Brain MR spectroscopic abnormalities in “MRI-negative” tuberous sclerosis complex patients

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ABSTRACT

Since approximately 5–10% of the ~50,000 tuberous sclerosis complex (TSC) patients in the US are “MRI-negative,” our goal was to test the hypothesis that they nevertheless exhibit metabolic abnormalities. To test this, we used proton MR spectroscopy to obtain and compare gray and white matter (GM and WM) levels of the neuronal marker, N-acetylaspartate (NAA), the glial marker, myo-inositol (mI), and its associated creatine (Cr), and choline (Cho) between two “MRI-negative” female TSC patients (ages 5 and 43 years) and their matched controls. The NAA, Cr, Cho and mI concentrations, 9.8, 6.3, 1.4, and 5.7 mM, in the pediatric control were similar to those of the patients, whereas the adult patient revealed a 17% WM NAA decrease and 16% WM Cho increase from their published means for healthy adults — both outside their respective 90% prediction intervals. These findings suggest that longer disease duration and/or TSC2 gene mutation may cause axonal dysfunction and demyelination.

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1. Introduction

Tuberous sclerosis complex (TSC), a neurocutaneous disorder affecting ~1:6000 live births, affects ~50,000 people in the US [1]. In 85% of cases, the disorder results from mutations in the TSC1 (9q34) or TSC2 (16p13.3) gene that codes for hamartin and tuberin, respectively. The latter is more common and causes more severe pathology [2]. Clinical symptoms and pathological findings in TSC are primarily from mammalian target of rapamycin complex 1 (mTORC1) hyperactivity. Tuberous sclerosis complex affects multiple organ systems, prominently involving the brain, skin, kidneys, and lungs [3,4]. Neurological disorders are probably the leading cause of morbidity and mortality [5] — seizures affect up to 90% of patients, often with early onset and medical intractability, and are associated with high rates of intellectual disability and autism [6–9]. Other neuropsychiatric disorders such as language delays, impaired social and emotional skills, aggressive behavior, attention-deficit, anxiety, and affective and motor disorders complicate TSC [9,10].

Tuberous sclerosis complex brain lesions include subependymal giant cell astrocytomas (SEGAs) and nodules that can occur throughout the brain (although predominant in the subcortical nuclei [11]), cortical and subcortical tubers, cerebral cortex and cerebellum developmental malformations composed of disorganized structures lacking six-layered lamination and containing dysplastic neurons, astrocytes, and/or giant cells. The tubers’ number and size correlate in some studies with autism, intellectual disability, and epilepsy severity [12,13]. In patients with treatment-refractory epilepsy and a single large tuber, its resection is associated with more than 90% reduction in seizures [14]. The relation between MRI lesion burden and neurologic phenotype, however, is imperfect: TSC patients with many tubers can have normal IQ, while others with just a few may suffer severe intellectual disability [13]. Moreover, some TSC patients with clinically symptomatic SEGAs have many cortical tubers but have no seizures and require no antiepileptic medication. Finally, TSC animal models have been able to replicate hyper-excitatory brains and seizures, but lack tubers [15–17].

Fluid-attenuated inversion recovery (FLAIR) MRI is the most sensitive sequence to identify the tubers, with a false-negative rate
of $\leq 0.5\%$ versus 21% for $T_2$-weighted MRI [18]. This is due to the long $T_2$-weighting and cerebrospinal fluid (CSF) signal suppression that enhances the sensitivity to small subcortical and gyral core (enlarged gyri with central hyperintensity) tubers, although in neonates and infants, MRI signal intensity changes may be opposite to those of older children or adults [19]. Despite FLAIR’s high sensitivity, only 90–95% of TSC patients exhibit brain tubers on MRI [18,19]. The remaining show none despite neuropsychiatric symptoms [18]. In these patients, mTORC1 pathway hyperactivation – the underlying pathogenic mechanism of TSC – may interfere with cortical development and contribute to functional impairments by producing giant cells, dysplastic neurons, and other MRI-occult microscopic changes [20,21].

