



Clinical and Genetic Study of Rett Syndrome in Portugal

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De acordo com o disposto no nº 2 do artigo 8º do Decreto-lei nº 388/70, nesta dissertação foram utilizados os resultados dos trabalhos publicados ou em preparação abaixo indicados. No cumprimento do disposto no referido Decreto-lei, a autora desta dissertação declara que interveio na concepção e execução dos trabalhos efectuados, na interpretação dos resultados e na redacção dos resultados publicados ou submetidos para publicação, sob o nome de Temudo T:

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“Quem nada cria, nada tem”

Anónimo

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Resumo

A síndrome de Rett (RTT) é uma doença neurológica do desenvolvimento e a segunda causa de atraso mental grave no sexo feminino; é causada, na maioria dos casos, por mutações *de novo* num gene situado no cromossoma X que codifica para a proteína de ligação às metil-CpG (*MECP2*). Foram encontradas mutações neste gene em mais de 90% dos probandos com a forma clássica de RTT. Os estudos de correlação genótipo-fenótipo na RTT clássica sugerem que o tipo de mutação e o padrão de inactivação do X têm um efeito na determinação da apresentação clínica e gravidade da doença.

Os objectivos da tese foram: (1) identificar um grande número de novos casos de RTT, em Portugal; (2) fazer o estudo genético molecular em doentes portugueses com RTT clássico e não-clássico, e identificar a frequência e tipo de mutações do *MECP2*; (3) estudar e classificar clinicamente os doentes identificados e, em particular, as alterações do movimento; (4) comparar doentes com e sem mutação identificada; (5) estudar correlações genótipo-fenótipo em doentes com mutação identificada; (6) estudar o papel dos neurotransmissores e folatos nos doentes com mutação identificada; e (7) pesquisar mutações no gene *MECP2* em doentes com outras patologias.

Com o objectivo de identificar o maior número possível de casos, contactámos todos os serviços de neuropediatria portugueses, de forma a identificarmos os doentes já diagnosticados; fizemos palestras de divulgação nos serviços de pediatria do Norte e nas reuniões anuais de pediatria, neuropediatria, neurologia e genética. Incentivámos a formação da Associação de RTT em Portugal, com quem mantivemos contacto estreito.

Observámos e filmámos as crianças com suspeita de RTT nos hospitais onde foram sinalizadas. As doentes observadas no HGSA foram seguidas por uma equipa multidisciplinar (neuropediatra, gastroenterologista, nutricionista, ortopedista e fisiatra).

Estabelecemos um banco de DNA para todos os casos identificados. Analisámos as sequências codificantes do gene *MECP2* em todos os casos de RTT e em doentes com patologias afins. Efectuámos também estudos bioquímicos no sangue e LCR.

Identificámos 88 casos com RTT em Portugal, tendo sido identificada mutação no gene *MECP2* em 60: 9 mutações ainda não estavam descritas.

Definimos como portadores de doença de Rett (RD) ou síndrome de Rett (RTT) os doentes com ou sem mutação identificada, respectivamente.

Comparámos as características clínicas destes dois grupos de doentes, de forma a redefinir o perfil da doença e a seleccionar melhor, no futuro, os candidatos a estudo genético. Estudámos a correlação genótipo-fenótipo e individualizámos 3 tipos clínicos de RD. Estudámos, em particular, as alterações de movimento dos doentes com RD e RTT e descrevemos vários sinais neurológicos que não tinham sido previamente descritos. Encontrámos diferenças em relação à marcha independente, distonia e tipo de tremor, quando comparámos doentes com mutações *missense* ou truncante; encontrámos diferenças em relação ao aparecimento, tipo e gravidade das alterações de movimento com o tempo de evolução da doença nos dois grupos. Documentámos em vídeo que os movimentos anormais estão presentes na RD antes da fase de regressão da doença. Analisámos os metabolitos da dopamina, serotonina e folato no LCR e efectuámos uma avaliação e intervenção gastrointestinal e nutricional em 25 doentes com RD.

Concluimos que um período de regressão nítido e a presença de 3 ou mais estereotipias diferentes, rigidez e marcha atáxica-rígida são úteis na distinção entre RD e RTT.

Doentes com mutações truncantes diferem daqueles com mutações *missense* em relação à aquisição de linguagem e marcha autónoma, antes do início da doença, e microcefalia, crescimento, tamanho do pé, distonia e gravidade global, à data de observação. Os doentes com mutação *R168X* apresentavam fenótipo mais grave e os portadores da mutação *R133C* o menos grave. Doentes com mutação *R294X* tinham um comportamento hiperactivo e maior número de estereotipias; aqueles com a mutação *T158M* pareciam ser particularmente atáxicos e rígidos.

As alterações de movimento são a “assinatura” da doença de Rett, sendo fundamentais para fazer o diagnóstico diferencial entre RD e RTT; para além disso, parecem reflectir a gravidade e progressão da doença. Doentes com mutações truncantes têm mais vezes e com maior gravidade distonia e síndrome rígido-acinético, quando comparados com doentes com mutação *missense* e o mesmo tempo de evolução da doença.

Os nossos resultados apoiaram a hipótese de o folato não ter um papel na patogénese da DR, mas reforçaram a opinião de os neurotransmissores terem um papel importante.

Os resultados da avaliação gastrointestinal demonstraram a necessidade de equipas multidisciplinares no tratamento das doentes com RD.

Propusemos ainda a revisão dos critérios de diagnóstico de RTT clássico e a abolição dos critérios de RTT variante, baseados na experiência clínica adquirida.

Abstract

Rett syndrome (RTT) is a neurodevelopmental disorder, caused in most cases by mutations in the methyl-CpG binding protein 2 (MeCP2) gene (*MECP2*), which maps to Xq28. It is considered the second most frequent cause of severe mental retardation in females. The reported detection rate for *MECP2* mutations in patients with clinically defined RTT is 60 to 95%, depending on the clinical parameters used to select the patients. Larger studies point to some correlation between genotype and phenotype. Another source of phenotypic variability seems to be X chromosome inactivation.

The aims of this thesis were: (1) to identify a large number of patients with RTT in Portugal and to study its phenotypic variability; (2) to analyze the entire coding sequence of *MECP2* in all patients and identify the most frequent mutations in our population; (3) to study in detail and classify clinically the patients identified, specially their movement disorders; (4) to compare patients with and without identified mutations; (5) to analyze genotype-phenotype correlation; (6) to study the role of cerebral folate and neurotransmitters in the physiopathology of the disease and (7) to study *MECP2* mutations in patients with diseases clinically overlapping Rett syndrome.

All paediatric neurology services were asked to refer their RTT patients, and conferences supported by videos were presented at the paediatric services of the North of Portugal and at the meetings of the national societies of paediatrics and neurology. A contribution was made toward organizing the Portuguese Rett Association.

The clinical study was based on all the information collected between 1996 and 2007. All patients referred by paediatric neurologists, neurologists or paediatricians were personally examined, and their fulfilment of the revised clinical criteria for RTT was reviewed.

All patients were observed and videotaped at the hospitals where they had been diagnosed. Patients followed at HGSA were also observed by a multidisciplinary team.

All coding sequences and the exon-intron boundaries of *MECP2* were analyzed in all cases, as well as in patients with clinical overlap with Rett syndrome. We also performed biochemical studies in blood and CSF, in 25 RTT patients with a *MECP2* mutation.

We studied 88 cases of RTT; *MECP2* mutations were identified in 60 (9 were novel).

Based on the presence or absence of a *MECP2* mutation, we defined two groups: patients with Rett disorder (RD) and with Rett syndrome (RTT). We compared these clinically, in order to redefine the clinical borders of the disorder and improve criteria for genetic ana-

lysis. We also studied in detail the phenotype-genotype correlation in RD and identified three clinical presentations. Movement disorders were analyzed in detail in RD and RTT; several neurological signs were described by the first time. Folate and neurotransmitters were analyzed in CSF and gastro-intestinal dysfunction was assessed in 25 RD patients.

We concluded that a clear regressive period (with loss of prehension and language, and deceleration of growth) and the presence of more than three stereotypies, rigidity and ataxic-rigid gait seemed to be helpful in differentiating RD from RTT.

Patients with truncating differed from those with missense mutations, regarding acquisition of propositive words and independent gait before the onset of disease, and microcephaly, growth, foot length, dystonia and severity score, at the time of observation. Patients with the R168X mutation had a more severe phenotype, whereas those with R133C showed a less severe one. Patients with R294X had hyperactive behaviour, while those with T158M seemed to be particularly ataxic and rigid.

Movement anomalies are the hallmark of RD and are essential to differentiate RD from RTT; furthermore, they seem to reflect severity and rate of progression of RD. Patients with truncating mutations presented more frequent and severe dystonia and rigid-akinetic syndrome, when comparing patients with similar time of disease evolution.

Folate deficit is not contributing to the pathogenesis of RD. In contrast, we report discrete and novel neurotransmitter anomalies, highlighting the need for further studies of CSF neurotransmitters, in clinically and genetically well characterized patients.

Management of Rett disorder requires a multidisciplinary team, including gastroenterologists. Individually designed feeding strategies are essential to achieve a good nutritional status. Early identification of nutritional and gastrointestinal disturbances and their correct management contributes to improve quality of life in these patients.

We suggested, based on the clinical experience acquired, that the international diagnostic criteria should be revised and defined in a stage-dependent manner, according to the clinical signs and symptoms present at the different stages of the disease, in a large series of patients with *MECP2* mutations. We also suggested that the only variant form that may need to be considered is the male form, as the others do not differ from the classical forms during the pseudo-stationary stage.

Résumé

La syndrome de Rett (RTT) est une maladie neurologique du développement et la deuxième cause de retard mental chez le sexe féminin; elle est causée, dans la plupart des cas, par des mutations de novo chez un gène situé au chromosome X, qui codifie pour la protéine de liaison aux methyl-CpG (*MECP2*). Des mutations dans ce gène ont été observées chez plus de 90% des sujets avec la forme classique de RTT. Les études de corrélation genotype-phenotype chez la RTT classique suggèrent que le type de mutation et le patron d'inactivation du chromosome X ont un effet sur la détermination de la présentation clinique et sur la sévérité de la maladie.

