

# Brain Metabolism in Rett Syndrome: Age, Clinical, and Genotype Correlations

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**Objective:** Brain metabolism, as studied by magnetic resonance spectroscopy (MRS), has been previously shown to be abnormal in Rett syndrome (RTT). This study reports the relation of MRS findings to age, disease severity, and genotype.

**Methods:** Forty RTT girls (1–14 years old) and 12 age-matched control subjects were examined. Single-voxel proton MRS of left frontal white matter was performed.

**Results:** NAA/Cr ratios decreased and myoinositol/Cr ratios increased with age in RTT patients (both  $p < 0.03$ ), whereas these ratios were stable in control. The mean glutamate and glutamine/Cr ratio was 36% greater in RTT patients than in control ( $p = 0.043$ ). The mean NAA/Cr ratio was 12.6% lower in RTT patients with seizures compared with those without seizures ( $p = 0.017$ ). NAA/Cr ratios decreased with increasing clinical severity score ( $p = 0.031$ ). Compared with patients with T158X, R255X, and R294X mutations, and C-terminal deletions, patients with the R168X mutation tended to have the greatest severity score ( $0.01 \leq p \leq 0.11$ ) and the lowest NAA/Cr ratio ( $0.029 \leq p < 0.14$ ).

**Interpretation:** Decreasing NAA/Cr and increasing myoinositol/Cr with age are suggestive of progressive axonal damage and astrogliosis in RTT, respectively, whereas increased glutamate and glutamine/Cr ratio may be secondary to increasing glutamate/glutamine cycling at the synaptic level. The relations between NAA/Cr, presence or absence of seizures, and disease severity suggest that MRS provides a noninvasive measure of cerebral involvement in RTT.

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Rett syndrome (RTT) (MIM 312750) is an X-linked neurodevelopmental disorder that primarily affects girls from early childhood.<sup>1,2</sup> Clinical features of RTT include acquired microcephaly, loss of purposeful hand use, respiratory irregularities during wakefulness, seizures, failure of speech development, varying degrees of intellectual disability, and behavioral changes.<sup>3,4</sup> Mutations in the methyl-CpG binding protein-2 (MeCP2) gene, located on chromosome Xq28, are identified in more than 80% of affected girls.<sup>5,6</sup> MeCP2 is a transcriptional regulator that appears to repress gene expression through binding to methylated DNA.<sup>5</sup> Its dysfunction contributes to synaptic pathology, mainly by affecting activity-dependent plasticity.<sup>7</sup> Multiple studies suggest that genotypic differences in RTT may account for differences in clinical profile and disease severity.<sup>8–13</sup>

Decline in brain development in RTT begins before the age of 1 year. Global reductions in gray and white matter volume, and regional variations in brain matu-

ration lead to microcephaly.<sup>14–16</sup> The frontal lobe appears to be particularly affected, by tissue reduction,<sup>17</sup> hypoperfusion,<sup>18–20</sup> and somewhat surprisingly, hypermetabolism.<sup>21</sup> In early RTT, proton magnetic resonance spectroscopy (MRS) detected lower concentration of *N*-acetylaspartate (NAA; a surrogate neuronal marker<sup>22</sup>) in the frontal lobe gray and white matter.<sup>23</sup> Neuroanatomic studies of cortical abnormalities in RTT have shown increased neuronal cell packing density, reduced size of neuronal bodies and dendrites, and astrocytic reaction.<sup>17,24,25</sup> Investigation of cortical white matter did not show any major qualitative changes in axons or myelin.<sup>26</sup>

In contrast with neuropathological studies, techniques of physiological magnetic resonance imaging (MRI) can probe axonal integrity and function over the entire brain. Because of the noninvasive nature of these techniques, neuronal tissue can be followed sequentially over the entire life span. One of the advanced methods of physiological imaging is proton

