

Original article

Evaluation of CSF neurotransmitters and folate in 25 patients with Rett disorder and effects of treatment[☆]

T. Temudo^{a,*}, M. Rios^a, C. Prior^a, I. Carrilho^b, M. Santos^b, P. Maciel^c, J. Sequeiros^d, M. Fonseca^e, J. Monteiro^e, P. Cabral^f, J.P. Vieira^g, A. Ormazabal^h, R. Artuch^h

^a *Unidade de Neuropediatria, Serviço de Pediatria, Hospital Geral de Santo António, SA, Largo Abel Salazar, 4099/001 Porto, Portugal*

^b *Serviço de Neuropediatria, Hospital de Crianças Maria Pia, Porto, Portugal*

^c *Instituto de Investigação em Ciências da Vida e da Saúde (ICVS), Escola de Ciências da Saúde, Universidade Minho, Braga, Portugal*

^d *UnIGENE, IBMC – Institute for Molecular and Cell Biology; UnIGENE, IBMC – Institute for Molecular and Cell Biology, Portugal*

^e *Serviço de Pediatria, Hospital Garcia da Horta, Almada, Portugal*

^f *Serviço de Neurologia, Hospital Egas Moniz, Lisboa, Portugal*

^g *Serviço de Neuropediatria, Hospital D. Estefânia, Lisboa, Portugal*

^h *Department of Clinical Chemistry, Hospital Sant Joan de Déu and CIBERER, Instituto de Salud Carlos III, Barcelona, Spain*

Received 4 February 2008; received in revised form 30 April 2008; accepted 10 May 2008

Abstract

Background: Rett disorder (RD) is a progressive neurodevelopmental entity caused by mutations in the *MECP2* gene. It has been postulated that there are alterations in the levels of certain neurotransmitters and folate in the pathogenesis of this disease. Here we re-evaluated this hypothesis. **Patients and methods:** We evaluated CSF folate, biogenic amines and pterines in 25 RD patients. Treatment with oral folinic acid was started in those cases with low folate. Patients were clinically evaluated and videotaped up to 6 months after therapy. **Results:** CSF folate was below the reference values in 32% of the patients. Six months after treatment no clinical improvement was observed. Three of the four patients with the R294X mutation had increased levels of a dopamine metabolite associated to a particular phenotype. Three patients had low levels of a serotonin metabolite. Two of them were treated with fluoxetine and one showed clinical improvement. No association was observed between CSF folate and these metabolites, after adjusting for the patients age and neopterin levels. **Conclusion:** Our results support that folinic acid supplementation has no significant effects on the course of the disease. We report discrete and novel neurotransmitter abnormalities that may contribute to the pathogenesis of RD highlighting the need for further studies on CSF neurotransmitters in clinically and genetically well characterized patients.

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Keywords: Rett syndrome; Neurotransmitters; Folate; Movement disorders

1. Introduction

Rett syndrome is a progressive neurodevelopmental disorder clinically defined by criteria revised in 2002

[1]. In 1999 its aetiology was first established: mutations of the methyl-CpG binding protein 2 (MeCP2) gene (*MECP2*), which maps to Xq28 [2]. Since then, patients meeting the revised diagnostic criteria can be divided in two groups: those with a detected *MECP2* mutation – Rett disorder patients (RD) and those without it – Rett syndrome patients (RTT).

Nomura et al. [3] were among the first to suggest that neurotransmitter anomalies could form the chemical

[☆] *Disclosure:* The authors have reported no conflicts of interest.

* Corresponding author. Tel.: +351 222077500/226106061; fax: +351 223320318.

E-mail address: teresatemudo@netcabo.pt (T. Temudo).

basis for RTT, postulating that its movement disorder and altered sleep structure could be related to a deficiency in brainstem dopamine and serotonin, respectively; nevertheless conflicting reports regarding the levels of biogenic amine metabolites and pterine values in the cerebrospinal fluid (CSF) have appeared from several laboratories [4–7].