Les objectifs de cette thèse ont été: (1) d'identifier un grand nombre de nouveaux cas de RTT au Portugal; (2) de faire l'étude génétique moléculaire de malades portugais(es) avec RTT classique et non-classique, et identifier la fréquence et le type de mutations du gène *MECP2*; (3) d'étudier et classer cliniquement les malades identifiés et, en particulier, ses altérations du mouvement; (4) de comparer des malades avec et sans mutation identifiée; (5) d'étudier des corrélations genotype-phénotype; (6) d'étudier le rôle des neurotransmetteurs et des folates chez les malades avec mutation identifiée; et (7) de chercher des mutations au gène *MECP2* chez de malades avec d'autres pathologies.

Avec le but d'identifier le plus grand nombre de cas possible, nous avons contacté tous les services de neuropédiatrie portugais; nous avons fait des exposés sur la maladie dans plusieurs services de pédiatrie du nord du pays et dans les réunions annuelles de pédiatrie, neuropédiatrie, neurologie et génétique nationales. Nous avons stimulé la formation de l'Association de RTT au Portugal, avec qui nous avons maintenu un contact proche. Nous avons observé et filmé les enfants avec soupçon clinique de RTT, dans les hôpitaux où ils ont été identifiés. Les malades observés à L'HGSA ont été suivis par une équipe multidisciplinaire. Nous avons établi une banque d'ADN pour tous les cas identifiés. Les séquences codifiantes du gène *MECP2* ont été analysées en tous les cas de RTT et chez des malades avec des pathologies similaires. Des études biochimiques du sang et LCR ont été faits.

Nous avons identifié 88 cas avec RTT au Portugal, et une mutation au gène *MECP2* a été identifiée en 60: 9 de ces mutations n'avaient jamais été décrites. Nous avons défini comme porteurs de la maladie de Rett (RD) ou syndrome de Rett (RTT) les malades avec ou sans mutation identifiée, respectivement.

Nous avons comparé les caractéristiques cliniques de ces deux groupes de malades, de façon à redéfinir le profil de la maladie et ainsi mieux sélectionner, au futur, les candidats à une étude génétique. On a étudié la corrélation génotype-phénotype et on a individualisé 3 types cliniques de RD. On a étudié en particulier les altérations de mouvement des malades avec RD et RTT et on a décrit plusieurs signes neurologiques qui n'avaient pas été préalablement décrits. Des différences ont été observées en ce qui concerne la marche indépendante, dystonie et type de tremblement, par comparaison entre patients avec des mutations *missense* et troncantes; on a observé des différences en ce qui concerne l'apparition, le type et la sévérité des altérations du mouvement avec le temps d'évolution des deux groupes. On a documenté en vidéo que les mouvements anormaux sont présents dans la RD avant la phase de régression de la maladie. Les métabolites de la dopamine, sérotonine et folate ont été analysés par LCR et une évaluation et intervention gastro-intestinale et nutritionnelle ont été faites en 25 malades avec RD. Nous avons conclu qu'une période de nette régression et la présence de 3 ou plus stéréotypies différentes, la rigidité et la marche ataxique-rigide sont utiles pour la distinction entre RD et RTT.

Les malades avec des mutations troncantes diffèrent de ceux avec mutations *missense* en ce qui concerne l'acquisition du langage et de la marche autonome, avant le début de la maladie ; ainsi que la microcéphalie, le grandissement, la taille du pied, la dystonie et la sévérité globale, à la date de l'observation. Les malades avec la mutation *R168X* présentaient un phénotype plus grave et les porteurs de la mutation *R133C* le moins grave. Les malades avec la mutation *R294X* avaient un comportement hyperactif et un nombre plus grand de stéréotypies; ceux avec la mutation *T158M* paraissaient particulièrement ataxiques et rigides.

Les altérations du mouvement sont la « signature » de la maladie de Rett, et sont fondamentales pour le diagnostic différentiel entre RD et RTT ; en plus, elles paraissent refléter la sévérité et la progression de la maladie.

Nos résultats ont appuyé l'hypothèse selon laquelle le folate n'a aucun rôle chez la pathogénèse de la RD, mais ont renforcé l'idée de que les neurotransmetteurs y jouent un rôle important.

Les résultats de l'évaluation gastro-intestinale ont indiqué le besoin d'équipes multidisciplinaires pour le traitement des malades avec RD.

Nous avons aussi proposé la révision des critères de diagnostic de RTT classique et l'abolition des critères de RTT variante, basées sur l'expérience clinique acquise.

Preface

Maybe those who will read this thesis will ask why I became interested by this particular disorder.

Our life is commanded by conscious and unconscious decisions, and ... by chance. What we do with our lives depends in a big part on the ability to seize new opportunities.

In 1996, when I had recently finished my training in Neuropediatrics, Karin Dias decided to organize a Pediatric Neurology meeting in Lisbon, and invited Bent Hagberg to give a conference on Rett syndrome. With the goal of doing a presentation about Rett cases in Portugal, she proposed me to make a questionnaire and send it to all the Paediatric neurology centres. At that time I collected 50 Rett syndrome cases from all the country. I studied all the scientific material and videotaped some girls with the disease.

As I presented my work in the morning and Bent Hagberg would present his conference only in the afternoon, I begun my lecture with a video of a typical Rett syndrome girl, followed by a very exhaustive introduction of the syndrome and, only after that, I showed the data I collected on the Portuguese Rett syndrome patients. I was so excited with this disorder that I exceeded the time scheduled for my presentation. Karin Dias was not very happy, as I didn't leave much for Bent Hagberg to tell about Rett syndrome... But, being a great person as he is, he understood my naive enthusiasm and, in an elegant way, he congratulated me by saying that, as I did most of his conference, it would then be easier for him to talk only on some particularities of the syndrome... One week later, I received a letter from him with some papers he wrote on RTT. He also encouraged me to continue...

In 2000, after the discovery of the MECP2 gene mutation as the cause of Rett syndrome, I decided to talk with Jorge Sequeiros about the possibilities of doing the genetic study of the Portuguese RTT girls. Some months later, he contacted me because he had a young PhD student in his group who became interested in that work. In March of 2001, we decided to ask for a grant to Fundação para a Ciência e Tecnologia and, in December of the same year, we got it, and this work began



1. General introduction

1.1. History of the disease

“One day in the spring of 1965, two mothers holding their children on their laps were sitting in the waiting room. Both children were swaying and the mothers held their arms. Both children, who were treated for epileptic seizures, were well known to me. That morning I passed by them repeatedly and, incidentally, the mothers let go of their children’s arms. At once, the children put their hands together and started nearly identically-looking washing movements. I asked the mothers not to stop these movements again, and was startled at the similarities. It was the same gaze, same facial expression, same weak muscles and the same stereotyped movements of their hands. Moreover, they were both females. At first, I was amazed by this enormous coincidence and thought I must have come across several other children with these symptoms. I asked my head nurse to look at these children and she immediately recalled some more names of children who behave similarly. We invited them, placed them next to each other and found that all of them showed the same kinds of movements. Further, all patients were female.”

(Andreas Rett,
unpublished letter to the Rett Syndrome Association,
June 7, 1985).

The original publication of Rett’s observations, which characterizes the neuro-developmental disorder that now bears his name, appeared in 1966 in the Austrian medical newsletter, *Wiener Medizinisch Wochenschrift* (1)

This clinical paper, like most of Andreas Rett’s publications was not widely circulated and remained unnoticed to the English-speaking world. Realizing the limitations of publishing in the German literature alone and having become convinced that this syndrome was a different entity, he prepared a film and a booklet, “A special kind of atrophy”, carrying it to pediatric meetings throughout Europe. The first English description of Rett syndrome appeared in the *Handbook of Clinical Neurology*, in 1977 (2).

In 1978, independently from Rett, Ishikawa and collaborators from Japan described, in a brief note, three girls with the same clinical symptoms and signs described by Rett (3). This publication also passed unnoticed.

In 1980, Karin Dias, a Portuguese paediatric neurologist invited Bent Hagberg to give a conference about cerebral palsy - a subject that was the main interest of this Swedish paediatric neurologist. During the coffee-break of the meeting, they walked along the gardens surrounding the conference building. Physicians go to congresses with the undiagnosed cases in their pockets, as children do with football players stickers. But,

unlike children, they are looking for repeated “stickers” and, when they find them, they describe a “new” disease. This was what happened then.

Karin (not knowing yet Rett’s chapter in the Handbook of Clinical Neurology) asked Bent Hagberg if he had ever seen a special type of girls with cerebral palsy, who had the particularity of continuous hand stereotypies. She had four cases, all similar. At that time, he answered that he had seen that clinical picture in both sexes, but as Karin insisted that only girls seemed to have such symptoms, he promised her that when he returned to Sweden he would check their record files. Some time later, he wrote her a letter confirming that he found only females with the above mentioned characteristics, and he presented the clinical data of 16 girls, in 1980, at a Child Neurology meeting, in Manchester.

Jean Aicardi, a famous French paediatric neurologist was in that meeting and he told Hagberg that he also had several girls with the same clinical findings. In November 1983, a paper was wrote, with 35 girls diagnosed as “Morbus Vesslan” (the nickname of the first Rett syndrome girl observed by Hagberg: Anne-Marie Vesslund) in Sweeden, Portugal and France.

It was a collaborator of Jean Aicardi, consulting one volume of the Handbook of Clinical Neurology (2), who noticed that the “Morbus Vessland” had already been described by Andreas Rett. Immediately, they changed the title’s name of the publication of this new entity to “Rett syndrome”.

The paper was published in a well-known neurological journal (4), and the diagnostic criteria for the syndrome were established at the second international Rett syndrome conference, in Vienna. While only three medical papers had been published, between 1961 and 1983, almost 300 appeared in the eight years after the publication in the Annals of Neurology. Rett syndrome (RTT) was recognized all over the world, seen in all ethnic groups, and known to appear with an ever widening pattern of clinical variability. Meanwhile, in the fall of 1983 a young woman called Houda Zoghbi was doing a fellowship in paediatric neurology, in Houston. She had read the first account of Rett syndrome in an American medical journal (4); within 2 weeks, she encountered two girls who had the classical symptoms of the disorder. Two years after first encountering patients with this disorder, Zoghbi sidelined her clinical career and formally trained as a researcher in Genetics in the hope of understanding the causes of diseases like Rett syndrome. In 1999, more than 30 years after the first clinical description, she discovered the gene responsible for Rett syndrome (5).

1.2. Clinical, genetic and neuropathological characteristics of Rett syndrome

1.2.1. Clinical overview

Rett syndrome (RTT) occurs worldwide, in all populations and ethnic groups, with an incidence between 1 in 10000 and 1 in 20000 female live births (6), and is considered the second most frequent cause of severe mental retardation in females (7).