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MRS, which can detect neurometabolites associated with neuronal integrity and function (NAA), cell membrane turnover (choline-containing compounds [Cho]), glial component of the brain tissue (myoinositol [mI]), and a major neurotransmitter, glutamate (Glu).<sup>22</sup> Assessment of these neurometabolites in RTT may potentially provide clinically valuable information. However, variability of technical approaches, differences in brain regions studied, and a relatively small number of RTT subjects in most studies have led to variable results.<sup>27,28</sup> As the genetic abnormality was identified in late 1999, some observations reported in earlier studies may also not be related to the *MeCP2* gene defect. NAA in RTT is decreased, both in the gray and white matter<sup>23,27</sup>; however, the decrease was observed only in certain brain regions,<sup>23,28</sup> including frontal lobes, parietal lobes, and the insula.<sup>23</sup> One study measured lower Glu concentrations compared with control subjects in both gray and white matter,<sup>27</sup> but later, slightly increased Glu/NAA ratio in gray matter was detected.<sup>29</sup> Mean Cho levels in RTT were reported to be slightly increased, in particular, in patients with seizures.<sup>23</sup> However, no significant increase in Cho concentration was documented in individual brain regions.<sup>23</sup> Levels of the glial marker, mI, were examined only in one study, and normal mI/Cr ratios were found.<sup>30</sup> To date, a detailed investigation of the relation between the levels of MRS-detected compounds and genetic impairment has not been conducted.

It is puzzling that despite findings of no major qualitative changes in myelin in RTT,<sup>26</sup> cortical white matter is affected by diffuse volume reduction and<sup>14</sup> metabolic abnormalities<sup>23</sup> indicative of impaired tissue integrity.<sup>31</sup> As discussed earlier in this section, there is a specific involvement of frontal lobe in RTT.<sup>17-21</sup> In agreement with these findings, frontal white matter impairment was detected in an earlier MRS imaging<sup>23</sup> and a preliminary diffusion tensor imaging study.<sup>31</sup> The goal of this MRS study was to examine metabolic impairment in the frontal white matter to further evaluate white matter pathology in the frontal lobe. Several hypotheses were considered. First, in RTT patients, white matter NAA levels will be decreased, consistent with axonal impairment; Cho and mI levels will be increased, suggesting presence of gliosis. Second, the concentration of the neuronal marker NAA will decrease with age, consistent with progression of symptoms. Third, Glu levels in RTT will be increased, particularly in younger subjects, in agreement with higher density of *N*-methyl-D-aspartate Glu receptors, demonstrated by postmortem autoradiography studies,<sup>32</sup> and increased CSF Glu levels.<sup>33</sup> Finally, the degree of metabolic impairment will be associated with disease severity and will vary among *MeCP2* mutation types.

## Subjects and Methods

### Study Population

Forty girls diagnosed with RTT (mean age, 6.1 years; range, 1.1–14.1 years; standard deviation, 3.1 years) and 12 healthy girls (mean age, 8.9 years; range, 3.6–14.7 years; standard deviation, 3.5 years) were examined. Diagnosis of RTT was confirmed by identification of the *MeCP2* mutation and presence of clinical features.<sup>34</sup> Symptom severity was classified by severity scores.<sup>12</sup> The list of mutations and corresponding age and severity scores ranges are presented in the Table. The girls in the control group were either healthy siblings of the RTT patients or unrelated healthy volunteers. During the MRS examination, all RTT patients were sedated with chloral hydrate; examination in all control subjects was performed without sedation. The study was approved by the local institutional review board, and all families provided written informed consent.

### Proton Magnetic Resonance Spectroscopy

Single-voxel proton MRS was performed with the Point Resolved Spectroscopy (PRESS) sequence (repetition time/TE = 1,500/30 milliseconds; 2,048 data points; 2,000Hz spectral width; 128 repetitions) at 1.5 Tesla. Single-voxel proton MRS was a component of an integrated MRI examination, including clinical MRI, volumetric MRI, and diffusion tensor imaging. Because of time constraints in sedated patients, proton MRS examination was limited to one region of interest. The 2.0L × 2.0L × 2.0cm<sup>3</sup> voxel was positioned in the left forceps minor and contained predominantly white matter. In 26 RTT patients, spectra without water suppression from the same voxel (acquired with 8 repetitions) were also obtained. LCModel (version 6.1)<sup>35</sup> was used for automatic analysis of the spectra. Individual metabolite ratios

**Table. MeCP2 Gene Mutations**

Mutation	n	Age Range (yr)	Severity Score
P101S	1	3.1	4
R106Q	1	8.4	8
R106W	1	3.2	5
R133C	1	4.3	5
P152R	1	5.1	6
T158M	9	2.5-13.1	4-12
R168X	5	1.1-8.5	5-13
R255X	4	1.1-7.7	3-7
R270X	3	6.6-14.1	6-15
R294X	5	4.3-10.4	4-12
R305R	1	6.6	6
R306C	1	5.0	8
R306H	1	9.3	8
P322L	1	5.7	9
C-terminal deletions	5	2.9-7.5	3-7

were obtained by the LCMoDel as ratios of NAA, mI, Glu, glutamate and glutamine (Glx), and total Cho concentrations to the creatine (Cr) concentration. It should be noted that concentration ratios are not necessarily the same as peak area ratios, which are reported by other software packages. In 26 RTT patients, apparent concentrations of individual metabolites (in arbitrary units) were also estimated based on white matter water concentration as an internal reference. Because of incomplete resolution of the Glx signals, the intensity of the complex Glx signal was measured. Good-quality spectra were obtained in all subjects. For a given metabolite, only data with the LCMoDel Cramer-Rao bounds  $\leq 20\%$  were included. In the Results section, the total number of subjects is noted for analyses with missing data on metabolite ratios or metabolite concentrations.

### Statistical Analysis

Normal distribution of all metabolite ratios was confirmed with the Kolmogorov–Smirnov test. General linear model (GLM) analysis of variance (ANOVA) was used to evaluate the differences in metabolite ratios between the RTT and control groups. Age and group (patients and control subjects) were the independent variables; an interaction term age  $\times$  group was also used. Linear regression of metabolite ratios and metabolite concentrations in RTT patients on age was used as a post hoc test to examine age-related differences in RTT patients and control subjects. GLM ANOVA was also applied to examine the differences in metabolite ratios between patients with and without seizures, among different types of *MeCP2* gene mutations, and to assess the relation between metabolite ratios and severity scores, controlling for age. Significance level was set to  $p < 0.05$ . Data are presented as age-adjusted (marginal) means  $\pm$  standard deviations.

### Results

Figure 1 shows the region of interest in the left frontal forceps minor and examples of spectra in a RTT patient and a healthy control subject.

The main GLM ANOVA of NAA/Cr ratios detected a significant interaction term: group  $\times$  age ( $p = 0.027$ ). In RTT patients, NAA/Cr ratios decreased with age ( $p = 0.021$ ), whereas stable NAA/Cr ratios with age were observed in control subjects (Fig 2). The differences in NAA/Cr ratios were more pronounced in older subjects; in RTT patients younger than 7 years, mean NAA/Cr ratio was similar to control subjects (group:  $p = 0.77$ ). The age-related decrease in NAA/Cr ratios in RTT was due to a decrease in NAA concentration ( $n = 26$ ;  $p = 0.05$ ); Cr concentration was stable ( $n = 26$ ;  $p = 0.32$ ) (Fig 3).

The main GLM ANOVA of mI/Cr ratios detected a significant effect of group ( $p < 0.0001$ ) and age ( $p = 0.003$ ). In RTT patients, mI/Cr ratios increased with age ( $n = 39$ ;  $p = 0.020$ ) but were stable in control subjects (see Fig 2). The age-related increase in mI/Cr ratios in RTT was due to an increase in mI concentration ( $n = 25$ ,  $p = 0.016$ ) (see Fig 3).

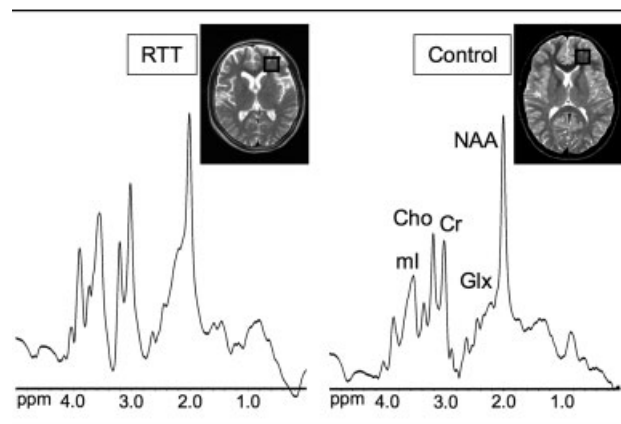


Fig 1. Proton magnetic resonance spectra of the left frontal white matter in a 10.4-year-old girl with Rett syndrome (RTT) and a 10-year-old healthy girl. The T2-weighted localizer images show the position of the region of interest. Microcephaly and prominent sulci are evident in the RTT patient. Lower N-acetylaspartate/creatine (NAA/Cr) and greater myoinositol (mI)/Cr and glutamate and glutamine (Glx)/Cr ratios are apparent in the RTT patient.

The mean Glx/Cr ratio in RTT patients was 36% greater compared with control subjects (group:  $p = 0.043$ ) (see Fig 2). Although no significant age-related differences in Glx/Cr ratios were detected, the observed group differences were due to greater Glx/Cr ratios in patients younger than 10 years ( $p = 0.024$ ). No difference in Glx/Cr ratios was detected between patients older than 10 years ( $n = 5$ ) and control subjects. No group or age differences in the Cho/Cr ratio were found (see Fig 2).

RTT patients with seizures had a 12.6% lower mean NAA/Cr ratio compared with patients without seizures ( $p = 0.017$ ). NAA/Cr decreased linearly with increasing severity score ( $p = 0.031$ ) (Fig 4). Because both NAA/Cr and severity score were age dependent, the effect of age was accounted for by using the residuals on the regression (of NAA/Cr and severity scores) with age. No relation between any other ratio and presence of seizures or severity score was detected.

The most frequent mutations in the examined RTT group were T158M, R168X, R255X, R294X, and C-terminal deletions (see the Table). The R168X mutation tended to be associated with the greatest mean severity score ( $0.01 \leq p \leq 0.11$ ) and the lowest NAA/Cr ratio ( $0.029 \leq p < 0.14$ ) (Fig 5). No significant difference in mean NAA/Cr, mI/Cr, or Glx/Cr ratio between the R168X and T158M mutations was detected (all  $p \geq 0.14$ ). Patients with C-terminal deletions tended to have the greatest mean Glx/Cr ratio compared with patients with the T158M, R168X, and R294X mutations ( $0.054 \leq p < 0.15$ ).

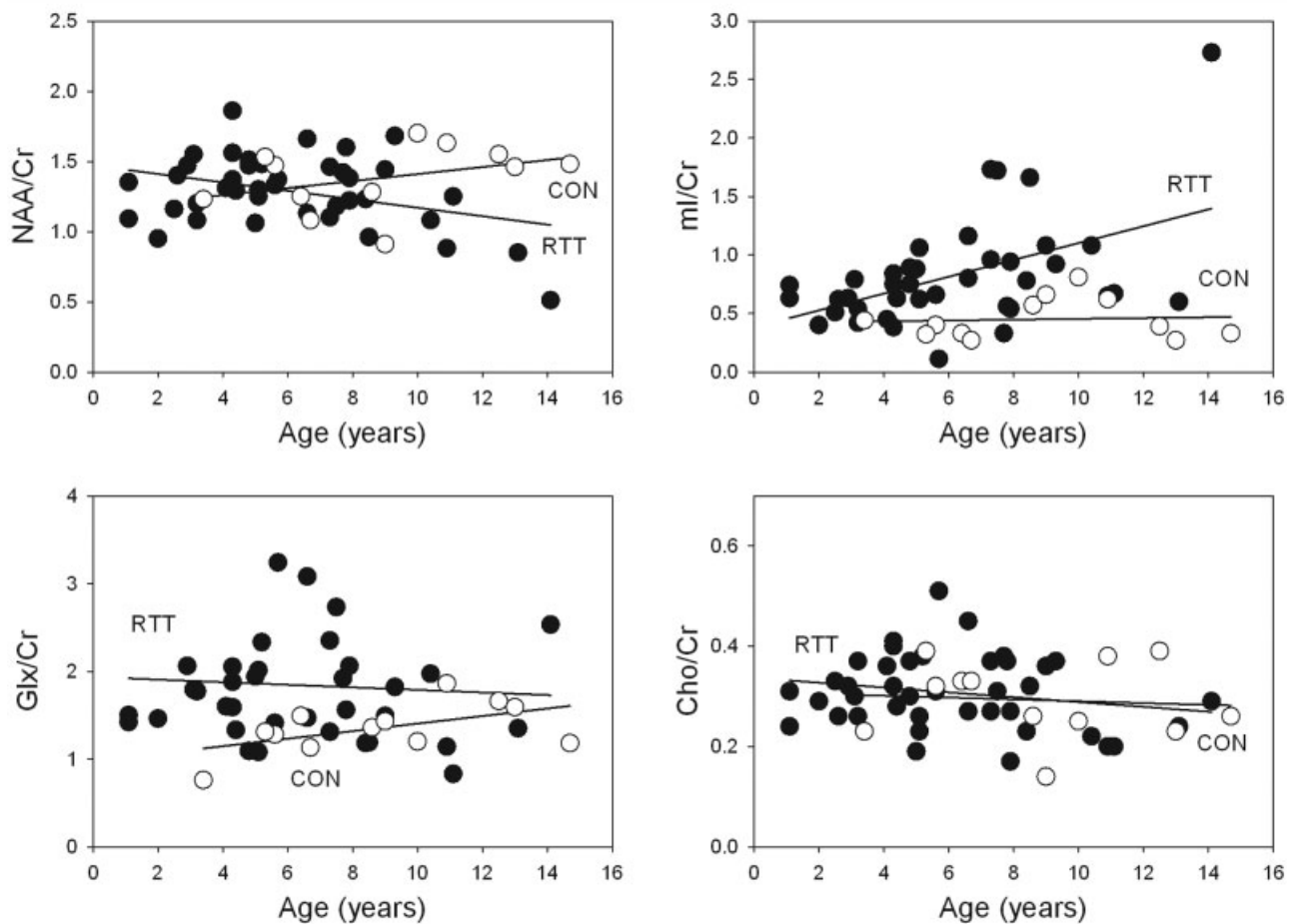


Fig 2. Age-related differences in frontal white matter N-acetylaspartate/creatine (NAA/Cr), myo-inositol (mI)/Cr, glutamate and glutamine (Glx)/Cr, and total choline (Cho)/Cr ratios in Rett syndrome (RTT) patients (filled circles) and healthy control subjects (open circles). NAA/Cr ratio decreased with age, and mI increased in age in RTT (both  $p < 0.03$ ), whereas stable ratios were detected in control subjects. Glx/Cr ratio was increased in RTT patients younger than 10 years ( $p = 0.024$ ). Chol/Cr ratio was stable across the examined age range in both groups.

## Discussion

The main findings of this study were detection of increased levels of mI, considered to be a glial marker, and low levels of NAA, a neuronal marker, in white matter in RTT patients. Compared with healthy children, the mean NAA/Cr ratio was 5% lower and mI/Cr ratio was 54% greater in RTT patients. The MRS results are, therefore, suggestive of mild white matter pathology, in agreement with the neuropathological findings that RTT is not associated with a recognizable degenerative, demyelinating, or gross malformative process.<sup>36</sup> Age-related decreases in NAA are consistent with clinical deterioration typically occurring in older subjects.<sup>37</sup> NAA/Cr decreased with increasing severity scores, was also low in patients with seizures, and tended to be low in patients with the R168X mutation, which was associated with the greatest mean severity score. Our results therefore suggest that NAA/Cr ratio may be a valuable quantitative marker of clinical status in RTT.

Increased white matter mI concentrations, which progressively increased with age, have not been previously reported in RTT. One earlier MRS study found normal mean white matter mI/Cr in seven girls with RTT, including four girls aged 4 to 10 years who had decreased NAA/Cr ratio.<sup>30</sup> The discrepancy may be because of differences in patient populations as the diagnosis of RTT in the previous study was based on clinical criteria alone. Low frontal white matter NAA concentrations and normal Cho concentrations in RTT reported in this study are in agreement with a previous proton MRS imaging study.<sup>23</sup> Although a 12% increase in mean Cho concentration was observed, no significant differences were detected in individual regions.<sup>23</sup> In this study,<sup>23</sup> an MRS imaging technique with higher spatial resolution was performed, but because of the use of a long echo time, mI was not detected. Increased mI concentration, normal Cho levels, and decreased NAA concentration in the frontal white matter may be interpreted as astrocytic