More recently, Raemaekers et al. [8] suggested that a disturbed transport of folate across the blood–brain barrier could contribute to the pathogenesis of RTT. Folate is involved in the biosynthesis of GTP, a precursor of tetrahydrobiopterine (BH₄), and therefore it could modulate phenylalanine catabolism and serotonin and dopamine biosynthesis. They studied 4 RTT patients (2 with and 2 without an identified mutation); all had low CSF levels of the folate metabolite 5-methyltetrahydrofolate (5-MTHF) and normal serum folate levels. After oral folinic acid supplementation, clinical improvement and normalization of CSF 5-MTHF levels were reported. Two years later, a Spanish study of 16 RTT patients (8 with a detected *MECP2* mutation) described low CSF 5-MTHF in 50% (3 with a *MECP2* mutation). All patients with low 5-MTHF had epilepsy; thus, a positive correlation between low CSF 5-MTHF and epilepsy was proposed. These researchers also reported an improvement in social contact, decreased stereotypic hand-washing movements and seizure control, one year after oral folinic acid supplementation [9]. Nevertheless, the largest study so far was conducted in North America and included 76 RTT patients (84% with detected *MECP2* mutations); only 2 patients (one with low serum folate and no detected *MECP2* mutation) had CSF 5-MTHF outside the reference range. The authors concluded that folate deficiency, or a disorder in folate transport across the blood–brain barrier, was not contributing to the pathogenesis of RTT; however, some patients were under folate supplementation before the study [10].

The aim of our study was (1) to analyze the correlation between the phenotype of RTT patients with detected mutation (RD), without previous folate supplementation, and CSF concentration of biogenic amines, pterins and 5-MTHF levels; we also aimed at (2) evaluating the clinical response to folinic acid supplementation in patients with low levels of 5-MTHF, in terms of social contact, behaviour, motor function and epilepsy control.

2. Patients and methods

We studied 25 female RD patients, all fulfilling the revised diagnostic criteria of RTT [1] and all with a detected mutation in the *MECP2* gene.

The protocol and the consent form were approved by the ethical committee of HGSA. Personal history, structured examination and lumbar puncture were performed

by the same experienced examiner (TT). Sedation was accomplished by intranasal midazolam (0.1–0.3 mg/kg). Samples were collected between 8:00 and 10:00 a.m., following a previously reported protocol [11], and were immediately frozen at –70 °C, until the moment of the analysis. Haematic samples were centrifuged immediately, and the clear CSF supernatant stored at –70 °C. Later on, all samples were transported at –70 °C to the Department of Clinical Chemistry, at Hospital Sant Joan de Deu, Barcelona, where the analyses followed a previously reported methodology [9]. In the CSF, we analyzed 5-MTHF, neopterin, biopterin, homovanillic acid (HVA), 5-hydroxyindoleacetic acid (5-HIAA), HVA/5-HIAA and HVA/MHPG. In peripheral blood, we analyzed haemoglobin, erythrocyte indices, folate and vitamin B12. Results of biochemical studies in CSF were compared with the reference values previously reported [9,11]. This reference ranges were established in 134 subjects (age range 11 days–18 years); average 4.1 years; 71 males and 63 females) whose CSF samples were submitted to our laboratory under suspicion of viral or bacterial meningitis, encephalitis or other neurological conditions of non metabolic origin.

Genomic DNA was extracted from leukocytes, using the Puregene DNA isolation kit (Gentra, Minneapolis, MN). The coding region and exon–intron boundaries of the *MECP2* gene were amplified by PCR and sequenced. RD-PCR was used, as described [12], for the detection of large rearrangements in the *MECP2* gene. Primers and PCR conditions are available upon request.

A treatment protocol with oral folinic acid supplementation was started with 0.8–1.5 mg/kg/day and 1 mg/kg of B12, 3 days in a week, after observation of low 5-MTHF concentrations in CSF. Patients were clinically evaluated and videotaped always by the same paediatric neurologist (TT), in baseline conditions, 3 and 6 months after folinic acid supplementation.

Since biogenic amines are strongly associated with age, multiple linear regression analysis was performed, with 5-HIAA and HVA as dependent variables; age, CSF 5-MTHF and pterin concentrations were the independent variables. The SPSS (v.14) statistical package was used to analyze the data. Statistical significance was considered with a $p < 0.05$.