The unique clinical profile of this syndrome was well delineated by Hagberg et al., in 1986 (8). RTT affects almost exclusively girls, with an apparently normal initial psychomotor development, during the first six months of life. Gestation and birth of the classic RTT infant are usually uneventful. Head circumference is normal at birth but, deceleration of head growth occurs later, approximately between 5 months and 4 years of age.

During the latter half of the first year, the first psychomotor anomalies appear, with a non-specific slowing down in development. In particular, the crawling ability is seldom achieved, and unsupported walk is usually delayed. Some patients never reach the stage of independent gait.

After this slow development stagnation occurs, later with loss (usually by the first or second year of life) of acquired fine motor, intellectual and communication abilities. Though a period of days, weeks or months, affected children usually lose interest in their surroundings, lose their ability to speak, manipulate objects and play, and repetitive movements with the hands are first noticed by their families.

Also, behaviour disturbances can occur, as unexpected screaming attacks, laughing spells and nocturnal insomnia. Through the following months of the regression phase, hand stereotypies become more frequent, and each girl develops her own personal type of movements (washing, clapping, wringing or tapping the hands). These stereotypies are considered the *signature* of this syndrome.

The regression period, with more or less apparent autistic traits, lasts for months or years. Following this phase – usually about 3 to 5 years later – most girls improve social contact and a few regain some of their original abilities, as the use of words, that had been acquired before the regression phase. Although profoundly mentally retarded, they usually have a particularly good eye contact.

At this phase, the combination of a previous personal history of developmental regression and deceleration of head growth, severe mental retardation, continuous hand

stereotypies, inability to use the hands and a particularly good eye gaze make the diagnosis of RTT obvious (8).

Bruxism, breathing dysfunction and peripheral vasomotor disturbances (9) are other characteristic signs; 85 to 90% of these patients have growth impairment. Seizures usually start during this stage, and the majority has epilepsy (9). Although considered a stationary period of the disease, neuromotor functions slowly, but steadily, decline in most patients: scoliosis, ataxia, limb wasting, dystonia, pyramidal signs, bradikinesia and rigidity may appear, after a variable time of evolution; however, 15 to 20% of these patients remain in an ambulatory stage until their middle age (7). Some RTT patients may never learn to walk, as they have a severe neurological impairment since the beginning of the disease.



Figure 1. Clinical characteristics of patients with Rett syndrome with identified MECP2 mutation

A - C : intense eye contact in three children; D: the same visual attention in an adult patient; E: abdominal distension, as a consequence of respiratory abnormalities; F - G: severe scoliosis; H: severe denutrition, in a 14 years old patient; I - K: small feet length in three adult patients; L: small hand length in an adult patient

The last stage (late motor deterioration stage) is exclusive of deteriorating girls, previous walkers, who have lost that capacity, or of those who never acquired independent gait and are older than ten years. At this stage, the frequency of hand stereotypies is usually lower, in spite of the maintenance of the same pattern of movement; patients present wasting, dystonia, and, in the older ones, Parkinsonian signs (hypomimia, bradikinesia, rigidity, tremor) are also evident.

Scoliosis is present in more than 50% of patients, and worsens with age, whereas epilepsy (present in more than 70%) and breath dysfunction usually become less disabling with disease evolution. Peripheral vasomotor disturbances, hypotrophic small and cold feet and growth retardation are the rule.

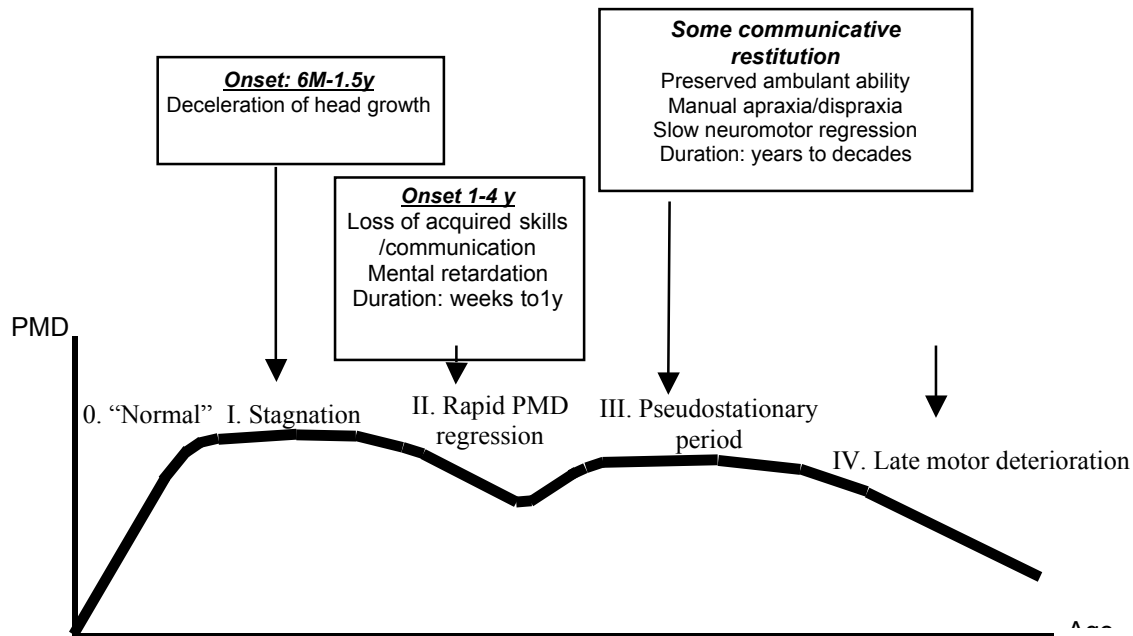


Figure 2. RTT disease profile: a regression stage followed by a recovery of interaction contrasting with slow neuromotor regression

PMD: psychomotor development

The almost disappearance of stereotypies in some patients at this stage is probably the cause of the under-diagnosis of RTT in adulthood, when the majority are wheelchair-dependent. At this stage, only a good clinical history can confirm the suspicion of this disorder. Although severely neurologically impaired, these patients can exceptionally last decades in this stage, and their age of death is quite variable. In one third of the cases, unexplained death occurs, during the first two decades of life.

On the whole, the above described symptoms and signs correspond to the diagnostic criteria, as defined by Hagberg et al., to diagnose the classic form of RTT (10), which were revised in 2002 (11) (Table 1):

Table 1. Revised diagnostic criteria for classical Rett syndrome (Hagberg et al. 2002) (11)

<i>Necessary criteria</i>
1. Apparently normal prenatal and perinatal history 2. Psychomotor development largely normal through the first 6 months or may be delayed from birth 3. Normal head circumference at birth 4. Postnatal deceleration of head growth for the majority 5. Loss of achieved purposeful hand skill between the ages of _ -2 _ years 6. Stereotypic hand movements such as hand wringing/squeezing, clapping/tapping, mouthing and washing/rubbing automatisms 7. Emerging social withdrawal, communication dysfunction, loss of learned words, and cognitive impairment 8. Impaired (dyspraxic) or failing locomotion
<i>Supportive criteria</i>
1. Awake disturbances of breathing (hyperventilation, breath-holding, forced expulsion of air or saliva, air swallowing) 2. Bruxism 3. Impaired sleep pattern from early infancy 4. Abnormal muscle tone successively associated with muscle wasting and dystonia 5. Peripheral vasomotor disturbances 6. Scoliosis/kyphosis progressing through childhood 7. Growth retardation 8. Hypotrophic small and cold feet; small, thin hands
<i>Exclusion criteria</i>
1. Organomegaly or other signs of storage disease 2. Retinopathy, optic atrophy, or cataract 3. Evidence of perinatal or postnatal brain damage 4. Existence of identifiable metabolic or other progressive neurological disorder 5. Acquired neurological disorders resulting from severe infections or head trauma

Rett variants

Some patients present with a core of symptoms of classical RTT, but show considerable variation in type and age of onset of the symptoms, severity of impairment and profile of clinical course. Based on their clinical experience, Bent Hagberg et al. delineated the clinical profile of five RTT variants (12), and also established diagnostic criteria (11) (Table 2).

- Congenital form

In this form, patients are retarded from the very first months of life. By the pseudostationary stage they have a complete clinical picture of RTT.

- Late - childhood regression

Usually, insidious psychomotor development regression occurs after the age of four years in a child with clinical features of non-specific mental retardation through late infancy to early school age.

- Infantile early onset seizure variant

Early - onset seizures are the first sign of the disease, and the most prominent one during the first year of life. After that period, patients may develop the signs of classical RTT.

- Forme fruste

Hagberg et al. (12) reserved this term for the variants with a milder, incomplete and protracted clinical course. Hand use may be partially preserved, hand stereotypies atypical or even absent, and there is only slight head growth deceleration.

- Preserved speech variant

Patients with this form, can speak some words, after the regression phase. It has been emphasised that the words or phrases pronounced are exclusively those acquired before the beginning of the disease.

- Male variant

Besides the five variant types described by Hagberg et al., RTT can also occur in males (13-20). The cases reported in the literature correspond to males with a 47, XXY karyotype (21), and in brothers of classical RTT girls with severe and lethal infantile

encephalopathy (22, 23). Exceptionally, males can present as a non-lethal encephalopathy with a clinical picture resembling RTT in females (24).



Figure 3 – Brother and sister with the same MECP2 mutation. The male (A, B) had a severe encephalopathy since birth, with major hypotonia, microcephaly and dysmorphic features. The girl had a classical form of RTT.

Table 2. Revised delineation of variant phenotypes (Hagberg B et al. 2002) (11)

<i>Inclusion criteria</i>
1. Meet at least 3 of 6 main criteria 2. Meet at least 5 of 11 supportive criteria
<i>Six main criteria</i>
1. Absence or reduction of hand skills 2. Reduction or loss of babble speech 3. Monotonous pattern of hand stereotypies 4. Reduction or loss of communication skills 5. Deceleration of head growth from first years of life 6. RTT disease profile: a regression stage followed by a recovery of interaction contrasting with slow neuromotor regression
<i>Eleven supportive criteria</i>
1. Breathing irregularities 2. Bloating/air swallowing 3. Bruxism, harsh sounding type 4. Abnormal locomotion 5. Scoliosis/kyphosis 6. Lower limb atrophy 7. Cold, purplish feet, usually growth-impaired 8. Sleep disturbances including night screaming outbursts 9. Laughing/screaming spells 10. Diminished response to pain 11. Intense eye contact/eye pointing

1.2.2. Genetics

It was speculated for many years that genetic defects in the X chromosome were involved in the pathogenesis of RTT, because almost only females were affected. It has been assumed that there is male lethality in this condition; nevertheless, a *de novo* mutation almost always occurs on the paternal X chromosome, what may explain the high preponderance of females with RTT. As most cases are sporadic, linkage studies were not possible until 1998, when a family identified with a maternal inheritance pattern of RTT permitted exclusion mapping studies to be performed, then defining the chromosome region Xq28 as the candidate one for the RTT gene (12). In 1999, Amir et al. (5) identified the first mutations in *MECP2* in 5 of 21 sporadic cases with RTT. *MECP2* was not one of the first candidate genes on chromosome Xq28 to be investigated, thus because it is expressed in all tissues and has no known brain-specific function (26).