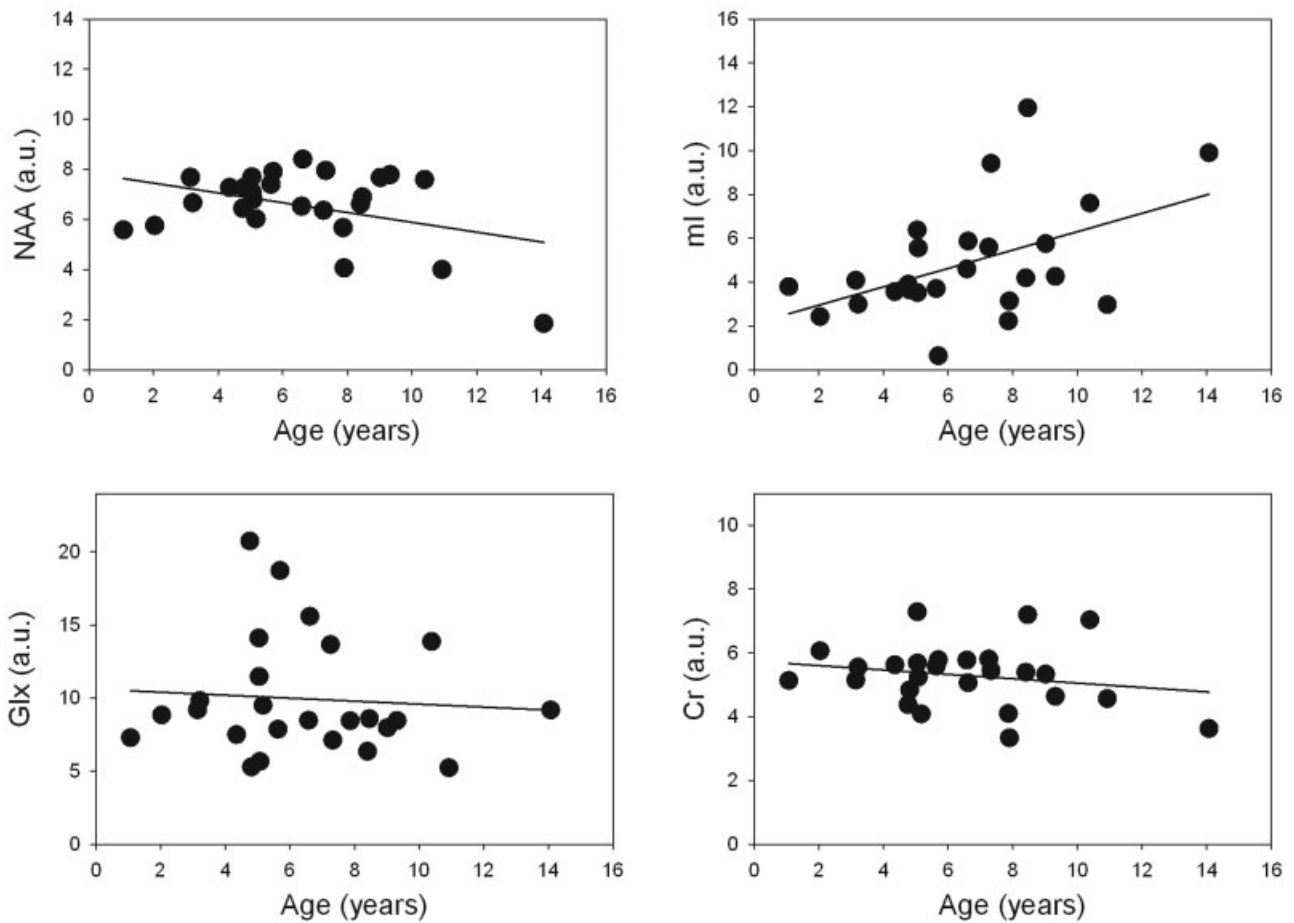


Fig 3. Age-related differences in frontal white matter N-acetylaspartate (NAA), myoinositol (mI), glutamate and glutamine (Glx), and creatine (Cr) concentrations (in arbitrary units [a.u.]) in Rett syndrome (RTT) patients. NAA concentration decreased ( $p = 0.05$ ) and mI concentration increased ( $p = 0.016$ ) with age. Stable Glx and Cr concentrations were detected.

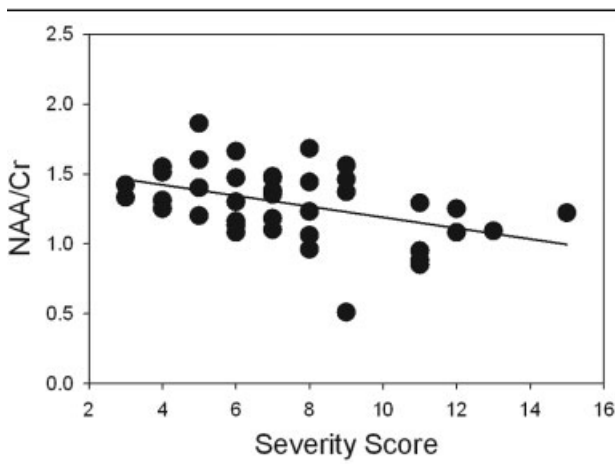


Fig 4. Relation between N-acetylaspartate/creatine (NAA/Cr) ratios in Rett syndrome (RTT) and severity scores. After controlling for age, NAA/Cr linearly decreased with increasing severity score ( $p = 0.035$ ).

reaction in the context of mild axonal disruption (or dysfunction). Astroglial proliferation appears as a less likely correlate, because of lack of concurrent increase in Cho and mI.<sup>38</sup> Also, as glial cells have relatively high concentrations of mI and Cr,<sup>39</sup> in gliosis, increase of Cr concentration may be present. However, in this study, Cr concentration in control subjects was not measured; thus, this cannot be confirmed. Although an 8% greater mean Cr concentration was previously found in the frontal white matter in the RTT group, the difference was not significant, because of a high variability in Cr levels in both RTT and control groups.<sup>23</sup> Involvement of glial metabolism was also suggested in an *in vivo* MRS study of *MeCP2*-null male mice, though deficit in *MeCP2* in these mice was associated with decreased mI, increased Cho, and decreased NAA concentration.<sup>40</sup> Comparison with the results of this study suggests a different causative factor for glial pathology in the *MeCP2*-null mouse than in human RTT female patients. Further experiments need to be performed to explain the difference. One of the

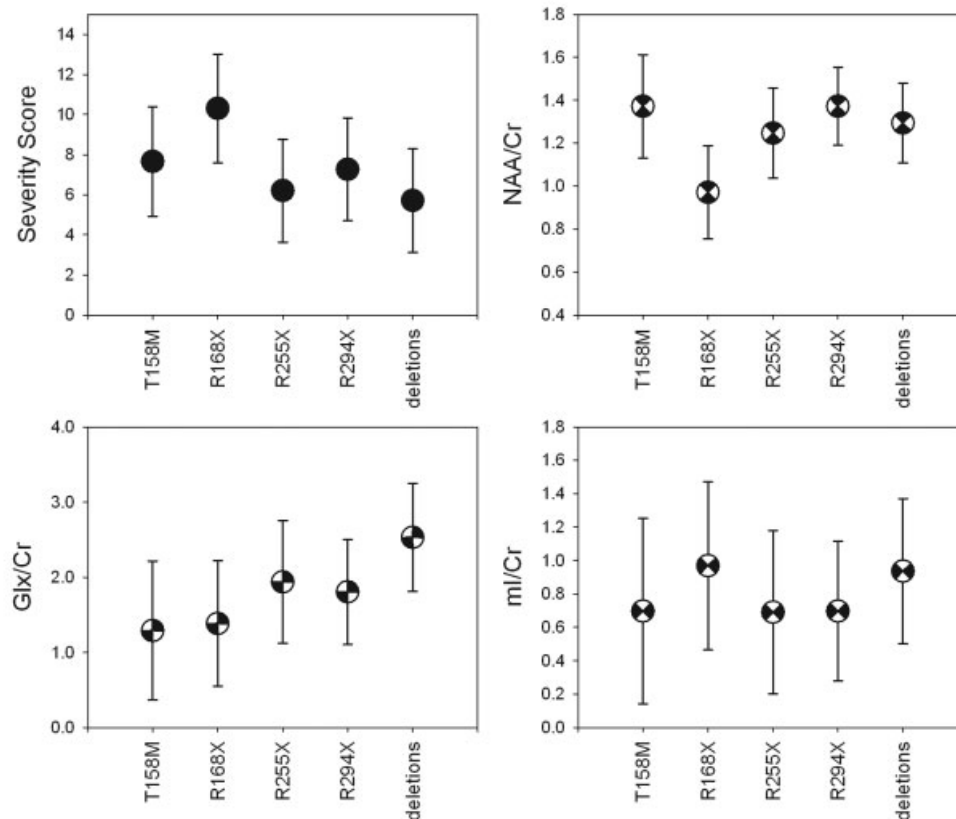


Fig 5. Age-adjusted (marginal) means of severity scores, N-acetylaspartate/creatine (NAA/Cr), glutamate and glutamine (Glx)/Cr, and myoinositol (ml)/Cr ratios in the most frequent mutations in the Rett syndrome (RTT) group: T158M ( $n = 9$ ), R168X ( $n = 5$ ), R255X ( $n = 4$ ), R294X ( $n = 5$ ), and e-terminal deletions ( $n = 5$ ). Patients with the R168X mutation tended to have the greatest severity score ( $0.010 < p < 0.11$ ) and the lowest NAA/Cr ratio ( $0.029 < p < 0.14$ ). A tendency to a high mean Glx/Cr ratio was observed in patients with C-terminal deletions compared with patients with the R168X, T158M, and R294X mutations ( $0.054 < p < 0.15$ ).

limitations of the *MeCP2*-null male mouse model studied, besides difference in magnitude of *MeCP2* gene involvement compared with human RTT subjects, is the severely restricted life span of the mice. *MeCP2*-null mice die at 10 weeks postnatal age, which precludes studies of later developmental stages. Neuropathological studies of RTT brain tissue reported high levels of glial fibrillary acidic protein immunoreactivity and prominent astrocytes<sup>25,41,42</sup>; however, gene expression profiles characterized by upregulation of astrocytic genes (eg, glial fibrillary acidic protein, alpha B crystalline, glial Excitatory Amino Acid Transporter 1 (EAAT1) suggest that the neuropathological abnormalities may not be typical astrocytic proliferation.<sup>41</sup>

Stable frontal white matter NAA concentrations in younger RTT patients were detected both in a previous study<sup>23</sup> and in this work. However, NAA levels were lower and decreased with age in older patients, whereas no age-related differences were detected in control subjects. One recent study including older patients also noted lower NAA/Cr ratio in frontal white matter compared with control subjects.<sup>30</sup> However, no age-

related differences were detected, probably because of a limited sample size (total of six subjects, 3–21 years old).<sup>30</sup> Lower NAA concentration in older RTT patients compared with younger patients was reported in one of the first MRS studies of RTT, which included nine patients aged 2.3 to 21.3 years old.<sup>27</sup> It was noted that the decrease in NAA was less pronounced in the white matter than in the gray matter.<sup>27</sup> To date, there are no MRS data from longitudinal studies, which could elucidate the time course of NAA concentration changes in individual patients.

The age-related differences in Glx/Cr found in this study are similar to the pattern of developmental differences between RTT patients and healthy children in Glu receptor binding in the frontal cortex.<sup>32</sup> A greater Glx/Cr ratio was found in RTT patients younger than 10 years compared with control subjects, but there was no difference in Glx/Cr between older patients and control subjects. In the Glu receptor binding study, the density of *N*-methyl-D-aspartate Glu receptors was higher in the frontal cortex of patients younger than 10 years but lower in the older RTT group.<sup>32</sup> Similar, but

not significant, age-dependent differences in  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), metabotropic, and GABA receptors were detected in the basal ganglia.<sup>43</sup> The findings of increased *N*-methyl-D-aspartate Glu receptor density, increased glucose utilization in the frontal lobe,<sup>21</sup> and increased Glu levels in the cerebrospinal fluid<sup>33</sup> are suggestive of enhanced excitatory neurotransmission in younger patients with RTT. A 4.1-Tesla MRS imaging study of six girls with early RTT (4.5–6 years old) reported increased Glu/NAA ratio in the gray matter and normal Glu/NAA in the white matter.<sup>29</sup> However, that study reported mean white matter Glu/NAA ratio in a slice at the level of the cingulate gyrus,<sup>29</sup> whereas our study examined only one specific region. Age-related differences for both Glx/Cr and Glu/Cr ratios observed in this study were similar (Glu/Cr results are not reported here); however, statistical significance was reached only for the Glx/Cr ratio because of the difficulty in separating Glu and glutamine at 1.5 Tesla. Increased accuracy in assessment of Glu may be achieved at higher magnetic fields, where the separation between Glu and glutamine resonances is improved.

Mutations in the *MeCP2* gene are associated with a wide range of severity.<sup>4,44</sup> The common mutations in RTT are the R106W, R133C, T158M, R168X, R255X, R270X, R294X, R306C, and small insertions and deletions toward the 3' end of the *MeCP2* gene, leading to C-terminal truncations.<sup>8,9,44</sup> In our RTT group, the R168X mutation tended to have the greatest mean severity scores and lowest mean NAA/Cr ratio. Subjects with C-terminal deletions had relatively greater mean Glx/Cr ratios. Despite its milder overall severity, this genotype has been linked to a distinctive clinical course characterized by early onset of dystonia.<sup>45</sup> Although our statistical analysis detected trend to significant differences in metabolites between common mutations, our results (obtained with a small number of patients in individual mutation groups) warrant studies in a larger series, which could permit more detailed understanding of association between genotype, phenotype, and concentration of neurochemicals detected with MRS.

Patients with the same mutation may manifest variable phenotypes likely because of differences in pattern of X-chromosome inactivation.<sup>13,46,47</sup> Because of the small number of cases, we could not evaluate the effect of X-chromosome inactivation on metabolite levels in individual mutations in this study.

In conclusion, proton MRS demonstrated presence of mild white matter pathological processes, which appear to be complex and progressive in nature. Therefore, future physiological MRI studies in RTT should examine interactions between the glial and neuronal components to evaluate the relation among metabolic, structural, clinical, and genetic impairment.

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