Rett syndrome: Neurologic and metabolic aspects
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Folinic acid supplementation in Rett syndrome patients does not influence the course of the disease: a randomized study

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Abstract

Rett syndrome is a neurodevelopmental disorder in girls, related to mutations in MECP2 gene. It has been postulated that low 5-methyltetrahydrofolate (5-MTHF) levels are present in cerebrospinal fluid. Folinic acid demonstrated clinical improvement. However, because studies have produced conflicting results, we performed a randomized, double-blind crossover, long-term, follow-up study on folinic acid. Eight Rett patients received both folinic acid and placebo, for 1 year each. Measurements included plasma folate, 5-MTHF, and clinical outcome scores like Rett Syndrome Motor Behavioral Assessment, Hand Apraxia Scale, and the parental Overall Well-Being Index.

In 2 patients, low 5-MTHF levels were present. Folinic acid supplementation increased cerebrospinal fluid 5-MTHF levels, but with no objective evidence of clinical improvement. The Overall Well-Being Index showed a significant difference in favor of folinic acid, not confirmed objectively.

In our double-blind randomized study, folinic acid supplementation resulted in increased 5-MTHF levels, but with no objective signs of clinical improvement.

Keywords: Rett syndrome, 5-methyltetrahydrofolate, folinic acid, Rett motor behavior assessment, hand apraxia scale, folate, vitamine B₁₂
Introduction

Rett syndrome is a neurodevelopmental disorder, in which girls develop various motor and behavioral features after initial normal development until the age of 6-18 months. Clinical characteristics are: loss of speech, social withdrawal, cognitive impairment, deterioration of purposeful hand function and abnormal deceleration of head growth. In addition, stereotypic hand movements and locomotor impairments develop. Seizures have an age-related onset and occurrence, and are associated with greater clinical severity and type of methyl-CpG-binding protein 2 gene (MECP2) mutations. Some features in Rett syndrome worsen throughout the lifespan, such as locomotor and orthopedic problem. Others, like feeding, autonomic disturbances and seizures, tend to become more stable. Therefore, evaluation of therapeutic efficacy in Rett syndrome requires long-term follow-up and the use of reproducible measurements over time.

Methyl-CpG-binding protein 2 gene mutations are identified in ≥ 80% of Rett patients, but the pathogenic mechanism is still unknown. Based on the possible role of a disturbed transport of folate across the blood-brain barrier in the pathogenesis of Rett syndrome, clinical studies have been performed. It was concluded that supplementation with folinic acid restored the 5-methyltetrahydrofolate (5-MTHF) levels in cerebrospinal fluid. In some Rett girls folinic acid supplementation resulted in improvement of social contact, mobility and seizure control. However, the clinical studies produced conflicting results, partly related to the study design, which was not blinded or placebo-controlled and/or the follow-up was short.

Therefore this prospective, randomized, double-blind, placebo-controlled crossover trial investigates the clinical and biochemical response to folinic acid treatment in patients with Rett syndrome, using specific outcome measures of clinical severity that are reproducible, objective, and applicable for patients with Rett syndrome.

Patients and Methods

A randomized, double-blind crossover study was performed at the department of Pediatric Neurology of the Academic Medical Center, Amsterdam. Eight females (aged, 2-30 years; mean, 11 years), all fulfilling the clinical criteria for Rett syndrome, participated in the trial.
The study protocol was approved by the Medical Ethical Committee of the Academic Medical Center; all parents of the participating children provided written informed consent. Exclusion criteria were the use of vitamin supplementation or other research treatment(s) for Rett syndrome with the exception of anti-epileptic drugs, and insufficient knowledge of the Dutch language by the caregivers.

**Biochemical Laboratory Testing**

Cerebrospinal fluid samples for measurement of 5-MTHF were collected in 3 fractions between 8:00 and 10:00 a.m. and immediately transported to the laboratory on crushed ice and frozen at -80 °C until analysis. The first fraction was used for cell count to exclude an infection, the second and third for metabolic testing. During the 2-year study period, per patient 3 cerebrospinal fluid samples were analyzed: once before start of the study, the second after the first year of therapy, and the third (after switching the therapeutic regimen) at the end of the second year of therapy.

5-MTHF was analyzed using HPLC with fluorescence detection essentially according to Huang et al. Reference values for 5-MTHF were established previously in our laboratory in cerebrospinal fluid samples analyzed for suspicion of infectious diseases or neurological conditions of non-metabolic origin.

For control of folate administration, folate levels in plasma were analyzed in the routine clinical chemistry laboratory using a Hitachi modular E-170 analyzer (Roche Diagnostics GmbH, Mannheim, Germany). At the same time schedule, plasma levels of total homocysteine, methionine, blood hemoglobin, erythrocyte counts, and vitamin B<sub>12</sub> were determined.

**Baseline measurements**

Rett syndrome patients were classified according to the Hagberg staging: stage I, early stagnation of development; stage II, regression of development (age 1-4 years, duration of months) in which Rett girls lose motor and verbal skills and develop stereotypic hand movements; stage III, pseudo-stationary period (time span of years or decades); and stage IV, late motor deterioration and non-ambulant Rett girls.<sup>1,10</sup> Rett patients aged ≤ 10 years, who are not able to walk, are classified as stage III-IV.

In addition, the modified symptom severity score was used for more detailed classification of severity. In the symptom severity score, individual clinical scores of 6 items such as onset of symptoms (maximum score 5), growth parameters (maximum 7), motor function (maximum 16), and communication function (maxi-
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...imum 8), Rett behavior disturbances (maximum 9) and seizures (maximum 5) are summed (score range 1-50). A higher score indicates earlier onset or greater severity.11

**Randomization**
Randomization, packaging, labeling, and dispensing of folinic acid and placebos were done by the Academic Medical Center pharmacy.

**Intervention**
The study consisted of 2 treatment periods of 1 year each with either placebo or folinic acid, to rule out temporary clinical or seizure frequency changes. After 1 year there was an intervening washout period of 2 months, after which the second treatment year was started. The principal investigator (EH), other participating researchers and the participants’ families were blinded for the study medication.

Capsules containing either folinic acid dosages (2 mg/kg/d, with a maximum dose of 50 mg/d) in combination with vitamin B_{12} (0.5 mg/d) or placebo capsules were prepared. Since folinic acid and vitamin B_{12} (cobalamins) are co-factors and cobalamin metabolism interacts with folate metabolism, vitamin B_{12} is necessary for optimal action of folinic acid and supplementation of both is required.

To evaluate compliance with folinic acid intake, plasma levels of folate and vitamin B_{12}, and levels of 5-MTHF in cerebrospinal fluid, were evaluated three times during the 2-year study period.

Tolerance of drugs was assessed by parent-reported and/or investigator-observed adverse events during the entire study period. When appropriate, anti-epileptic drugs were adjusted.

**Follow-up measurements**
A standardized neurological examination and detailed history from parental report was taken every 3 months by the same pediatric neurologist (EH). The neurological examination contained a 9-point neurological assessment schedule, based on a multidisciplinary clinical assessment of Rett girls,4 to standardize the neurological findings. Neurological Score A measured eye contact, oromotor skills and communication skills. Neurological Score B evaluated hand use and motor abilities including sitting, standing and walking (scores 0-4, maximum score 36; a higher score = better motor performance).

In Rett syndrome girls, since purposeful hand use is often replaced by stereo-
typic movements, changes in voluntary hand use are extremely informative. This was monitored by the validated 10-point Hand Apraxia Scale (maximum score 20, demonstrating total inability to use the hand). A dependency scale (a self-developed 4-point assessment scale) was used to measure change in the assistance needed for feeding, drinking, dressing and toilet use (minimum score 4, maximum score 16=indicating total independence).