The *MECP2* gene is thus located on the long arm of the X chromosome, at band q28, and is subject to X-chromosome inactivation (27, 28). The gene consists of four exons, which code for two different isoforms of the MeCP2 protein. Until early 2004, it was thought that only one isoform existed. For this isoform, a start codon in exon two is used, while exon 1 is noncoding, explaining why, until recently, exon 1 was not analyzed in the routine mutation screening; however, it has since been found that there is a second isoform of the protein, in which exon 1 includes coding sequences and exon 2 is eliminated by alternative splicing. The protein isoform that was first described was named MECPe2, and the newly described isoform MECPe1 (29).

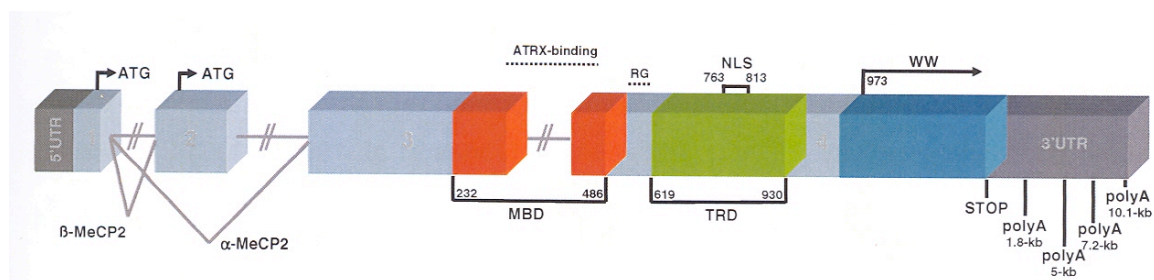


Figure 4. Schematic representation of the structure of the *MECP2* gene. MBD – methyl – CpG binding domain, TRD – transcription repression domain, NLS – nuclear localization signal, WW – group II WW domain, poly A – polyadenylation site, kb – kilobase, 3'/5' – untranslated region, ATG – start codon

The MeCP2 protein was first described in 1992 (30). It is one of five known proteins sharing a methyl-CpG binding domain, which allows them to bind to methylated CpG islands in the DNA. Through different mechanisms, binding leads to transcriptional

repression of genes in the area of these binding sites. MeCP2 dysfunction could thus disrupt the normal development program of gene silencing, but how this might result in a predominantly neurological phenotype has been a pressing question.

It is interesting that MeCP2 is more abundant in brain tissue than in most peripheral tissues, is expressed in neurons but not in glia, and is localized to cell nuclei (31, 32, 33). Even more interesting, MeCP2 levels increase in cortical neurons throughout development (34, 35). This expression pattern suggests that MeCP2 might help maintain or modulate neuronal maturity and plasticity.

Studies of MeCP2 expression in primate prefrontal cortex demonstrate increases during development, with expression expanding from the deeper cortical layers in 110 day embryos, to robust expression throughout the prefrontal cortex in adult monkeys (33). Moreover, increased MeCP2 expression appears to be associated with neuronal maturation, particular in the hippocampus, cortex and cerebellum, the brain regions primarily affected in RTT (36, 37). These combined expression data suggest that MeCP2 has specific functions in neuronal cells of the central nervous system. Indeed, Aber et al. (38) demonstrated that MeCP2 links the regulation of transcription to synaptic activity.

Recently, two papers have identified one possible MeCP2 target in this pathway in mammals (39, 40). These authors found that MeCP2 binds specifically to *BDNF* (brain derived neurotrophic factor) promoter III in rats and promoter IV in mouse, respectively, thereby repressing *BDNF* transcription in resting neuronal cells.

There are two (possibly three) domains of the protein that are essential for its function (Fig 4). The methyl CpGs binding domain is encoded by exons 3 and 4, and binds to the methylated DNA (41). The second important domain is the transcriptional repression domain, which contains binding sites for the corepressor complex, and is therefore involved in the repression process (42). The third domain is not yet well defined: it is located in the C-terminus of the protein, and is thought to facilitate the binding both to naked DNA and to the nucleosome core (43).

In RTT patients, more than 200 different *MECP2* mutations have been described until now (44). Eight *MECP2* mutation hot spots are located at CpG dinucleotides, at transitions from cytosine to thymine: they comprise 65% of all mutations (44). The other *MECP2* mutations are less frequent and some have been found only once or twice.

Most missense mutations are located in the methyl-CpG binding domain and in the last part of the repression domain, whereas the nonsense mutations are located mainly

between the methyl-CpG binding and the transcriptional repression domains, or in the first part of the transcriptional repression domain.

It was noted early that there is a region at the C-terminus that harbours a hot spot for deletions. The proximal breakpoint of these deletion, which can be found in about 10% of the patients, is located in a section with repetitive sequence elements between nucleotides 1050 and 1200 (45). Furthermore, many of the larger rearrangements have one breakpoint in this region (46). They were detected in 16% of patients with classical RTT, in whom sequencing had not revealed any mutations (46).

In the literature, the reported detection rate for *MECP2* mutations, in patients with clinically defined RTT, is between 60 and 95%, depending on the clinical parameters used to select the patients for genetic analysis (47).

Despite advances in molecular diagnosis, the relationship between *MECP2* mutations and RTT remains complex. A small proportion of RTT patients do not have any detected *MECP2* mutation; conversely, males and females with mutations in this gene do not always present with RTT features.

The clinical spectrum of phenotypes in girls with mutations in *MECP2* is wide: some patients had never been normal since birth, but others maintain language and hand use after the regression stage of the disorder; however, by the pseudostationary stage, patients accomplish the majority of the necessary criteria for RTT.

The speculation that other neurodevelopmental disorders could be caused by mutations in *MECP2* has not proved to be conclusive. Although some autism cases have been reported, large studies have ruled out *MECP2* mutations as playing a major role in the pathogenesis of autism (48, 49). The same was true for patients with an Angelman-like syndrome (50).

To this date, more than 60 male patients with mutations in the *MECP2* gene have been reported. They can be separated clinically into three groups: (1) patients with a severe epileptic encephalopathy, who have the *MECP2* mutations found in female patients with RTT; (2) patients with a phenotype that resembles female patients with RTT and have mosaic form mutations or the Klinefelter syndrome; (3) patients with a clinical picture that does not resemble RTT and have *MECP2* mutations infrequently found in female RTT patients (51, 52).

Sporadic cases of RTT are the rule, with 99.5% being single occurrences within the family (53). The new mutation almost always occurs on the paternal X chromosome, which may

explain the high preponderance of females with RTT; however, rare cases of germline mosaicism of *MECP2* (54) and of asymptomatic mothers passing on *MECP2* mutations (55) occur, accounting for familial cases. Genetic counselling should thus be offered to all families regarding recurrence risk, including prenatal testing if so desired by the families.

1.2.3. Neuropathological studies

In spite of a predictable sequence of clinical symptoms that suggests a progressive pathology, the processes characterizing degeneration associated with a progressive brain disorder have not been recognized in RTT: there is no continuing deterioration in serial clinical examinations (56), no progressive alteration in the magnetic resonance imaging (57), no evident progressive deterioration in the electroencephalogram (EEG) (58), nor progression of brain atrophy as defined by brain weight (59). Also, in the central nervous system there is no recognizable malformation, degeneration or inflammatory process, nor any consistent evidence of a cellular disorder involving the cytoskeleton, lysosomes or the myelin. The alterations appear rather to be a deficiency of the dendritic and synaptic apparatus of selected neurons, their neurotransmitters and, possibly, some cellular proteins.

In RTT, the early decrease in head growth rate is followed by a decrease in height and body weight, with extremely small feet. At autopsy, all organs (excluding the lungs and adrenal glands) weigh less than those of age-matched controls. The brain weight is reduced by 12 to 34%, being lower than the 95% confidence limits of age-matched controls (60). This is not related to age, since there is no progressive decrease in brain weight, after the age of 2.5 years (61). There is a generalized decrease of neuronal size with increased cell-packing density in the cerebral cortex, thalamus, basal ganglia, amygdala and hippocampus (62).

Other studies revealed a decline in neuronal numbers in the frontal and temporal cortex without decrease in cortical thickness, primarily involving the large pyramidal cells, more prominent in layers II and III than in deep layers, and preservation of the visual cortex (63). These changes were associated with a lack of area specialization in the orientation patterns of dendrites and axons, decreased dendritic branching (61), small neurons with increased neuronal packing (64), loss of dendrites of pyramidal cells in the frontal, motor and subicular areas (65), shortening of the apical and basilar dendritic branches in layers 3 and 5 of the frontal, motor and inferior temporal cortex (66), and “naked dendrites” without spines in pyramidal neurons, indicating a reduction in synaptic contacts from

afferent neurons (67). The Golgi studies have identified a deficiency of dendrites in frontal, motor and temporal cortex, brain regions that are associated with many of the functions deficient in RTT. Similar abnormalities characterize the medial temporal lobe structures in infantile autism (68).

There is atrophy in the frontal and temporal regions, with a reduction of up to 30% of the corpus callosum (2). Some RTT brains show a pale substantia nigra, and tyrosine hydroxylase staining has been reported to be diminished (69, 70, 71).

Jellinger observed gliosis of the basal ganglia, in his first study of RTT brains (72). Gliosis in caudate, striatum, claustrum and nucleus basalis has been observed inconsistently (73).

Certain symptoms and signs in RTT suggest a dysfunction of the autonomic nervous system: small, cold blue feet; breathing irregularities; sleep disturbances; prolonged QT intervals; altered heart rate variability; constipation and bloating. Julu (74) has documented abnormalities of breathing patterns and of vagal tone, which he relates to an immaturity of the integrative processes in the brainstem autonomic centers. These include the nucleus of the solitary tract, the nucleus ambiguus, and the bulbar reticular formation. Several chemo-architectonic studies of these regions suggest that the brainstem is immature; a study of substance P immunoreactivity revealed an infant-like pattern of staining in the spinal cord and brain stem with a consistent reduction of substance P immunoreactivity, in the nucleus of the solitary tract and the reticular formation (75). In another study, serotonin receptor binding was increased, approaching that of the normal infant, in many brain stem nuclei involved in motor control, including autonomic centers (76).

Oldfors *et al.* reported a progressive loss of neurons in the cerebellum of five RTT brains, from patients aged 5 to 20 years. They observed a gross atrophy of all lobules of the vermis, in older patients, and suggested that there was a progressive loss of Purkinje cells (77). In 3 cases, Bauman and Kemper reported a reduction of Purkinje cells and simplified principal olives, suggesting that these changes begin before birth (78).

Degeneration of the corticospinal tracts and lower than normal numbers of anterior horn cells in two RTT patients aged 20 and 30 years, were also reported. In younger cases, gliosis in the corticospinal tracts has also been observed (73). A mild distal axonal neuropathy was reported in RTT cases by Haas (79) and Jellinger (80). Muscle biopsies showed type I or II atrophy, in a minority of patients (79).

In conclusion, the neuropathology of RTT points to arrested neuronal development rather than neurodegeneration or severe malformation of nervous tissue. It should be noted, however, that the majority of these data result from small samples of post-mortem tissue and none of these changes are diagnostic or occur in every patient. Furthermore, the majority of these studies were performed before the discovery of *MECP2* mutations as the cause of RTT, and the neuropathological studies in humans were all performed well after the onset of the symptoms. It would be interesting to determine, in the existing animal models, if alterations exist prior to the onset of symptoms.

1.2.4. Phenotype-genotype correlation studies

Since 1999, after the description of mutation in the *MECP2* gene as the cause of RTT (5), several series of patients with genetic studies have been published and tentative phenotype-genotype correlations made. The results have not been conclusive, because different methodologies were applied in the various studies, concerning clinical and mutation classification, and severity scoring systems. For example, the scores of Percy and Kerr (81, 82) increase with age, but this is not so evident with the scale of Pineda, which was more based on developmental data than on the clinical features at the date of observation (83); however, the larger studies point to a positive correlation between mutation type and location with phenotype (84).

Two different genetic factors are likely to influence the phenotype in RTT: the type and location of the mutations and the X inactivation pattern. Some research has found that patients with missense *MECP2* mutations may have a milder phenotype than those with truncating mutations (83,85). Weaving et al. (86) found that age at onset of hand stereotypies was higher, whereas speech and height (but not head growth) were slightly closer to normal, in patients with missense mutations. In contrast, Nielsen et al. (87) found no difference in severity between mutation types. In the study of Amir et al. (82), breathing abnormalities were found to be more commonly associated with truncating mutations, and scoliosis with missense mutations. Hoffbuhr et al. (88) concluded that patients with missense mutations in the methyl binding domain (MBD) and mutations truncating the entire transcription repression domain (TRD) were more severely affected than those with missense and nonsense mutations in the TRD and C terminal segment. Similarly, in another study, a milder phenotype was associated with late rather than early truncating mutations (89). In a recent publication, Huppke et al. (84) considered mutations in the nuclear localization signal (NLS) as a separate category from the other truncating mutations. Overall, they found that cases with mutations leading to a partial or complete

truncation of the NLS were more severe than those with mutations downstream of the TRD.

Examining common individual mutations, several works (90-93) showed that patients with the R133C and R306C mutations have better function, overall. In general, the most severe outcomes were found in patients with the R270X and R255X mutations (92). Mutations toward the N terminus, including T158M and R168X, were also associated to a more severe phenotype (93). However, until now, no specific neurological or behavioural findings were identified in these groups of mutations, with the exception of an association of fear and anxiety in those with R133C and R306C, and mood difficulties in those with R294X (94).

1.2.5. Main objectives of the study

A - Identification of the RTT patients in Portugal

The incidence of RTT in females is 1:10000 to 1:20000 worldwide. Our goal was not to perform an epidemiological study, but to identify a high number of patients with RTT in Portugal, in order to obtain a sample large enough to study in detail the phenotypic variability of this disorder.

B - To perform a detailed clinical study of the selected patients

The goal was to observe all suspected cases and to perform:

- a general medical examination with particular attention to dysmorphisms and anthropometry;
- a neurological examination with a register of frequency and severity of neurological signs;
- a videotape register (10-15 minutes);
- to offer all patients the possibility to be assessed by an orthopaedist, physiatrist and a gastroenterologist;
- an evaluation of nutritional status and of their phospho-calcic metabolism; and
- a CSF analysis, in order to evaluate folate and neurotransmitters levels.

Our goal was also to establish a DNA bank from all cases identified, in order to obtain material for molecular studies.

C - To analyze the entire coding sequence of *MECP2* gene in all patients and families; to identify the most frequent *MECP2* mutations in the Portuguese population

We aimed to analyze the entire coding sequence (four exons that code for two different isoforms of the MECP2 protein) of the MECP2 gene, for all RTT patients and families selected for the study. (1) Initially, testing of most coding areas of MECP2, by DNA sequencing and mutation scanning, for point mutations, should detect mutations in more than 80% of the cases with classical RTT; (2) then, testing for large deletions, which detects an additional 10-16% of cases; and (3) later, to sequence mutations in exon 1, which should detect mutations in about 1% of RTT patients.

D - To be able to offer counselling and prenatal diagnosis for subsequent pregnancies of mothers of patients with an identified MECP2 mutation

Though sporadic causes of RTT are the rule, some rare cases may be recurrent. Cases of germline mosaicism of MECP2 and asymptomatic mothers passing on MECP2 mutations may be responsible for familial cases. Genetic counselling should be offered to all families regarding recurrence risks, and prenatal testing may be offered to those willing to have it.

E - To redefine more restrictive diagnostic criteria, based on the comparison of the groups of patients with and without MECP2 mutations

F - To analyze phenotype-genotype correlations in the group of patients with detected mutations

Our aim was to try quantify the phenotype severity using a severity scale, and also to identify specific neurological signs, and to correlate these findings with type and localization of the mutation. We also studied the X inactivation pattern in all the patients with an identified MECP2 mutation.

G - To clarify the clinical individuality of some clinical presentations of RD

H - To perform the study of *MECP2* in subsets of patients with clinical overlap with Rett syndrome

Although other neurodevelopmental disorders with phenotypic overlapping with RTT are rare, they provide important insight into the phenotypic consequences of mutations in *MECP2*.

We decided to study the *MECP2* also in the following groups of patients: (1) children with idiopathic autism; and (2) males with a RTT phenotype.

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2. General subjects and methods

2.1. Strategy for identification of the cases

As described above, in 1996 we made an attempt to know the prevalence of Rett syndrome (RTT) in Portugal. The goal of this study was to start a national register of the disorder, to divulge scientific information about RTT and, as a consequence, to ameliorate life quality of these patients and their families.

We then sent standardized questionnaires to all the paediatric neurologists in Portugal. The questionnaire had 50 questions with 3 options (yes, no, unknown) and 18 questions to provide an answer. At that time, we received 50 fulfilled questionnaires, from five Neuropaediatric Services: the prevalence based in these results would be very low, (1/100,000), and we concluded that the syndrome was under-diagnosed. Through the clinical information of the questionnaires, we classified 75% of these cases as *classical* forms, and 25% as RTT *variants*. Since then, I continued to collect information on other RTT patients, but only in March of 2001, a delineation of this study was made.

In order to identify the maximum number of RTT cases in Portugal, I used a strategy of information through conferences supported by videos of typical and atypical forms of RTT. I presented these conferences in all the Paediatric services of the north of Portugal and at the annual meetings of the national societies of Paediatrics and Neurology. I also contacted some families of RTT patients, who wanted to create a Portuguese RTT association. In 2002, this association was formally constituted under the name of ANPAR and, since then, I maintained a close contact with them. The president of ANPAR knows the structure of the multidisciplinary consultation of RTT that I co-ordinate in Hospital Geral de Santo António and all the ongoing research projects, and contacts their members in order to offer them the possibility to have access to a specialized centre and to cooperate with our research. I also sent (by regular mail) to all the Paediatric services in the north of Portugal a published revision article, which included the description of the syndrome and the inclusion and exclusion clinical criteria for typical and atypical forms of RTT.

For 6 years (2001-2007), I contacted periodically, by phone or mail, all the paediatric neurologists in Portugal, proposing to observe their new cases identified and re-observe some previously diagnosed.

There are striking similarities between autism and Rett syndrome, especially during its regressive stage. The timing of disease onset is similar, both entities manifest abnormal social and communication behaviour and movement disorders.

Thus, we also studied *MECP2* gene mutations in 100 idiopathic autistic children, selected and studied by Dr Guiomar Oliveira. The expected results were to find, among these, 10 patients with a *MECP2* mutation.

As referred in the introduction, *MECP2*-related disorders in males cover a wide range of severity, but may be divided into three main categories. (1) Males with mosaicism or Klinefelter syndrome have a similar clinical course and *MECP2* mutations as classical RTT females. (2) The second group of males, with the same mutations as RTT females, manifests a severe progressive encephalopathy with epilepsy, and die by age 3 years. (3) Males with different *MECP2* mutations may have less severe mental retardation, or psychiatric or neurologic problems.

Our initial goal was to study 25 male patients with an epileptogenic encephalopathy of unknown origin, with beginning in the first year of life, or progressive encephalopathy of unknown origin, and a clinical profile that shared some neurological signs with RTT.

2. 2. Clinical study of the patients

The clinical study was based on all the clinical information collected between 1996 and 2007. The only criterion of selection of the patients to observe was their signaling from paediatric neurologists, neurologists or paediatricians.

Informed consent was obtained from all parents to collect blood, as well as to take and use the video and photographic material.

The majority of the suspected RTT patients, identified by other paediatric neurologists, were observed by me, at their services (Lisbon, Coimbra and Porto). Some suspected patients, observed by paediatricians and patients from the Azores, were signalled by phone and were later observed at Hospital Geral de Santo António. Two families of adult RTT patients contacted me directly by phone, and were observed and sampled at home.

The inclusion of the patients in the study was based on their fulfilment of the revised clinical criteria for RTT (Table 1 and 2).

All patients were thus observed and videotaped by me, one or more times, and a clinical checklist for RTT was filled (see attachments). The anamnesis was collected by me, and only at the end of my observation, the file of the patient was consulted, when available, in order to complete the information, to obtain information about previous neurological examinations, treatments and to obtain laboratory data.

2. 2.1. Clinical database

Each patient was registered in a protocol clinical checklist for RTT and an Access database was filled. The register consisted in the patient's identification, date of observation, obstetrical and perinatal data, and family history of the proband, with particular attention to mental retardation, autism, epilepsy, psychomotor development milestones, age of stagnation and/or regression of psychomotor development, onset of specific signs and symptoms of RTT, at the different stages of the disease. Patients who had follow-up visits, had several registers of general and neurological examination for each observation in the database. For statistical analysis, however, only one of these was selected (at or above 5 years of age). The database had several fields where the data collected from the patient files were noted: the particularities of each patient and some subjective annotations were also introduced.

A video-database was also organized with segments of the films of the RTT patients. This material allowed the possibility of reviewing the clinical features several times, to compare patients and to observe their clinical evolution.

2. 2. 2. Scoring and scales

Cases were scored, using the Pineda general severity scale (2) (see attachments).

A motor-behavioral assessment scale was used to rate the different characteristics on a 0 to 4 scale (0= normal or never, 1= mild or rare, 2= moderate or occasional, 3= marked or frequent, and 4= very severe or constant).

Dystonia was classified according to distribution (focal, segmental, multifocal and generalized).

A classification of stereotypies modified from Jankovick (3) and Fernandez-Alvarez & Aicardi (4), was used (see attachments).

2. 2. 3. Evaluation by other medical specialities

Patients with a follow-up at Hospital Geral de Santo António (n=30) were also observed by other medical specialities: orthopaedics, physiatry and gastroenterology. Physical examination of the patients in the paediatric gastroenterology consultation was performed with special attention to signs of malnutrition and digestive disturbances. Body mass index (BMI) was determined in all cases.

Laboratory analyses were also performed, including haemogram, iron and lipid profiles, liver aminotransferases, total serum proteins and albumin, renal function, sodium, potassium, chloride, calcium and phosphorus, as well as vitamins D, B12 and folate.

A therapeutic plan was established and a follow-up in the consultation was offered to the patients with identified gastrointestinal and/or nutritional disturbances.

2.3. Genetic study

Blood samples from patients and their parents were received at the laboratory and genomic DNA was extracted using the Puregene DNA isolation kit (Gentra, Minneapolis, MN). The coding region and exon-intron boundaries of the *MECP2* gene were amplified by PCR, using one pair of primers, for exons 1, 2 and 3, and 3 pairs of primers, for exon 4. After PCR amplification, the different fragments were sequenced, using the same primers, and analysed using an automated sequencer. The sequence changes were confirmed by re-amplification of genomic DNA and by sequencing in the opposite direction. The RD-PCR method (5) (see attachments: [Article 14](#)). was used for the detection of large rearrangements in the *MECP2* gene. Primers used for amplification, as well as the PCR conditions used, are available upon request.

2.4. Other blood analyses

In peripheral blood, we analyzed haemoglobin, erythrocyte indices, folate and vitamin B12. Results of biochemical studies in CSF and plasma were compared to the reference ranges, obtained in controls of similar age.

2.5. Study of LCR

Lumbar punctures were always performed by me. Conscious 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 (6), and were immediately frozen at -70°C, until the moment of the analysis. Haematic samples were centrifuged immediately, and the clear CSF supernatant was stored at -70°C. Later on, all samples were transported in dry ice to the Department of Clinical Chemistry of Hospital Sant Joan de Deu, Barcelona, where the analyses followed a previously reported methodology (7). In the CSF, we analyzed 5MTHF, neopterin, biopterin, 3-O-methyldopa, homovanillic acid (HVA), 3-methoxy-4-hydroxy phenylglycol (MHPG), 5-hydroxytryptophan, 5-hydroxy-indoleacetic acid (5-HIAA), HVA/5-HIAA and HVA/MHPG.

2.6. Statistical analysis

Contingency tables were obtained and a Chi-square and the Fisher's exact test used. Groups were compared by Student's *t* test. The SPSS (v.14) statistical package was used to analyse the data. Further details are provided in each chapter/article.

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3. Results

3.1. Redefining phenotypes and genotypic correlations

The results described in this chapter are included in the following manuscripts:

Article 1 - Rett Syndrome and Rett disorder: an attempt to redefine phenotypes, 2007 (submitted)

Article 2 - Nutritional and gastrointestinal dysfunction in Rett disorder: importance of early intervention, 2007 (submitted)

Article 3 - Mutations in the MECP2 are not a major cause of Rett-like phenotype in males, 2007 (submitted)

3.1.1. Introduction

The diagnosis of Rett syndrome (RTT) is based on a set of clinical criteria, irrespective of the mutation status. Since 1999, after the description of mutations in the *MECP2* gene, we can distinguish Rett disorder (RD), a disease of known aetiology from Rett syndrome (RTT), a constellation of clinical signs, which we speculate may include, in addition to RD, different diseases currently under the large umbrella of RTT.

Several studies examined previously the genotype-phenotype correlation in patients with Rett syndrome and detected mutations; however, some of them were performed based on different clinical criteria and the observations of various neurologists, geneticists or paediatricians.

On the other side, some studies preceded the identification of the *MECP2* as the gene mutated in most RTT cases, while others continue to analyze phenotypic characteristics in collections containing both patients with known and unknown mutation status (RD and RTT).

The aims of this study were: (1) to define the clinical differences existing between patients with RD (when there is a known *MECP2* mutation) and RTT (when aetiology is unknown), in order to achieve a better definition of both conditions, based on the clinical observation always by the same paediatric neurologist; (2) to characterize the phenotypes associated with the more common *MECP2* mutations; (3) to study in detail gastrointestinal dysfunction and nutritional status in a selected group of patients with RD; and (4) to analyze the clinical characteristics of a group of RTT males.

3.1.2. Rett syndrome and Rett disorder: an attempt to redefine phenotypes

(Article1)

Rett syndrome and Rett disorder: an attempt to redefine phenotypes

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Abstract

The diagnosis of Rett syndrome (RTT) is based on a set of clinical criteria, irrespective of mutation status. The aims of this study were (1) to define the clinical differences existing between patients with Rett disorder (RD), when there is a known *MECP2* mutation, and Rett syndrome (RTT), when aetiology is unknown, and (2) to characterize the phenotypes associated with the more common *MECP2* mutations.

We analyzed 88 patients fulfilling the clinical criteria for RTT. All were observed and videotaped by the same paediatric neurologist. Seven common mutations were considered separately, and associated clinical features analysed.

Comparing RD and RTT, we found differences concerning psychomotor development prior to onset, acquisition of propositive manipulation and language, and evolving autistic traits. Based on age at observation, we found differences in eye pointing, microcephaly, growth, number of stereotypies, rigidity, ataxia and ataxic-rigid gait, and severity score. Patients with truncating differed from those with missense mutations regarding acquisition of propositive words and independent gait, before the beginning of the disease, and microcephaly, growth, foot length, dystonia and severity score, at the time of observation. Patients with the R168X mutation had a more severe phenotype, whereas those with R133C showed a less severe one. Patients with R294X had a hyperactive behaviour, and those with T158M seemed to be particularly ataxic and rigid.

A clear regressive period (with loss of prehension and language, deceleration of growth) and the presence of more than three different stereotypies, rigidity and ataxic-rigid gait seemed to be very helpful in differentiating RD from RTT.

Introduction

Rett syndrome (RTT) was first described by Andreas Rett, in 1966 (1), and rediscovered in 1984, by Bent Hagberg et al. (2). Since 1999, after the description of mutations in the *MECP2* gene as the cause of RTT (3), we know that more than 90% of the patients with classical, but only 30% with variant RTT have a *MECP2* mutation. Thus, we can consider a Rett disorder (RD), a disorder of known aetiology, and a Rett syndrome (RTT), a constellation of clinical signs, and speculate that maybe, in addition to RD, different diseases are currently under the large RTT umbrella. Widespread availability of the molecular diagnosis creates a great opportunity for this to be clarified, based on clinical and genetic grounds.

Several series of patients with genetic studies have been published, and attempts of phenotype-genotype correlations made. The results, however, are not conclusive, as different methodologies were applied in the various studies, concerning clinical and mutation classifications and severity score systems (4, 5, 6). Nevertheless, the larger studies pointed to a broad correlation between type of mutation and phenotype, patients with truncating mutations having a more severe phenotype, than those with missense mutations (5-10).

Two different genetic factors are likely to influence the phenotype in RTT: the type and location of the mutations, and the X inactivation pattern (11). Concerning the phenotype of specific mutations, several studies (12-15) showed that patients with the R133C and R306C mutations have better overall function. In general, the most severe outcomes were found with the R270X and R255X mutations (14). Mutations toward the N terminus, including T158M and R168X, were also associated with a more severe phenotype (15); however, no specific neurological or behavioural findings were identified in patients with these mutations, with the exception of an association of fear and anxiety with R133C and R306C (16), and mood abnormalities in patients with R294X (16).

The aims of this study were (1) to define the clinical differences between patients with (RD) and without (RTT) known mutations in the *MECP2* gene, in order to delineate the clinical boundaries of the disease, and to redefine criteria for RD diagnosis; (2) to analyze the genotype-phenotype correlation in patients with RD and characterize phenotypes associated with the more common *MECP2* mutations; and (3) to identify sub-groups of patients with RTT that might be candidates for mutational search in other genes.

Subjects and methods

All Portuguese paediatric neurologists were asked to indicate their patients with possible RTT. Patients included in the study (n=88) were observed and videotaped by the same paediatric neurologist (TT), and a clinical checklist for RTT was completed. Informed consent was obtained from all parents, for blood sampling and to take and use videos and photographs; 36 patients were observed and videotaped at least twice, at 6 months intervals.

Blood samples from patients and their parents were received at the laboratory, and genomic DNA was extracted 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. The RD-PCR method was used, as described (17), for the detection of large rearrangements in the *MECP2* gene. Primers and PCR conditions are available upon request.

X-chromosome inactivation (XCI) assays were performed with genomic DNA isolated from leukocytes of peripheral blood. The assay was based on a previously described method (18), which allows the determination of the X-inactivation status, using a trinucleotide repeat polymorphism in the androgen receptor gene (*AR*, RefSeq ID: NM_000044.2), flanked by two methylation-sensitive restriction enzyme sites. The restriction enzyme (*HhaI*) will hydrolyze only the unmethylated alleles. Scoring of the XCI pattern was made by densitometry of the amplified DNA bands.

MECP2 mutations were categorized and grouped for analysis according to: (1) type of sequence change - missense or truncating (including nonsense and frameshift, but also large deletions) and (2) location of the mutation (affected domain) - TRD, MBD. The seven most common mutations were considered individually, and their associated features analysed.

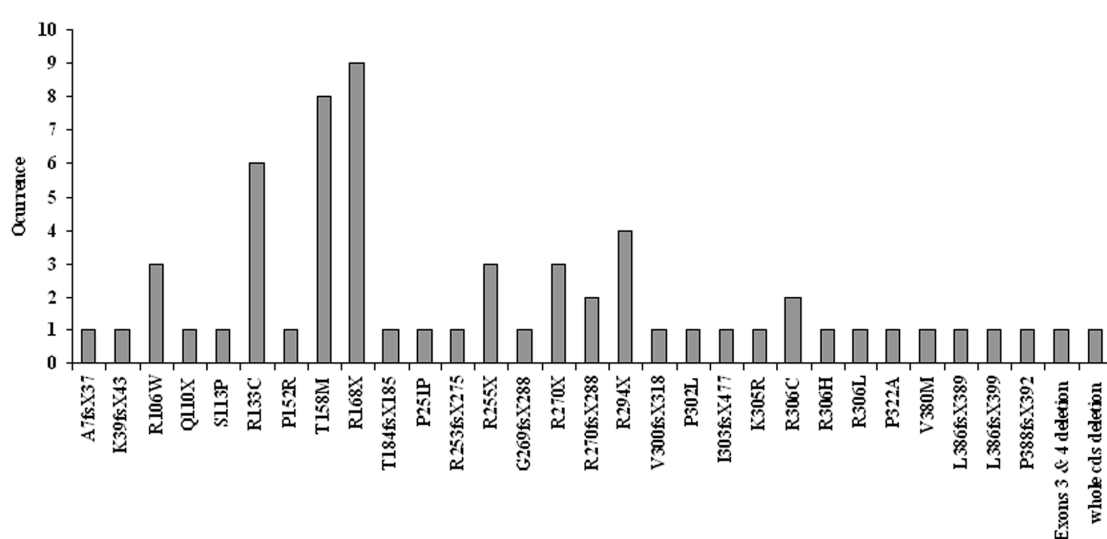
For statistical analysis, patients were divided into two groups: those with (RD) and without a molecular diagnosis (RTT). Within the mutation-positive group, comparisons were made between patients carrying missense and truncating mutations. Cases were scored using the Pineda severity scale (6). The SPSS (v.14) statistical package was used to analyse the data. Proportions were compared using Chi-square and Fisher's exact test. Quantitative variables were compared using the Mann-Whitney test and were presented as median (25th percentile - 75th percentile).

Results

In this study we analyzed the entire coding sequence of the *MECP2* gene in 88 patients, who fulfilled the revised clinical criteria for RTT (19). Cases were classified as classical (58 %) and variant (42 %) forms (congenital, preserved speech, *forme fruste*, early epileptic encephalopathy and male patients) (20).

Clinical differences between RD and RTT patients

Mutations in *MECP2* were found in 68.2% (n=60) of all patients, corresponding to 96.2% of classical forms and of 29.7% variants (seven congenital forms; three preserved speech and one male). Two of the patients were siblings (one female and one male). We identified a total of 29 different mutations in *MECP2* including two large deletions and a mutation in exon 1; most mutations were located in exons 3 and 4 (figure 1). Nine of these mutations were never described before: A7fsX37, K39fsX43, Q110X, S113P, T184fsX185, R253fsX275, I303fsX399, V380M and L386fsX399. All were *de novo* mutations, except V380M which was present in the unaffected mother (who had a balanced XCI pattern) and in 4 X chromosomes in a control population of Portuguese origin including 230 X chromosomes. We assumed the truncating mutations as causative of the disease; the new missense mutation S113P was excluded in the parents and in a control population of Portuguese origin including 230 X chromosomes.



Legend of figure 1 – *MECP2* mutations found in 60 patients with Rett Disorder

XCI patterns were analysed for all patients carrying a *MECP2* mutation, though data was not informative for 14; among the 46 patients remaining, only two showed a skewed XCI pattern.

Median age at first observation and median duration of the disease were not significantly different between RD and RTT, neither between groups with missense and truncating mutations. A significant difference was found when comparing clinical stage: more patients with RD, especially those with truncating mutations, were in stage IV of the disease, which may mean that their rate of evolution was faster.

Comparison between RD and RTT showed some important clinical differences since birth (Table 1). Dystocic delivery (caesarean or forceps) seemed to be more common in RD than RTT patients, though this did not reach significance. Additionally, none of the RTT patients presented microcephaly at birth, but 6 (12.2%) of group I had an occipitofrontal circumference (OFC) below the 5th percentile (3 truncating, 3 missense mutations). Also, 63.6% of RTT patients presented an OFC at birth above the 25th percentile, against 26.6% of RD patients. Stagnation of OFC growth occurred in 71.7% of RD and 53.6% of RTT, at a median age of 6 months in both groups. Later, microcephaly was acquired in 46.7% of RD (67.6% among truncating, 19.2% for missense mutations) and 39.3% of RTT patients. Interestingly, 13.3% of group I patients presented an OFC above the 25th percentile (3% above the 75th percentile); 26.9% of patients with missense mutations presented an OFC above the 25th percentile, against only 2.9% of those with truncating mutations. Differences were also found for height and weight, comparing the two groups, and for head size, height, weight and foot length, comparing the group of patients with missense versus truncating mutations (Table 1).

Clinical classification in these groups was also different: 60.7% of RD were classified as classical, against only 11.6% of RTT patients. Significant differences were found when comparing psychomotor development before the onset of the disease: in a higher percentage of RTT patients, this was not considered to be normal, by the parents, before the age of 12 months; a clear regression period was evident in 85% of RD, but only 25.0% of RTT patients; acquisition of propositive words was more common in patients with RD, specially those with missense mutations; evolving of autistic traits (loss of social communication) was described in 81.7% of RD and 50% of RTT, and it occurred earlier in life in RTT; in 42.9% of RTT, propositive manipulation was never acquired (against 8.3% in RD); independent gait was acquired by 80.8% of patients with missense, but only in 47.1% of those with truncating mutations (Table 1).

Table 1. Clinical data of RD and RTT groups of patients

	Group I (n = 60)	Group II (n = 28)	p	Missense (n = 26)	Truncating (n = 34)	p
Median age (years)*	7.0 (5.0-13.5)	7.5 (5.0-12.0)	0.857	7.0 (4.7-10.5)	10.5 (5.0-14.3)	0.185
Median duration of disease (months)*	92 (55-140)	82 (35-139)	0.354	72 (26-111)	122 (40-156)	0.325
Stage (%)						
Stage II	10.0	3.6	0.156	7.7	11.8	0.010
Stage III	60.0	82.1		80.8	44.1	
Stage IV A and IV B	30.0	14.3		11.5	44.1	
Perinatal data (%)						
Abnormal delivery	30.0	21.4	0.452	23.1	35.3	0.398
Microcephaly at birth						
Yes	10.0	0.0	0.261	11.5	8.8	0.999
Missing information	16.7	21.4		15.4	17.6	
Reported psychomotor development (%)						
Normal until 12 months of age	58.3	17.9	<0.001	69.2	50.0	0.188
Stagnation	91.7	92.9	0.999	92.3	91.2	0.999
Median age of stagnation (months)*	12.0 (10.0-18.0)	6.0 (4.0-11.3)	<0.001	16.0 (10.0-18.0)	12.0 (9.0-18.0)	0.172
Regression	85.0	25.0	<0.001	84.6	85.3	0.999
Median age of regression (months)*	18.00 (14.0-24.0)	14.00 (11.0-24.0)	0.202	18.0 (18.0-24.5)	18.0 (13.0-23.0)	0.160
Acquisition of autistic traits	81.7	50.0	0.003	76.9	85.3	0.507
Median age of acquisition of autistic traits	18.0 (12.5-18.0)	7.5 (3.75-18.0)	0.005	18.0 (14.0-21.0)	15.0 (12.0-18.0)	0.175
Propositive manipulation	91.5	57.1	<0.001	92.3	90.0	0.999
Median age of acquisition of stereotypies	20.0 (14.5-25.0)	24.0 (14.5-36.0)	0.425	24.0 (18.0-28.5)	18.00 (13.3-24.0)	0.086
Propositive words	70.0	17.9	<0.001	88.5	55.9	0.015
Acquisition of independent gait	63.3	50.00	0.341	84.6	47.10	0.007
Data at observation						
Eye pointing	95.0	57.1	<0.001	92.3	97.1	0.574
Microcephaly at time of observation	46.7	39.3	0.646	19.2	67.6	<0.001
Low weight (< 5 th percentile)	43.3	18.5	0.031	23.1	58.8	0.008
Low height (< 5 th percentile)	55.9	30.8	0.046	38.5	69.7	0.012
Low foot length (< 3 th percentile)	67.9	45.8	0.112	47.8	83.3	0.014
Agitation	56.7	67.9	0.444	50.0	61.8	0.517
Laughing spells	68.3	53.6	0.180	61.5	73.5	0.322
Abnormal sleep pattern	56.7	53.6	0.785	46.2	64.7	0.240
Hyperpnea/Apnea	76.7	82.1	0.762	69.2	82.4	0.377
Epilepsy	56.7	75.0	0.155	46.2	64.7	0.193
controlled	57.6	76.2	0.270	66.7	52.4	0.665
Vaso-motor disturbances	70.0	78.6	0.452	53.8	82.4	0.024
Neurogenic muscle atrophy	48.3	28.6	0.106	42.3	52.9	0.446
Chewing difficulties	76.7	71.4	0.792	65.4	85.3	0.134
Pyramidal signs	28.3	39.3	0.333	23.1	32.4	0.566
Ataxia	35.0	10.7	0.034	46.2	26.5	0.190
Number of stereotypies per patient*	4.00 (3.0-6.0)	3.00 (2.2-4.0)	0.050	3.50 (2.8-7.0)	4.00 (3.0-6.0)	0.999
Dystonia	63.3	75.0	0.336	46.2	76.5	0.029
Rigidity	48.3	7.1	<0.001	34.6	58.8	0.074
Tremor	48.3	35.7	0.358	50.0	47.1	0.999
Ataxic/rigid gait	43.6	0.0	0.001	36.8	50.0	0.523
Scoliosis	71.7	67.9	0.803	69.2	73.5	0.777
Gastro – intestinal (%)						
Gastro-esophageal reflux	13.3	14.3	0.904	11.5	14.7	0.999
Pineda's scale*	13 (9-15)	14 (12-16)	0.048	11 (8-13)	14 (11-16)	0.001

*Data presented as median (25th percentile- 75th percentile)

Besides growth failure, we found other important differences concerning clinical data of the patients at observation: eye pointing was present in 95% of RD, but only in 57% of RTT patients; rigidity and ataxia were more frequent in RD, and an ataxic-rigid gait was observed only in them. They also displayed a greater number of stereotypies. Dystonia and oral motor difficulties were more frequent in patients with truncating mutations (Table1). We found no significant differences between the two groups concerning pyramidal signs, dystonia, neurogenic muscle atrophy, vasomotor disturbances, scoliosis, epilepsy or gastro-intestinal disturbances. Abnormal sleep pattern, agitation, laughing spells and hyperpnea/apnea were also not more frequent in patients with known mutations.

The Pineda's severity score showed that the median severity was greater in the RD group and, within this group, in those with truncating mutations (Table1).

Clinical presentation among RD patients

In general, we identified three neurological presentations in RD patients: (1) Some had a *severe form* (n=28; 46.6 % of all RD patients), with major axial hypotonia, in which dystonia and rigidity were present after few years of evolution (median, 11 years). These patients either regressed earlier (median, 15.0; range, 6-36 months), or never had been normal (18.5%); 75.0% did not acquire independent gait; those who did, lost it at a median age of 66 months (range, 24-120). Epilepsy was present in 67.8% of the cases. Among patients with the severe form, 82.1% had truncating mutations, and all but one with the R168X mutation presented it (video 1); they showed a median severity score of 16 (range, 12-20).

(2) In an *intermediate form*, ataxia predominated (n=17; 28.3% of all RD patients). Most acquired an independent gait (94.1%), but this was both ataxic and rigid; they regressed at a median age of 18 months (range, 13-16), and 52.9% had epilepsy. Their median severity score was 11 (range, 8-14); 68.8% of the patients with this presentation had missense mutations, particularly T158M (video 2).

(3) A *milder form* was present in 15 (25%) of RD patients, with few neurological signs except for mental retardation and autistic features. All acquired independent gait and maintained it during the period of follow-up; 40% developed focal dystonia after some years of evolution, and gait became rigid in two patients (one with the R133C mutation, older than 20 years; one six years old with the R294X mutation). Epilepsy was present in

33.3% of these patients. They regressed at a median age of 25 months (range, 13-60), and had a median severity score of 9 (range, 4-11); 60% had missense mutations. Most of our patients with the R133C mutation illustrate this presentation well. Three patients with R294X mutation also presented this phenotype (video 3).

Interestingly, the distribution of mutation types was significantly different between these clinical groups. In the milder group, 52.3% of the patients had missense mutations. In the intermediate group, 75% of the patients had missense mutations; and in the more severe group 81.5% of the cases had a truncating mutation. The frequency of missense *versus* truncating mutations was significantly different between the three clinical subtypes (Fisher's exact test, $p=0.001$) (figure 2).

The missense mutations present in patients with the milder form of the disease were all described to have a milder effect, if any effect at all. The majority of truncating mutations in the less severe group did not affect the NLS (29.4%). Additionally, contributing to this group is the R270X mutation that affects only the last aminoacid of the NLS, and so it may not impair its function or have a milder effect. The only exceptions to this pattern were the R106W and R255X mutations, given their predictably severe effect; surprisingly, our study of the XCI patterns of the patients with these mutations did not reveal a significant bias of inactivation, at least in lymphocytes.

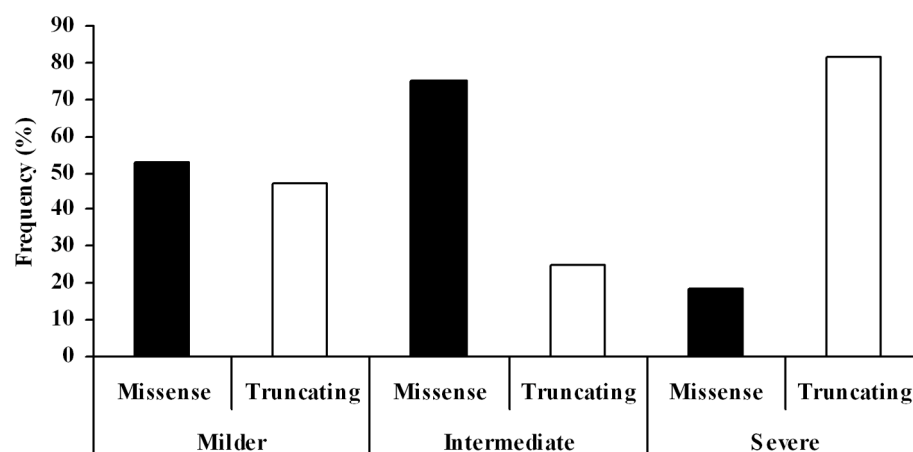
Interestingly, the T158M mutation was predominant in the intermediate group, which could suggest some specificity of the effect of this mutation upon MeCP2 function. In this group, all truncating mutations disrupted the NLS.

In the more severe group, the majority of truncating mutations affected the NLS (66.7%), the R168X mutation being predominant. The exceptions were two patients with mutations P152R and R294X, and three patients with very late truncating mutations - L386fsX389, L386fsX399 and P388fsX392, none of which presented an unbalanced pattern of XCI, although these mutations would be predicted to have milder effects.

Table 2. Clinical characteristics of patients with the seven most frequent mutations

	R168X n=9	T158M n=8	R133C n=6	R294X n=4	R270X n=3	R106W n=3	R255X n=3
Median age (years) [†] (<i>range</i>)	11 (2-23)	6 (1-16)	7 (5-31)	6.5 (5-11)	7 (2-24)	7 (3-7)	15 (2-21)
Median age of regression (months) [†] (<i>range</i>)	12 (6-23)	18 (14-27)	34 (18-60)	33 (18-36)	14 (13-21)	18 (all 18)	15 (13-28)
Clinical classification	8 classical 1 co-natal	8 classical	3 classical	4 classical	3 classical	3 classical	3 classical
Microcephaly (%)	66.6	25	0.0	50	66.6	66.6	33.3
Low weight (%)	77.7	37.5	16.6	25	33.3	0.0	66.6
Low height (%)	66.6	50	50	25	66.6	33.3	66.6
Acquisition of independent gait (%)	22.2 *	87.5*	100*	100*	66.6	33.3	33.3
Independent ataxic-rigid gait (n)	1/2	5/7	1/5	2/4	0/2	0/1	1/1
Ability to speak (%)	0.0	0.0	50	0.0	0.0	0.0	0.0
Propositive prehension (%)	11	50	83.3	75	33.3	33.3	33.3
N° of stereotypies [†]	3 (2-8)	4.50 (2-11)	3 (2-10)	9.50 (5-12)	3 (9-3)	3 (3-4)	3 (3-4)
Motor agitation (%)	0.0	37.5	50	100**	66.6	66.6	66.6
HPP/Apnoea (%)	88.8	75	66.6	100	66.6	66.6	66.6
Rigidity (%)	66.6	62.5	16.6	100	33.3	33.3	33.3
Epilepsy (%)	66.6	37.5	16.6	100	66.6	66.6	33.3
Scoliosis or kyphosis (%)	77.7	62.5	66.6	50	66.6	66.6	66.6
Score in Pineda's scale [†]	16 (9-19)	11 (7-14)	7.5 (4-11)	10 (8-13)	11 (10-16)	13 (8-15)	12 (11-14)

*One patient lost independent gait, 1 year after acquisition; **very agitated; [†]data presented as median (25th percentile-75th percentile); PS - preserved speech



Legend of figure 2- Frequency of MECP2 mutation type by RD clinical subtypes

Clinical presentation associated with specific MECP2 mutations

The clinical features associated with specific mutation groups found in RD patients are presented in Table 2. Overall, we observed that those with the *R168X mutation* presented the most severe phenotype. Only two of nine patients with this mutation acquired independent gait; one lost it one year later, while the other had a very rigid and ataxic gait. Two presented microcephaly at birth and six developed it within the 2nd year of life. The majority had low height and weight, and epilepsy. They also had continuous hands-apart stereotypies.

RD patients with the *R294X mutation* seemed to have a peculiar phenotype with an extremely unusual hyperactive motor behaviour. They had a lot of different and exuberant hand stereotypies and with other topographies (including vocal), and all had epilepsy. All displayed focal or segmental dystonia, and, in three, gait became rigid and broad-based, in the earlier stages of the disease; however, they maintained a good eye contact, and seemed less profoundly retarded than other patients with better motor function.

Patients with the *R133C mutation* had the mildest phenotype among all those within the RD group. None had microcephaly, and all but one acquired and maintained independent gait. Three could make phrases, albeit with a peculiar voice tone, and answered to simple questions, after a considerable delay of time. Also, only one had epilepsy (the same patient who lost independent gait), and five maintained propositive prehension (three fed themselves with a spoon).

Some particular clinical features were also found in patients with the *T158M mutation*; the majority could walk without support, but their gait tended to be very rigid and ataxic, since

the beginning of the disease; postural and intention tremor was evident, and they had many different stereotypies.

Discussion

1. Clinical features: redefining RD and RTT

The diagnosis of Rett syndrome remains clinical, and this may lead clinicians to decide whether or not, to request a laborious *MECP2* mutation screening. Both from a scientific and economic point of view, it is very important to define more restrictive criteria, to improve the rate of finding a *MECP2* mutation, and to identify groups of patients that may have a different disease.

Comparing patients *with* and *without* a *MECP2* mutation (RD and RTT), we concluded that there are significant clinical differences between them. Among RTT patients, only a minority had a normal development during the first year of life, and a regressive period was not evident to their parents; also, autistic behaviour, when noticed, occurred very early in life, which means that these children may never have been normal.

Propositive manipulation was acquired in the vast majority of RD, but less frequently in RTT patients. On the whole, we observed that patients with no detected *MECP2* mutation had worse social contact and less stereotypies. Our results suggest that eye pointing seems to be an important sign to suspect for RD. Rigidity, and specially the presence of an ataxic-rigid gait, seems to be very specific of RD: none of the patients without a *MECP2* mutation presented it. