0.28-0.66)	0.941

B, Median ratio (IQR)

Metabolite	CIS-untreated (n=23)	HC (n=28)	P-value
tNAA/tCr(th)	0.78 (0.53-0.96)	0.876 (0.52-1.08)	0.513
tNAA/tCr(cs)	1.22 (0.92-1.39)	1.26 (1.03-1.46)	0.840
tCho/tCr(th)	0.26 (0.21-0.30)	0.29 (0.27-0.33)	0.015[a]
tCho/tCr(cs)	0.38 (0.35-0.42)	0.38 (0.36-0.40)	0.875
mIns/tCr(th)	0.58 (0.45-0.72)	0.48 (0.35-0.73)	0.456
mIns/tCr(cs)	0.57 (0.41-0.76)	0.58 (0.51-0.68)	0.760
Gln/tCr(th)	0.34 (0.00-0.56)	0.62 (0.45-0.90)	0.004[a]
Gln/tCr(cs)	0.40 (0.00-0.59)	0.55 (0.47-0.91)	0.267
Glu/tCr(th)	0.56 (0.24-1.04)	0.90 (0.10-1.36)	0.272
Glu/tCr(cs)	0.62 (0.54-0.78)	0.87 (0.69-1.11)	0.021[a]
Glx/tCr(th)	0.94 (0.54-1.78)	1.40 (0.89-2.01)	0.041[a]
Glx/tCr(cs)	0.85 (0.62-1.37)	1.52 (1.25-1.78)	0.003[a]
Glth/tCr(th)	0.14 (0.06-0.23)	0.07 (0.00-0.20)	0.064
Glth/tCr(cs)	0.10 (0.07-0.14)	0.09 (0.06-0.13)	0.626
Glx/tNAA(th)	1.12 (0.89-2.37)	1.95 (1.28-2.74)	0.030[a]
Glx/tNAA(cs)	0.87 (0.65-1.28)	1.35 (0.93-1.59)	0.015[a]
Glth/tNAA(th)	0.18 (0.07-0.28)	0.08 (0.00-0.20)	0.086
Glth/tNAA(cs)	0.08 (0.06-0.13)	0.07 (0.04-0.12)	0.552

[a]P<0.05. CIS, clinically isolated syndrome; HC, healthy control; ^{1}H-MRS, proton magnetic resonance spectroscopy; IQR, interquartile range; (th), thalamic-voxel; (cs), centrum semiovale-voxel; tNAA, total N-acetyl aspartate; tCr, total creatine; tCho, total choline; mIns; myoinositol; Gln, glutamine; Glu, glutamate; Glx, glutamate + glutamine; Glth, glutathione.

increased levels of these two markers: Srinivasan *et al* (22) reported an elevation of Glu in acute lesions (contrast-enhancing) and NAWM areas, but no significant elevation in chronic lesions. Moreover, Tisell *et al* (49) used 1.5 T ^{1}H-MRS and observed that Glx concentration was higher in the NAWM of patients with MS compared with that in healthy individuals, showing a positive correlation with the MS severity score, therefore suggesting that Glx in the NAWM may be associated with disease progression. Another 3.0 T ^{1}H-MRS study of NAWM in patients with SPMS revealed annual declines of

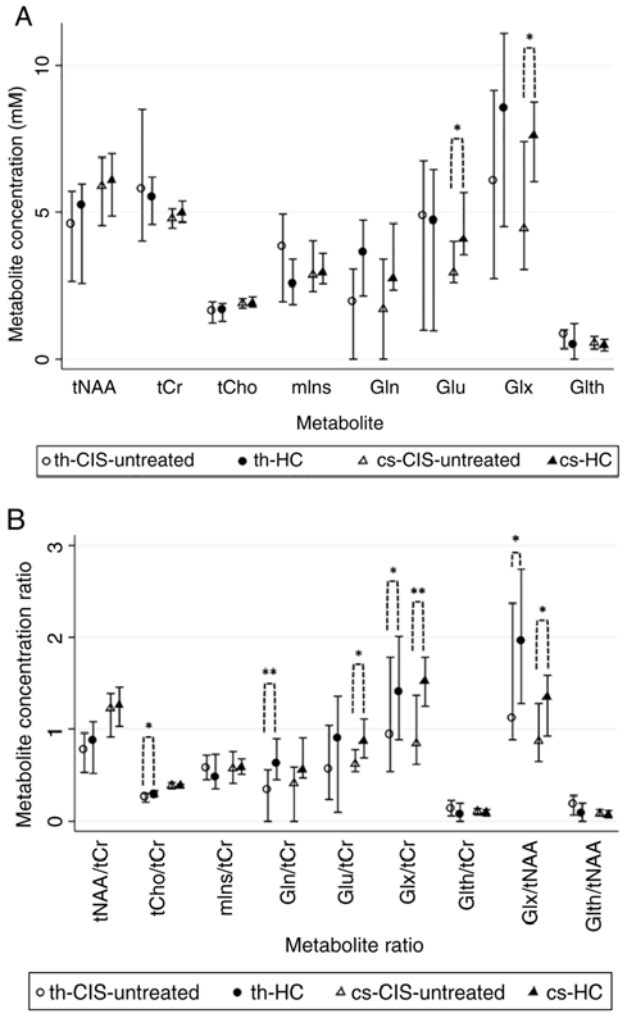

Figure 4. ^{1}H-MRS metabolite concentrations and ratios in the CIS-untreated and HC groups. (A) Metabolite concentrations (median ± IQR) in th and cs. (B) Estimated metabolite concentration ratios (median ± IQR). *P<0.05 and **P<0.01. ^{1}H-MRS, proton magnetic resonance spectroscopy; CIS, clinically isolated syndrome; HC, healthy control; tNAA, total N-acetyl aspartate; tCr, total creatine; tCho, total choline; mIns, myoinositol; Gln, glutamine; Glu, glutamate; Glx, glutamate + glutamine; Glth, glutathione; th, thalamic-voxel; cs, centrum semiovale-voxel; IQR, interquartile range.

Glu and Gln levels within a 2-year period follow-up, implying that these metabolic changes may be considered biomarkers of MS disease progression (50). Azevedo *et al* (51) used multi-voxel ^{1}H-MRS of mixed tissue of NAWM plus GM and concluded that higher Glu concentrations increased the rate of NAA decline and a higher Glu/NAA ratio in the NAWM increased the rate of the decrease in brain volume. Although Fernando *et al* (35) and Wattjes *et al* (32) reported higher mIns in the NAWM of patients with CIS than in HCs, the present study did not confirm such a difference.

In the present study, several metabolite ratios were also calculated and showed heterogeneity between the CIS and the HC groups; significantly reduced ratios of tCho/tCr(th), Glu/tCr(cs), Glx/tCr(cs), Glx/tNAA(th) and Glx/tNAA(cs) were found in the CIS group. In *in vivo* ^{1}H-MRS studies, Cr is commonly considered a relatively stable marker of intact brain energy metabolism; therefore it has been frequently used as the reference molecule for the ^{1}H-MRS metabolite ratios (17,52). Moreover, Cho is considered a marker of cell wall integrity as it is a precursor of the cellular membranes (20). Therefore, decreased tCho/tCr(th) ratio that was found in the CIS group may be mainly attributed to the lower Cho levels in the thalamus. This could reflect increased uptake of Cho from the free phase for the building of cell membranes and therefore may be associated with the onset of healing of brain tissue (53). Mathiesen *et al* (54) reported reduced Cho/Cr ratio within the cortical GM in patients with MS compared with that in HCs. These results in the NAGM may also indicate reduced cellularity during MS pathogenesis. In further accordance with the present results, which demonstrated reduced Glu/tCr(cs) and Glx/tCr(cs) ratios, Wattjes *et al* (32) indicated that the Glx/Cr ratio decreased by 13.2% in the parietal NAWM of patients with CIS compared with that in the HC group.

Furthermore, the present results demonstrated that tNAA(cs) was not affected in the CIS-cohort, which was in accordance with Fernando *et al* (35) and Brex *et al* (33); this may be indicative of a chemical environment characterized by lack of severe impairment of axonal and neuronal integrity (55). Nevertheless, other ^{1}H-MRS studies described lower tNAA in the parietal NAWM in patients with CIS than that in HCs (32,34). The present Glx/tNAA ratio was found to be decreased in the thalamus and CS of the CIS group compared with that in the HC group; this may suggest disruption of glutamate homeostasis rather than neuronal/axonal damage (56) and could be because the compensatory capacity of the CNS for axonal disruption is not significantly compromised at the early stages of the MS pathogenic process. Glx/NAA ratio of the hypothalamus in patients with RRMS was assessed by Polacek *et al* (57); the increased ratio is associated with an increase in the MS severity scale score and disease severity. Low Glx/tNAA was observed in the NAWM and NAGM of the present CIS group; this could be attributed to their mild disease severity, since all the participants in the CIS group were at the early clinical stage of MS, with <6 months from the first clinical episode and a low median EDSS score of 1 (IQR, 1.0-1.5).

The present analysis compared ^{1}H-MRS results between the CIS-untreated and the HC group, thus excluding the CIS-treated participants to examine for potential early impact of DMTs on the biochemical brain content. Differences between the CIS-untreated and HC group yielded statistically stronger differences than testing between the CIS vs. the HC group for Glx(cs), tCho/tCr(th), Glu/tCr(cs) Glx/tCr(cs), Glx/tNAA(th) and Glx/tNAA(cs). These findings were in alignment with the hypothesis of glutaminergic impairment and altered glutamate homeostasis in early MS (58,59). Glu(cs), Gln/tCr(th) and Glx/tCr(th) were significantly decreased in the CIS-untreated group compared with HCs, reflecting the aforementioned imbalance of Glu and its metabolites in the NAWM and NAGM.

CNS demyelination is associated with increased energy demand; Witte *et al* (60) observed enhanced mitochondrial density in axons and astrocytes in active MS lesions. An elevated number of mitochondria is also reported in the NAGM of patients with MS (61). Additionally, mitochondrial dysfunction is observed in the NAWM of MS (62). Consequently, the ^{1}H-MRS quantification of tNAA, which is located in neurons and axons, may provide not only information on axonal integrity but also on mitochondrial function (11,63). The present comparison between the CIS-treated and the CIS-untreated

groups indicated a higher tNAA(cs) in the CIS-treated group; this could reflect an early protective treatment effect on the chemical environment of brain WM tissue as increased levels of NAA contribute to the enhancement of mitochondrial energy production and membrane lipid production and thus to the survival of neurons (56).

Glth, is hypothesized to be a key antioxidant in neuroprotection by interacting with the reactive oxygen species, which are increased during MS pathogenesis (64) and are generated by activated macrophages during inflammation (63), thus leading to cellular damage and tissue injury. Glth is involved in the Glu-Gln cycle in astrocytes and neurons of the brain (65), and its synthesis depends on extracellular Glu levels, hence possibly contributing to the minimization of Glu toxicity by the conversion of Glu to Glth (66,67). Here, Glth(cs) and Glth(th) were estimated to be higher in the CIS than in the HC group and also in the CIS-untreated group than in HCs; however, these differences did not reach statistical significance. Differences in Glth ratios were also observed. Increased Glth/tCr and Glth/tNAA were evidenced in the thalamus and CS of the CIS-untreated group compared with those in the HC group with the thalamic voxel ratios demonstrating a trend toward significance for both ratios. Considering the decrease in concentrations of Glx that was observed in the CIS group of the present study, which may be attributed to the inhibition of Glu synthesis or to its rapid transformation to other metabolites such as Glth, the higher levels of Glth could reflect an adequate compensatory mechanism for diminishing Glu levels in the neuronal environment in the early clinical stage of MS as the CIS.

In the present study, certain results demonstrated only a trend toward statistical significance; a larger sample size could lead to significant results. Comparison of the CIS treated and untreated groups decreased the number of patients per sub-group, however, it revealed additional multiple significant associations and hence indicated strong differences. Additionally, heterogeneity with the results of previous ^{1}H-MRS MS studies may be attributed to the following factors: different technical and methodological approaches and discrepancies within various spectroscopy acquisition protocols; differences in the applied magnetic field strength (1.5, 3.0 or 7.0 T); use of different [short (35 msec) or intermediate (144 msec)] TEs; inhomogeneity of tissue selection during voxel placement (such as inclusion of mixed areas of NAWM with GM or ventricles, or inclusion of regions with demyelinating lesions); different methods for spectral processing and quantification; heterogeneity in the definition of the diagnosis of early or progressive MS, and treatment influence on the levels of neurometabolites. Consequently, ^{1}H-MRS acquisition and data processing protocols must be standardized to achieve reliable results and expand its clinical utility in MS and other diseases affecting the CNS. Follow-up ^{1}H-MRS measurements in both CIS and HC groups should be performed to evaluate changes in metabolite levels in the long term.

In conclusion, the current findings suggested that 3.0 T ^{1}H-MRS may provide novel insights into the metabolic alterations that could occur during the pathogenesis of MS and at the very early clinical phase of the disease. It could be considered a useful and advanced method to non-invasively evaluate and quantify brain metabolite concentrations. 3.0 T ^{1}H-MRS was capable of detecting early biochemical changes in NAWM and NAGM before lesion formation became evident on conventional MRI, reflecting an imbalance caused by the immunological mechanism of MS. Moreover, an early indirect therapeutic impact of DMTs on the biochemical profile of NAWM and NAGM in the CIS group was also observed, despite the relatively small number of patients in the sub-group comparisons. The observed therapeutic effect indicated the need for early initiation of immunotherapy, aiming for rapid disease control to ameliorate tissue damage, since a biochemical shift may be mediated even in a few weeks from treatment onset, as shown in the present study. Therefore, in the future, ^{1}H-MRS might be incorporated both into the monitoring of treatment efficacy and therapeutic decision-making process in MS.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding authors on reasonable request.

Authors' contributions

DT, AK, EK, GV, CP, CK and EA conceived and designed the study. DT, AK, EK, GV, JST, SAS, PT, IE, GT, CP, CK and EA acquired, analyzed and interpreted the data. DT and AK confirm the authenticity of the raw data. DT, AK, EK and EA drafted the manuscript. All authors critically reviewed the manuscript for important intellectual content. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Approval was obtained from the Ethics Committee of Eginition Hospital (approval no. 518/5.10.2015). Informed written consent to participate was obtained from all the participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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