A Dutch version of the Rett syndrome Motor Behavioral Assessment scale was used, in which the major clinical symptoms of Rett girls are analysed to define objective numerical ratings in 3 main areas: behavioral and social skills (maximum score 64); orofacial and respiratory signs (maximum score 28) based on parental reporting; and motor and physical signs as assessed by the principal investigator (pediatric neurologist EH) (maximum score 56). 13,14

In addition to the Rett syndrome Motor Behavioral Assessment scale, parental reports included a patient Overall Well-Being Index: this is a questionnaire previously used in Rett syndrome girls in which parents rate the energy level, physical activities, appetite, mental alertness and school performances from 1-10 (10=the best score). These individual items are summed and a mean score is calculated. 13,15 In earlier studies improvement in social contact during treatment with folinic acid was often reported. 6-7 Therefore we paid special attention to the item of mental alertness in the Patient Overall Well-Being Index, and also selected items in the Rett syndrome Motor Behavioral/social Assessment concerning speech, contact, interaction and communication (items 2, 3, 7, 8) to evaluate changes in social contact.

Outcome measures at baseline and at the end of each 3-month interval consisted of neurological examination, the Hand Apraxia Scale, the Dependency scale, the Rett syndrome Motor Behavioral Assessment scale, and the parental Overall Well-Being Index.

Finally, the Global Outcome Score is a questionnaire in which parents qualify the global change of their child on a 6-point scale (range: complete recovery to many more complaints). 16 This questionnaire was completed at the end of each year of treatment (i.e. twice during the study period).

Data analysis
To estimate the therapeutic efficacy on the outcome measures, linear mixed models were made in SPSS version 16.0.2. This technique allows for repeated measurements in individuals, thereby making use of all available information per patient.
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The final multivariate model contained intervention as a fixed factor, time, and the interaction term of time and intervention, as random factors. The covariance structure was chosen based on Akaike’s Information Criterion, and the fit of the final model was verified by investigating the distribution of the residuals. A Wilcoxon signed rank test was performed to estimate the effect of intervention on the General Outcome Score. Related samples Friedman’s 2-way analysis of variance by ranks was used to evaluate changes of baseline plasma folate and of 5-MTHF in cerebrospinal fluid during both folinic acid and placebo therapy. Spearman’s correlation coefficient was used to evaluate the relationship between plasma folate and 5-MTHF in cerebrospinal fluid Pearson’s correlation coefficient was used to evaluate the relationships between the parental Overall Well-Being Index questionnaire and the Rett Motor Behavioral Score. Statistical significance was set at \( p <0.05 \).

Results

Eight clinically diagnosed Rett patients (aged, 2-30 years; average, 11 years) entered the study, 5 of whom were classified as stage III (IV) and 3 as stage IV (non-ambulatory).\(^1,10\) In 6 of the 8 patients mutations in the Methyl-CpG-binding protein 2 (MECP2) gene was demonstrated. Since the two MECP2-negative patients showed all the distinctive features of typical Rett syndrome, and diagnosis was confirmed by at least one other pediatric neurologist (not participating in this study), both were included in this study. Extensive investigations ruled out other causes of psychomotor retardation. Pre-treatment anti-epileptic drugs (mostly multiple) were used by 7 of the patients. Table 1 presents the clinical characteristics and biochemical parameters of the group. Initially all but 1 patient (patient 8) had normal levels of plasma vitamin B\(_{12}\) and folate. None of them used vitamin supplements. In 1 adult (patient 5) and one 4-year-old patient (patient 1) baseline 5-MTHF was low, despite normal levels of plasma folate (folate not available in patient 1), homocysteine, amino acids, blood hemoglobin, and erythrocyte count number (data not shown). In another patient (patient 6) 5-MTHF levels were in the lower reference range. Relatively low folate, correlated with low 5-MTHF (Spearman’s correlation coefficient at baseline 0.93; \( P =0.003 \)). During folinic acid use, plasma folate and CSF 5-MTHF levels increased significantly (related samples Friedman’s 2-way analysis of variance: \( P=0.030 \) and \( P=0.008 \), respectively).
Table 1: Clinical characteristics and laboratory results

<table>
<thead>
<tr>
<th>Patient ID no.</th>
<th>Age, Y</th>
<th>Stage&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Folate&lt;sup&gt;b&lt;/sup&gt; baseline</th>
<th>Folate&lt;sup&gt;c&lt;/sup&gt; suppl</th>
<th>Folate placebo</th>
<th>Reference range</th>
<th>5MTHF&lt;sup&gt;b&lt;/sup&gt; baseline</th>
<th>5MTHF&lt;sup&gt;c&lt;/sup&gt; suppl</th>
<th>5MTHF placebo</th>
<th>Reference range</th>
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<tr>
<td>1</td>
<td>4</td>
<td>III</td>
<td>n.a.</td>
<td>&gt;45.5</td>
<td>15.1</td>
<td>7-39</td>
<td>34(&lt;sup&gt;c&lt;/sup&gt;)</td>
<td>132</td>
<td>72</td>
<td>43-129</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>III-IV</td>
<td>30.0</td>
<td>n.a.</td>
<td>38.9</td>
<td>7-39</td>
<td>114</td>
<td>224</td>
<td>79</td>
<td>43-129</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
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<td>25.8</td>
<td>&gt;45.5</td>
<td>29.9</td>
<td>7-39</td>
<td>71</td>
<td>114</td>
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<tr>
<td>4</td>
<td>11</td>
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<td>n.a.</td>
<td>16.2</td>
<td>7-39</td>
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<td>145</td>
<td>66</td>
<td>40-156</td>
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<tr>
<td>5</td>
<td>20</td>
<td>IV</td>
<td>15.6</td>
<td>&gt;45.5</td>
<td>15.6</td>
<td>7-39</td>
<td>34(&lt;sup&gt;c&lt;/sup&gt;)</td>
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<tr>
<td>6</td>
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<td>7-39</td>
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<td>43-102</td>
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<tr>
<td>7</td>
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<td>III-IV</td>
<td>&gt;45.5</td>
<td>&gt;45.5</td>
<td>38.9</td>
<td>7-39</td>
<td>126</td>
<td>156</td>
<td>160</td>
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<tr>
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<td>7-39</td>
<td>95</td>
<td>138</td>
<td>62</td>
<td>40-156</td>
</tr>
</tbody>
</table>

Abbreviations: suppl, supplementation; 5MTHF, 5-methyltetrahydrofolate; n.a., not available.

<sup>a</sup> Stage (Hagberg): III, pseudo-stationary period; III-IV: pseudo-stationary period, <10 years and non-ambulant. IV: late motor deterioration, non-ambulant.

<sup>b</sup> in nmol/l.

<sup>c</sup> on folinic acid supplementation.
Table 2 presents the clinical baseline characteristics of the 8 patients; both treatment groups were similar. Although more stage IV Rett syndrome girls were present in the first folinic acid group, the mean symptom severity scores of both groups were similar and clinical severity was therefore comparable. Figure 1 shows the means and standard deviations of all outcomes measurements per 3 months per intervention group. Table 3 presents the results of the linear mixed models. No significant differences were found between the folinic acid and placebo groups for neurological features, the Hand Apraxia scale, Dependency, and the Rett Syndrome Motor Behavioral Assessment. The only (borderline) significant beneficial effects concerned the parental Overall Well-Being Index, with a sum score mean difference of 3.6 (95% CI 0.0-7.3). We considered a change of score > 5 points to be clinically relevant, since the maximum score of this 5-item scale is 50.
Table 3. Results of the linear mixed models*

<table>
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<th>Measure</th>
<th>Mean difference between folinic acid and placebo phase</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hand apraxia total score</td>
<td>0.1</td>
<td>-0.6 — 0.9</td>
</tr>
<tr>
<td>Neurological score A</td>
<td>-0.3</td>
<td>-1.8 — 1.1</td>
</tr>
<tr>
<td>Neurological score B</td>
<td>0.6</td>
<td>-1.1 — 2.2</td>
</tr>
<tr>
<td>Dependency scale total score</td>
<td>0.0</td>
<td>-0.8 — 0.8</td>
</tr>
<tr>
<td>OWBI total score</td>
<td>3.6</td>
<td>0.0 — 7.3</td>
</tr>
<tr>
<td>OWBI mean score</td>
<td>0.7</td>
<td>0.0 — 1.5</td>
</tr>
<tr>
<td>OWBI Mental</td>
<td>0.5</td>
<td>-0.3 — 1.4</td>
</tr>
<tr>
<td>RS total score</td>
<td>-2.6</td>
<td>-8.1 — 2.8</td>
</tr>
</tbody>
</table>

Legend table 3: OWBI, Overall Well-Being Index, RS total score, total of the different items of the Rett syndrome Motor Behavioral Assessment scales.

*The final models contained intervention as a fixed factor, time, and the interaction term of time and intervention as random factors.

No significant change was found on the mental item of the parental Overall Well-Being Index. The Pearson correlation coefficient between the Overall Well-Being Index and the Rett Score Behavioral/Social Assessment items, evaluating speech, contact, interaction and communication (items 2, 3, 7, 8, not corrected for dependence) was 0.134 (P=0.334). No improvement of social contact was found during the use of folinic acid.

Analysis of the parental questionnaires assessing the Global Outcome Score revealed that, at the end of the folinic acid treatment, 75% of the parents reported some improvement. Interestingly, 62.5% of all parents also mentioned some improvement during the placebo treatment. The Global Outcome Score showed no significant difference between the placebo and folinic acid groups (P=0.564).

The parents of one 30-year-old patient (patient 6; stage III-IV) reported a deterioration in the Global Outcome Score during both treatment periods, because the patient became less ambulant. No adverse effects related to folinic acid were reported. One adult patient with low 5-MTHF value (patient 5) demonstrated irritability and aggressive behavior at the end of the folinic treatment, which continued after folinic discontinuation during the 2-year post-treatment follow-up. This patient was previously known with behavior disturbances.
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Figure 1. Means and standard deviations of all outcomes measured every 3 months per intervention group. Circles/solid lines represent the group with placebo first; squares/dashed lines represent the group with folinic acid first. The vertical line is positioned at 12 months, after which the treatment is changed for the following year.

Neurological score A = contact, communication; neurological score B = hand use and motor performance; Apraxia sum = evaluating hand use. Dependency scale evaluating the need for assistance in daily life; RS sum = sum score of the different items of the Rett syndrome Motor Behavioral Assessment scales; OWBI = parental Overall Well-Being index.
Discussion

This study examined the hypothesis that folinic acid supplementation has a positive effect on the course of the disease in Rett syndrome. Biochemical evaluation showed that only 2 of our 8 Rett patients had a low baseline level of cerebrospinal fluid 5-MTHF. Supplementation of folinic acid led to a significant increase in levels of plasma folate and cerebrospinal fluid 5-MTHF, without any clinical side-effects. However, there were no differences between the folinic acid and the placebo groups for motor and behavioral outcomes. The only (borderline) significant beneficial effect was for 1 of the 8 outcome measures on the parental questionnaire.

An appreciable clinical improvement and/or seizure reduction and electroencephalography improvements following folinic acid supplementation were reported in a total of 12 Rett syndrome patients. However, this result was not confirmed in a short-lasting 6-months prospective uncontrolled Portuguese study of 7 Rett syndrome patients, in which there was no improvement of seizure control, global motor function or diminishment of hand stereotypes. Our earlier randomized, placebo-controlled double-blind crossover trial showed in a minority of 12 Rett syndrome patients limited effects of folinic acid in seizure control and electroencephalography recordings. Since folinic acid did not prevent antiepileptic drug adjustments, we do not recommend folinic acid treatment in Rett syndrome patients with epilepsy.

In a recent well designed study, 73 (Methyl-CpG-binding protein 2 mutation positive) patients with Rett syndrome were monitored with respect to breathing, electroencephalography, hand stereotype and clinical neurological changes. Patients were randomized to receive folate-betaine or placebo treatment for 12 months; the authors aimed to increase DNA methylation by supplying substrates such as like folate and betaine. However, since no cerebrospinal fluid was collected, there is no information on the transport of these substrates over the blood-brain barrier. Our study focused on increasing the availability of cerebral folate and, although repeated cerebrospinal fluid 5-MTHF measurements were made, no objective evidence of improvement during folinic acid treatment was found. In the above-mentioned study, however, based on parental questionnaires that assessed changes with respect to sleep, breathing problems, oral facial problems and hand skills, some improvement was noted in children aged ≤ 5 years.

A beneficial response involving social contact, communication and mental alert-
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In the present study, the parental Rett Behavior/Social skills assessment (especially the items concerning contact and communication) and the parental Global Outcome Score showed no significant alterations during folinic acid treatment. Neurological investigation (part A) evaluating eye contact, communication skills and speech, demonstrated no significant change during the 2-year study period. Thus, there was no beneficial effect of folinic acid on mental alertness and social contact in our population.

In the present (double blinded) study, during folinic acid supplementation 75% of the parents reported a subjective improvement in the parental Global Outcome Score and 62.5% during placebo treatment (not significant). The (clinically not relevant) improvement in parental Overall Well-Being Index during folinic acid supplementation, could not be confirmed in our objective data obtained from the patients. We attribute these seemingly positive results to the effect of long-term management and care of the Rett girls and their parents, rather than to a ‘real’ change caused by the folinic acid treatment.

Special attention was given to transfer/walking abilities and purposeful hand function (Hand Apraxia scale) since this is a characteristic of Rett girls and always severely impaired. No improvements were seen during folinic acid treatment. Increasing the ability for self-care would diminish nursing demands on parents and/or caregivers; however, the Dependency score remained in the lower range, indicating total dependency. Overall, no beneficial effect of folinic acid treatment on the clinical course of this group of Rett patients was observed.

Ramaekers et al. were the first to demonstrate the presence of 5-MTHF disturbances in Rett syndrome and this finding was considered very important, especially in these girls; at that time we designed our randomized double-blind crossover trial. However, it was later shown that low levels of 5-MTHF and encephalopathy symptomatology are also present in the infantile-onset cerebral folate deficiency syndrome and mitochondrial encephalopathy. Therefore, at the moment it remains unclear to what extent the presence of 5-MTHF disturbances in Rett syndrome contribute to its pathophysiology.

A double-blind placebo-controlled randomized, crossover study is the only reliable approach to evaluate the efficacy of a novel/experimental therapy in any disease condition, after such an approach has received the approval of an ethical committee. All patients in the present study were clinically diagnosed as having typical Rett syndrome. More importantly, in 75% of our group a Methyl-CpG-
binding protein 2 mutation was present. Because it is established that clear differences in clinical severity exist between the different mutations in Rett syndrome\textsuperscript{5}, we used a crossover design and compared individuals during folinic acid or placebo use, to rule out the influence of disease severity and age.\textsuperscript{5} Moreover, the patients were evaluated for $\geq 2$ years to rule out temporary changes.

We believe this is the first adequately randomized study with folinic acid and placebo supplementation to evaluate the long-term therapeutic efficacy of folinic acid treatment. The outcome measures included cerebrospinal fluid 5-MTHF to evaluate the biochemical effect of folinic acid supplementation. The outcome measurements were carefully selected and are relevant to the clinical features of Rett syndrome; in addition, they are quantifiable. Despite the relatively small number of patients in the present study, the results are in agreement with others who reported no significant beneficial effects of folinic acid treatment. For future investigations, our study demonstrates the importance of randomization for placebo versus pharmacological treatment, the use of specific outcome measurements to objectively evaluate therapeutic efficacy, and the use of parental questionnaires in the evaluation of Rett syndrome.

More long-term studies in a larger Methyl-CpG-binding protein 2 mutation-positive Rett population are recommended.

**Declaration of conflicting interests**

Folinic acid was provided by the pharmaceutical company TEVA Pharmachemie, Haarlem The Netherlands. This company was not involved in study protocol design, follow up or study evaluation.
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