Aberrent neuronal and glial function in TSC may be monitored non-invasively through their proton MR spectroscopy ($^1$H MRS)–observed markers: $N$-acetylaspartate (NAA) for neurons [22], myoinositol (ml) for the astroglia [23,24], and creatine (Cr) and choline (Cho) that are more abundant in the latter [25]. Our goal, therefore, was to test whether “MRI-negative” TSC patients exhibit the following: (a) increases in the astroglial metabolites: ml, Cr, and Cho; and (b) NAA levels similar to those of controls, since mTORC1 processes produce dysfunctional neurons but do not damage existing ones. Towards these ends and since TSC pathology is diffuse, we performed three-dimensional (3D) $^1$H MRS on the brains of two patients with MRI-negative TSC and their healthy matched controls.

2. Materials and methods

2.1. Human subjects

Two female TSC patients, ages 5 and 43 years, and their age- and gender-matched controls were prospectively enrolled. The 5-year-old patient did not have either TSC1 or TSC2 mutation although she met criteria for “clinically-definite” TSC [26] and was diagnosed with cardiac rhabdomyomas by birth. Seizures began at age 2 years, and she developed medically refractory partial (simple and complex partial, status), and gait ataxia. Bilateral subdural strip studies revealed multifocal and symmetric cardiac rhabdomyomas at birth. Seizures began at age 2 years, and the patient was diagnosed with TSC2 [26] and was diagnosed with TSC1/2-negative TSC2 mutation although she met criteria for “clinically-definite” TSC [26] and was diagnosed with cardiac rhabdomyomas by birth. Seizures began at age 2 years, and she developed medically refractory partial (simple and complex partial, secondary generalized) and symptomatic generalized (myoclonic, atypical absence, and tonic) seizures. Electroencephalogram showed generalized spike-wave and left occipital discharges. Her examination revealed five hypopigmented macules, global developmental delay, and gait ataxia. Bilateral subdural strip studies revealed multifocal and diffuse seizure onsets that were inoperable. The 43-year-old woman was diagnosed with a TSC2 gene mutation, facial angiofibromas, and renal angiomyolipomas, but had no history of neurological symptoms. Both patients and controls had “unremarkable” brain MRI. The children’s parents and the adults gave written Institutional Review Board-approved informed consent.

2.2. MR acquisition

Measurements on the pediatric subjects were done in a 1.5 T MRI scanner with its standard transmit–receive circularly-polarized head coil (AVANTO®, Siemens AG, Erlangen Germany). Axial $T_2$-weighted turbo-spin echo (TSE; $TE/TR = 107/4770$ ms) MRI were acquired at $220 \times 220$ mm$^2$ field-of-view (FOV), $256 \times 256$ matrix, and 5.0 mm slice thickness. Measurements on the adults were done in a 3 T MRI scanner (TRIO®, Siemens AG, Erlangen Germany) with a transmit–receive circularly-polarized head coil (TEM3000, MR Instruments, Minneapolis, MN). The TSE MRI were acquired at $256 \times 256$ mm$^2$ FOV, $512 \times 512$ matrix, and 3.7 mm slice thickness.

In the children, following our chemical shift imaging (CSI)-based autoshim procedure [27], and depending on their brain size, a 9–10 cm anterior–posterior (AP) by 7–8 cm left–right (LR) by 4 cm inferior–superior (IS) = 252–320 cm$^3$. $^1$H MRS volume-of-interest (VOI) was centered on the corpus callosum, as shown in Fig. 1. It was excited with $TE/TR = 30/1380$ ms PRESS and partitioned using Hadamard spectroscopic imaging into four axial slices, each encoded with $16 \times 16$ 2D-CSI over a $16 \times 16$ cm$^2$ (LR × AP) FOV [28]. The VOI was defined in these slices’ planes with two 8 ms numerically optimized 180° pulses (6.3 kHz bandwidth) to yield 252–320 voxels, 1.0 cm$^3$ each. The MRS signal was acquired for 512 ms at ±0.5 kHz bandwidth. At three averages, the $^1$H MRS signal took 35 min and the protocol was 3 h/4.

In the two adults, a $10 \times 8 \times 4.5$ cm$^3$ = 360 cm$^3$ (AP × LR × IS) $^1$H MRS VOI was used, as shown in Fig. 2, and excited with $TE/TR = 35/1800$ ms PRESS. It was partitioned into six axial slices with three-second-order Hadamard encoded slabs [28]. These were encoded with $16 \times 16$ 2D-CSI over a $16 \times 16$ cm$^2$ (LR × AP) FOV, and the VOI was defined in the slices’ planes by two 11.2 ms optimized 180° pulses (4.5 kHz bandwidth) yielding $8 \times 10 \times 6 = 480$ voxels, 0.75 cm$^3$ each. The $^1$H MRS signal was acquired for 256 ms at ±1 kHz bandwidth; at two averages, it took 31 min.

2.3. Metabolite quantification

Processing was done with in-house software that removed the residual water signal in the time domain, zero-filled the data from 512 to 2048 points, voxel-shifted the data to align the CSI grid with the NAA signal within the VOI, Fourier transformed in the time, AP, and LR dimensions, and Hadamard reconstructed along the IS direction. The spectra were frequency-aligned and phased in reference to the NAA peak in each voxel [29], and the relative level of the i-th metabolite = NAA, Cr, Cho or ml in each voxel was estimated from its peak area, $S_i$, using prior knowledge modeling software [30]. These were scaled into absolute concentrations, $C_i$ relative to their signals from a 2 L reference sphere of $C_NAA = 12.5$, 10.0, 3.0, and 7.5 mM NAA, Cr, Cho and ml in water at physiological ionic strength:

$$C_i = C_i^{\text{in vivo}} S_i \frac{p^{180}}{p_R^{180}} \left( \frac{p^{180}}{p_R^{180}} \right)^{1/2} \frac{1}{T_i^{\text{in vivo}}},$$

(1)

where $S_i$ is the reference metabolites’ signal and $T_i$ the T1 VOI tissue fraction, $p^{180}$ and $p_R^{180}$ the radio-frequency (RF) powers for a non-selective 1 ms 180° pulse on subject and reference and $f_j$s the factors that correct for different relaxation times in vivo ($T_1^{\text{in vivo}}$, $T_2^{\text{in vivo}}$) and in the reference ($T_1^{\text{in vitro}}$, $T_2^{\text{in vitro}}$):

$$f_j = \frac{\exp\left(-TE/T_1^{\text{in vivo}}\right)}{\exp\left(-TE/T_1^{\text{in vitro}}\right)} \times \frac{1-\exp\left(-TR/T_1^{\text{in vivo}}\right)}{1-\exp\left(-TR/T_1^{\text{in vitro}}\right)}.$$  

(2)

We used the reported 3 T $T_1^{\text{in vivo}}$ values of 1360, 1300, 1145, and 1170 ms for NAA, Cr, Cho, and ml [31] and $T_2^{\text{in vivo}}$ values of 350, 174, 251, and 200 ms [32,33]. The 1.5 T metabolite $T_2^{\text{in vivo}}$ values used were 357, 216, 332, and 200 ms [34]. Since the corresponding $T_1^{\text{in vivo}}$ values are reported not to significantly differ from 3 T [34], the same values were used at both fields.

The global concentration of the i-th metabolite in the VOI of a subject, $C_i$, was obtained by summing all the spectra in the VOI. This strategy is appropriate for diffuse disorders, retains the individual (narrow) spectra linewidth, and dramatically improves the signal-to-noise-ratio (SNR), i.e., the precision [35], by the square root of the number of voxels, $\approx 20$-fold in this study [36]:

$$C_i = \frac{\sum_j n_j \cdot Q_j}{\sum_j f_j (V_j^{180} + V_j^{180})} f_j \text{mM/g wet weight},$$

(3)

where $n$ is the number of voxels in the VOI and $f_j$ is given by Eq. (2) for the i-th metabolite.
2.4. Brain volumetry

The TSE images were segmented with our FireVoxel package [37] that also works well on pediatric brains, as shown in Fig. 3. It first corrects the images for non-uniform intensities due to the coil’s RF inhomogeneity, using the common histogram devolution method of Sled et al. [38]. Next, a white matter (WM) signal intensity, \( I_{WM} \), is selected in a periventricular seed region. Following automatic detection of all pixels at or above 50% (but below 192.5% to exclude the CSF) of \( I_{WM} \), every slice’s tissue-mask is constructed in three steps: morphological erosion, recursive region growth retaining pixels connected to the seed, and morphological inflation to reverse the erosion effect. Pixels of intensity above 192.5% of \( I_{WM} \) are classified as CSF, above 130% (but below 192.5%) of \( I_{WM} \) classified as part of the GM mask, and under 130% (but over 50% to exclude air cavities) \( I_{WM} \) classified as WM, as shown in Figs. 3b, c and d. The in-house software (MATLAB, The Mathworks Inc., Natick, MA) estimated each mask’s volumes in every \( j \)-th voxel in the \( k \)-th subject (\( V_{ijk}^{GM} \), \( V_{ijk}^{WM} \), and \( V_{ijk}^{CSF} \)). The overall VOI, GM, and WM tissue fractions (\( T_{ijk} \), \( GM_{ijk} \), and \( WM_{ijk} \)) were obtained by dividing \( V_{ijk}^{GM} + V_{ijk}^{WM} \), \( V_{ijk}^{GM} \), and \( V_{ijk}^{WM} \) by the VOI volume.

2.5. Global GM and WM concentrations

Since the CSF does not contribute to the \(^1\text{H} \) MRS signal, the \( i \)-th metabolite amount in the \( j \)-th voxel in the \( k \)-th subject can be modeled as the sum of two compartments’ (GM and WM) amounts:

\[
Q_{ijk} = Q_{ijk}^{GM} f_{ij}^{GM} + Q_{ijk}^{WM} f_{ij}^{WM} = C_{ik}^{GM} V_{ijk}^{GM} f_{ij}^{GM} + C_{ik}^{WM} V_{ijk}^{WM} f_{ij}^{WM},
\]

(4)

where \( C_{ik}^{GM} \) and \( C_{ik}^{WM} \) are the (unknown) global GM and WM metabolites’ concentrations and the \( f_{ij} \)-s are given by Eq. (2). Since no significant GM and WM \( T_1^{1\text{H}} \) differences are reported between 1.5 and 3 T [34], we used the values below Eq. (2). For NAA, Cr, Cho, and mI \( T_2^{1\text{H}} \) values at 1.5 T, we used 317, 208, 300, and 130 ms for the GM and 361, 215, 330, and 110 ms for WM [34,39]. At 3 T, the \( T_2^{1\text{H}} \) values used were 275, 157, 241, and 200 ms in GM and 400, 185, 258, and 200 ms in WM [32,33]. Although \( C_{ik}^{GM} \) and \( C_{ik}^{WM} \) cannot both be derived from Eq. (4), since the brain’s GM and WM spatial heterogeneity is on a scale much smaller than the voxels, each has different, independent \( V_{ijk}^{GM} \) and \( V_{ijk}^{WM} \) coefficients. The resulting over-determined system of equations in \( C_{ik}^{GM} \) and \( C_{ik}^{WM} \) was solved...
with linear regression. The inter-subject coefficient of variation (CV = standard deviation/mean) of this approach has been shown to be under 15% [40].

2.6. Statistical analysis

Due to the small sample size, there was not enough statistical power to formally test patient to control values for statistical significance. Therefore, a sample of 18 previously-studied healthy adults was used to derive 90% prediction intervals [40]. Each metabolite concentration’s (or tissue fraction’s) mean, \( \bar{X}_i \), and standard deviation, \( S_i \) in the VOI and in its GM and WM moieties for a sample of N controls can be used to derive a 90% prediction interval for their value in a randomly-selected individual from the same population as:

\[
\bar{X}_i \pm 1.645 \cdot \sqrt{1 + 1/N \cdot S_i}.
\]

Consequently, if a new subject is observed to have a measured metric outside its prediction interval, either he or she is one of the 10% for whom this metric falls outside this interval, or, more likely, came from a population with a different distribution of values.

3. Results

Our automatic shimming adjusted the whole-head water linewidth to 14 and 27 Hz at 1.5 and 3 T, yielding 12 and 22 Hz in the VOI without further adjustments. The VOI size and placement and \(^1\)H spectra for the patients and controls are shown in Figs. 1 and 2. The spectral fit quality is reflected by <20% mean Cramér–Rao lower bounds for all metabolites in the 1.0 cm\(^3\) voxels and <5.5% in the averaged VOI spectra, shown in Fig. 4. The global VOI and its GM and WM metabolite concentrations are compiled in Table 1. The NAA, Cr, Cho, and mI concentrations’ 90% prediction intervals for the adults were obtained using Eq. (5) from their published values: 7.7 ± 0.5, 5.4 ± 0.5, 1.3 ± 0.1, and 4.8 ± 0.6 mM in the VOI; 7.6 ± 0.5, 4.8 ± 0.4, 1.4 ± 0.2, and 4.6 ± 0.7 mM in its WM; and 8.4 ± 0.7, 6.7 ± 0.6, 1.2 ± 0.2, and 5.4 ± 0.7 mM in the GM [40].

The mean tissue fractions from the 18-subject sample were 91 ± 2%, 39 ± 2%, and 52 ± 3% in VOI, GM, and WM, and the resulting
prediction intervals are also given in Table 1. In the adult patient, we observed 25% and 16% higher Cho concentration in the VOI and its WM, respectively, and 17% lower NAA concentration in WM compared with the controls’ mean. All differences were outside their respective 90% prediction intervals [40]. No metabolite was observed outside its 90% prediction interval for the adult control. For the children, no meaningful differences were observed between the TSC patient and her control for any metabolite or tissue compartment, as shown in Table 1 and Fig. 4. Based on the reported similarity of pediatric spectra at this age to adults’ [41,42], we assume that because the differences between the patient and her control were relatively small (<10% for any metabolite), the patient is within the 90% prediction interval of a representative healthy pediatric population.

Global VOI, GM, and WM tissue fractions, T_0, GM, and WM_0, are also shown in Table 1 for all subjects. For the adults, no tissue fraction was outside its relevant 90% prediction interval.

4. Discussion

The low to modest correlation between brain tubers on clinical MRI and the neurologic phenotype of TSC strongly suggests that this modality does not define the full range of pathologic abnormalities. We therefore sought to use 1H MRS in MRI-negative TSC patients. The presence of MRS abnormalities in the adult TSC brain but not in the child is paradoxical since the child has developmental delay and treatment-refractory epilepsy, whereas the adult is neurologically normal. Several explanations may account for this double dissociation. First, the child did not have either TSC1 or TSC2 mutation on genetic testing although she met criteria for clinically-definite TSC [26]. The cause for TSC in patients without TSC1 or TSC2 mutation remains unknown and may relate to either a third gene and/or regulatory DNA controlling TSC1 or TSC2 [43]. Thus, the pathophysiology of her disorder, although clinically parallel to patients with TSC1/TSC2 mutation, may result from another genetic disorder acting via a non-mTORC1 pathway. Second, the presence (or lack) of a TSC1/TSC2 mutation may be functionally dissociated from neurological symptoms; approximately 15% of children and adolescents with TSC have no CNS complications [43].

The MRS abnormalities in the adult, despite being neurologically asymptomatic, suggest that longer disease duration and/or TSC2 gene mutation can cause: (i) axonal dysfunction, reflected by decreased WM NAA and elevated VOI and WM Cho suggesting demyelination that may lead to subsequent neuronal loss by Wallerian degeneration; and (ii) aberrant glial growth, reflected by elevated
shift scales. Note the excellent SNRs and resolution (compared with the single voxels model functions (thick gray lines), on common intensity (at each 324
Fig. 4. Real part of the aligned and globally-averaged \(^1\)H spectra from all VOI voxels (thin black lines) representing Eq. [3], for each subject, superimposed with their fitted model functions (thick gray lines), on common intensity (at each field) and chemical shift scales. Note the excellent SNRs and resolution (compared with the single voxels in Figs. 1 and 2) and observable Cho, Cr, and ml increases in the adults (See Table 1).

ml and Cr in the VOI and its GM (though not outside their 90% prediction intervals), possibly due to mTORC1 hyperactivity.

Several other neurologic processes could account for the dissociations between our pediatric and adult TSC subjects. Lack of abnormalities in the pediatric TSC brain may reflect ongoing development, e.g., myelination may prevent or offset the demyelinating effects of hamartomas. Indeed, previous studies of the diffusion characteristics showed focal microstructural abnormalities limited to hamartomas, i.e., no apparent diffusion coefficient differences in TSC patients versus controls in normal-appearing WM [44]. In addition, cortical remodeling could also reverse or prevent changes to neurons and glia. Either mechanism, if substantiated, could have implications for therapy. Another possibility is that the underlying neuropathology in the child is in the 80–90% of cortex outside the VOI (Fig. 1) missed in our study.

It is noteworthy that although not outside their 90% prediction intervals, GM used lower and WM was higher in both patients than those of their controls (cf. Table 1). Although a curiosity, given the small N, and that TSC pathology is known to affect the cortex, it is perhaps not surprising that possible microscopic lesions and/or atrophy (that leaves healthy neurons intact) is responsible for both GM reductions. If deep GM nuclei within the VOI, e.g., caudate, thalamus, etc., shrink, the surrounding WM that would collapse inward to replace that lost volume may be responsible for the observed increased WM.

Admittedly, this study is also subject to several limitations: First, only two patients were enrolled, each belonging to a different age group and each representing a different genotype. However, given the rarity of MRI-negative TSC cases nationwide (about 1:10\(^7\)), the Manhattan (2011 census population: 1.6 million [45]) area served by our epilepsy center would expect to see at most about sixteen patients. Since 10–20% of TSC patients do not experience seizures [43] and thus would not require an epilepsy center, the actual number of MRI-negative TSC patients available will usually underestimate the true prevalence. making two out of (at most) sixteen a reasonable yield given our patient base [4]. Second, each patient represents a subset not typically found in TSC. While these are rare conditions, their exploratory study is important for wider understanding of gene-function and/or its biomarker correlates. One patient without TSC1/TSC2 mutation expresses a neurological phenotype while the other with the TSC2 mutation had no neurological phenotype; this “double dissociation” points to the possible conclusion that mutation by itself has little bearing on neurologic outcome. It is noteworthy that approximately 15% of TSC patients have no CNS complications [43]. Our MRS findings seem to support this functional dissociation. For instance, the asymptomatic adult showed lower WM NAA and higher WM Cho, which suggest neuro-axonal abnormalities associated with mutation, but not with symptoms. Meanwhile, similar MRS profiles between the children suggest that perhaps a different cause is needed to explain the patient’s neurological symptoms. Either scenario, if substantiated, could have implications for treatment. Third, due to lipid contamination and signal “bleed” into surrounding voxels near/ on lateral surfaces of the cerebral cortex, as well as poor shimming in those areas, our cortical coverage within the VOI was limited to cortex near the midline, e.g., anterior and posterior cingulate.

Future studies with larger samples are needed to define the role of different genotypic features, e.g., TSC1 versus TSC2 versus non-TSC1/ TSC2 specific mutations, and their relationships with phenotypic features, e.g., age, disease duration and severity, and with specific clinical features such as intellectual disability, autism, epilepsy, and their correlation(s) with MRS abnormalities. Further investigation of the relationship of MRS abnormalities to neurologic phenotype in TSC could potentially provide diagnostic and prognostic information regarding brain development and function. It may also provide data relevant to defining epileptogenic versus non-epileptogenic brain tissue that could influence the identification and localization of seizure foci. Lastly, MRS could reveal new diagnostic biomarkers of mTOR1 brain dysfunction, which may help identify patients most likely to respond to mTOR inhibitors and help monitor their response to therapy.

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### Table 1

<table>
<thead>
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<th>Adult(^a)</th>
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<tr>
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<td>Control(^b)</td>
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\(^a\) Millimoles/kg wet weight.

\(^b\) [lower bound, upper bound] is the 90% prediction interval (Eq. (5)) for each metabolite concentration/tissue fraction derived from a sample of 18 healthy adults [40].

\(^c\) Percent (%). Bold type indicates a value outside its 90% prediction interval.

\(^d\) Absolute metabolite concentrations and tissue fraction for each region for both TSC patients and their matched controls.

\(^e\) TSC2 versus non-TSC1/TSC2 specific mutations, and their relationships with phenotypic features, e.g., age, disease duration and severity, and with specific clinical features such as intellectual disability, autism, epilepsy, and their correlation(s) with MRS abnormalities.