3. Results

The clinical and genetic characteristics and the biochemical findings of the 25 patients are presented in Table 1 and Fig. 1. The median age of the patients was 7.2 years (range, 22 months–16.3 years).

3.1. RD and CSF folate

All patients had normal plasma B12 vitamin and folate values, but in 8 (32%), CSF folate was below

Table 1
Clinical, genetic, biochemical, and treatment data of 25 RD patients

Patient	Age (years)	Stage	MECP2 mutation	Independent gait	Agitation	Dystonia	Epilepsy	Antiepileptic drugs	5-MTHF (nmol/L)	Plasma folate (ng/mL)	NP (nmol/L)	BP (nmol/L)	5-HIAA (nmol/L)	HVA (nmol/L)	HVA/5-HIAA	Folate + B12 Tx	Epilepsy control	↑ social contact
1	1,9	III	R270X	+	+	–	–	NA	74	12,6	9	25	135(↓)	506	3,75(↑)	NA	NA	NA
2	2,4	II–III	S113P;P251P	–	–	+	+	CMZ, VPA	80	15,6	13	20	122(↑)	411	3,37	NA	NA	NA
3	2,4	III	R168X	–	+	–	–	NA	57	9,4	8	24	134(↓)	459	3,43	NA	NA	NA
4	2,7	II–III	T158M	–	+	–	–	NA	58	8,7	22	17	236	460	1,95	NA	NA	NA
5	3,6	III	R294 X	+	+++	+	+	CMZ	42(↓)	17,9	22	14	234	500	2,14	NA	NA	NA
6	4,0	III	R270fsX288	+	+	–	+	VPA	95	16,3	12	28	122(↑)	401	3,29	NA	NA	NA
7	4,2	III	R306H	+	+	–	–	NA	84	4,6	16	17	110	393	3,57(↑)	NA	NA	NA
8	4,8	III	R294X	+	+++	+	–	NA	43(↓)	10,1	31	21	352(↑)	666(↑)	1,89(↓)	Yes	NA	Yes
9	4,8	+	R133C	+	–	–	–	NA	63	9,7	13	26	133	510	3,83(↑)	NA	NA	NA
10	5,6	III	R294X	+	+++	+	–	NA-	46(↓)	8,9	24	16	283	714(↑)	2,52	Yes	NA	Yes
11	6,4	III	Del (7 pb) V300frX318	+	++	++	–	NA	62	12,7	14	17	78(↓)	259(↓)	3,32	NA	NA	NA
12	6,6	III	T158M	+	+	+	+	CMZ, VPA	61	11,3	25	19	114	614	5,39(↑)	NA	NA	NA
13	7,2	III	R168X	–	+	+	+	CMZ, VPA	40(↓)	7,6	11	10	98	226	2,31	Yes	No	No
14	7,2	III	Del (exon 3 and 4)	+	–	–	+	CMZ	69	7,1	14	22	119	445	3,74(↑)	NA	NA	NA
15	7,3	III	R106W	–	–	+	+	CMZ	72	16,1	14	29	158	502	3,18	NA	NA	NA
16	7,4	III	T158M	+	–	–	+	CMZ	59	12,9	17	21	132	424	3,21	NA	NA	NA
17	7,5	III	R306C	+	–	–	–	NA	56	5,6	14	17	90	277	3,08	NA	NA	NA
18	7,7	III	R133C	+	–	–	–	NA	37(↓↓)	6,5	13	18	192	570	2,97	Yes	NA	No
19	8,7	III	K305R	+	–	+	–	NA	45	12,1	14	12	182	331	1,82	NA	NA	NA
20	9,9	III	R168X	+	+	–	–	NA	37(↓)	5,9	13	21	120	382	3,18	Yes	NA	No
21	10,8	III	T158M	+	+	–	–	NA	41(↓)	9,1	12	18	174	347	2,36	Yes	NA	Yes
22	11,2	IVA	R294X	+	+++	+	+	LVT, CMZ	82	6,5	10(↓)	20	130	453(↑)	3,48(↑)	NA	NA	NA
23	12,3	III	R133C	+	–	–	–	NA	50	3,2	11	23	125	300	2,4	NA	NA	NA
24	15,5	IVB	K39fsX43	–	–	+	+	CMZ, VPA	42(↓)	6,4	11	8(↓)	105	154(↓)	1,47	Yes	No	No
25	16,3	IVB	R168X	–	–	+	+	CMZ	54	9	12	13	203(↑)	299	1,47(↓)	NA	NA	NA
Controls	0.5–2 (n = 38)								48–127	2,6–20	8–43	8–54	170–490	344–906	1,11–3,48			
	3–6 (n = 24)								56–124		7–55	10–52	106–316	304–658	1,92–3,44			
	7–10 (n = 20)								45–94		10–46	8,2–68	87–366	202–596	1,20–3,45			
	11–16 (n = 12)								45–94		10–24	13–36	63–185	156–410	1,44–3,17			
	>16 (n = 7)								45–94		9–27	11–30	55–130	98–376	1,50–3,50			

5-MTHF, 5-methyltetrahydrofolate; NP, neopterin; BP, biopterin; 5-HIAA, 5-hydroxyindoleacetic acid; HVA, homovanillic acid; LVT, levetiracetam; CMZ, carbamazepine; VPA, valproic acid; NA, not applicable.

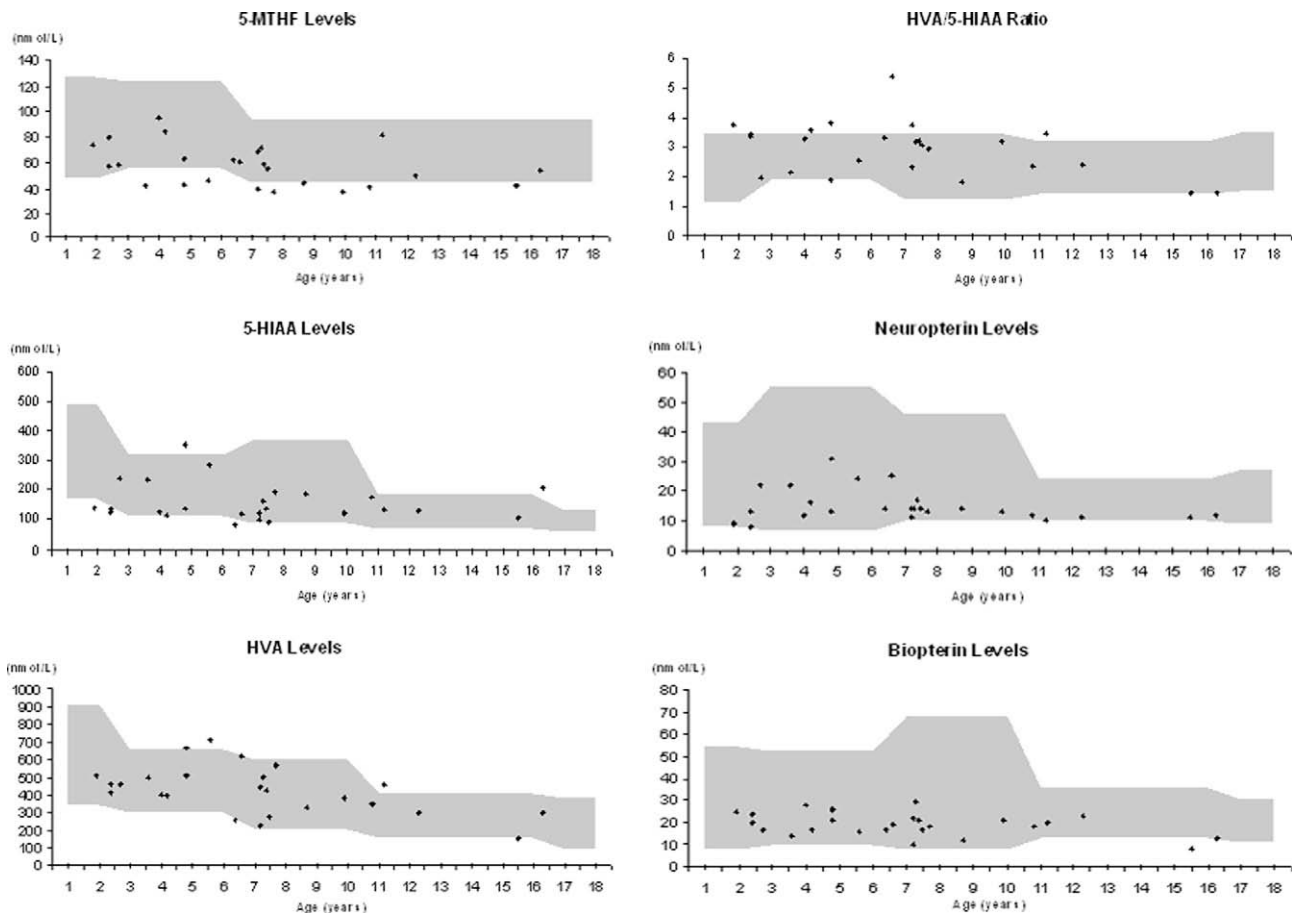


Fig. 1. CSF folate, biogenic amine metabolites and pterines in 25 patients with RD. Grey bars represent normal values – range between mean minus 2SD and plus 2SD in the control population.

the reference values (mean 41, median 41.5, range 37–46 nmol/L). Two patients with low CSF 5-MTHF levels had epilepsy, both being resistant to the prescribed antiepileptic drugs (Valproate and Carbamazepine). Supplementation with folic acid and B12 vitamin was applied to all patients with low CSF folate. After 6 months of treatment, no improvement in seizure control, global motor function, or reduction of stereotypies was observed.

3.2. RD and CSF neurotransmitters

Three of the four patients with the R294X mutation (patients 8, 10, 22) had levels of HVA above the reference range. They had a median age of 5.6 years (range, 4.8–11); all had a particular phenotype, with unusual motor agitation and a high number of various stereotypies. Moreover, four patients with a median age of 7 years (range 2–7) had high HVA/5-HIAA (patients 1, 5, 12, 14).

Three patients (two aged under 3 years and one aged 6) had low levels of 5 HIAA (patients 1, 3, 11). Patient 11 also had low levels of HVA and suffered from segmental dystonia of the right inferior limb, which had

developed in the last 18 months. In patients 1 and 3 with low levels of 5 HIAA, a clinical trial of 10 mg/day of fluoxetine was initiated. In one of them the self-injurious stereotypies decreased considerably after one month of treatment.

No association was observed between CSF 5-MTHF and 5-HIAA or HVA concentrations, after adjusting for the patients' age and neopterin levels.

4. Discussion

4.1. RD and CSF folate

It has been suggested that a deficit in cerebral folate could be contributory to the pathogenesis of RD and that folic acid supplementation might be effective in symptom management in these patients [8,9], however this remains controversial [10].

The present study may contribute to clarify this, as we studied a very homogeneous sample of RD patients, all with a *MECP2* mutation, and who had not been previously submitted to oral folate supplementation. Furthermore, all patients were evaluated by the same paediatric neurologist (TT).

In spite of the finding that 32% of the RD patients studied had low CSF folate values, we did not find a positive association between epilepsy and decreased CSF folate values as the Spanish study did [9], nor could we observe control of seizures with folate supplementation. Also, none of the patients ameliorated their motor functions or showed a decrease of stereotypies after oral folate supplementation. However, two of the three Spanish patients with *MECP2* mutations showed a more severe CSF folate deficiency than ours, who showed only mild CSF folate deficiency, which could explain the different response to supplementation. As we did not verify a clinical improvement after folic acid supplementation, lumbar puncture was not repeated for ethical reasons. Our results support that folic acid supplementation has no significant effects on the course of the disease. More studies with RD patients may be needed to clarify this further.

4.2. CSF neurotransmitters in RTT and RD

Among the hypotheses proposed to explain the mechanisms that lead to the neurological symptoms of RD, several authors suggested an alteration in the bioaminergic function, although the evidence existing is not conclusive. The disagreement around the levels of specific markers may be related to the inclusion of patients with and without detected *MECP2* mutations, age at examination or variable degree of impairment. Interestingly, we found that some patients had abnormal values of biogenic amines, not correlated to low 5-MTHF levels.

Other authors reported low CSF metabolites of serotonin in RTT patients [5]. We found low CSF 5-HIAA in 12% of the patients (2 patients below 3 years of age). Serotonin deficiency could explain some symptoms of RD like sleep, breathing and autonomic anomalies, insensitivity to pain, early autistic-like behaviour and the particularly compulsive behaviour of stereotypies, more exuberant in the first decade of life of these patients. One of the two patients in our study with low 5-HIAA received treatment with fluoxetine, since she had continuous self-injuring stereotypies of hand biting, leading to hand wounds. After 1 month of treatment, these stereotypies decreased considerably and the use of protective gloves was no longer needed. The other patient under treatment with fluoxetine was in the regressive period, which hampered the clinical evaluation of the drug effects; however, she did not present sleep abnormalities neither irritability, two frequent symptoms of the regression stage.

Recently, several studies suggested impairment in serotonin synthesis or transport in the aetiology of autism [13–19]. Serotonergic receptor-binding was found to be increased in the brainstem nuclei in RTT, which may reflect decreased serotonergic input with a compensa-

tory upregulation of receptor binding and/or binding affinity [20].

Concerning the metabolites of dopamine, only one of our patients had low levels of HVA in the CSF and lost independent gait due to the development of severe segmental dystonia in her left leg. Studies of CSF neurotransmitters in RTT patients without genetic studies have reported contradictory results of dopamine levels [5,21,22], but neuropathological studies found a significant reduction of the metabolites of dopamine and norepinephrine in the *Substantia nigra*, more prominent in the older RTT females, and the possibility of age-related abnormality was considered [23–24]. The progressive reduction of dopamine with age could explain the decreased intensity of the stereotypies and the appearance of dystonia and Parkinsonian symptoms.

To our knowledge, increased CSF dopamine metabolites have never been reported in RTT. Recently, Toda et al. reported increased stereotyped behaviour, hyperactivity and temper exacerbation, in four out of 12 children with autism in whom high levels of CSF HVA were found after treatment with secretin [25]. Intriguingly, in our study, three of the four patients with the R294X mutation (patients 8, 10, 22) had HVA levels above the reference range. All had a particular phenotype, with unusual motor agitation and more stereotypies than the median found in RD [26]. Robertson et al. [27] reported that RD patients with the R294X mutation were more likely to have mood difficulties, but they did not describe hyperactive motor behaviour; however, they also reported that these patients had less frequent hand stereotypies, which is in contrast with our results. Further studies are required in order to confirm the association of the R294X mutation with high dopamine metabolites, stereotypies and hyperactive behaviour. The remaining four patients presenting an increased HVA/5-HIAA ratio support the hypothesis that impaired dopamine neurotransmission might have a role in the pathophysiology of RD.

With the discovery of *MECP2* gene mutations as a cause of RD, monoamine metabolites and pterines are no longer useful as biochemical markers of the disorder. Nevertheless, questions remain regarding the fundamental mechanisms of RD: *MECP2* gene mutations probably cause a deregulation of target genes controlling neurotransmitters. In our opinion, supplementation with serotonin and dopamine may have some potential for clinical intervention in this disorder in patients with altered levels of the metabolites of these neurotransmitters; therefore, more CSF neurotransmitter studies should be performed in RD, preferably using clinically and genetically well-characterized samples.

Acknowledgements

To all patients and families who participated in this study. To Júlio Teixeira and Filomena Loureiro for technical support. To Mónica Santos for the molecular genetic analysis. Research in Rett syndrome is supported by FSE/FEDER and Fundação para a Ciência e Tecnologia (FCT, Portugal), Grant No. POCTI 41416/2001.

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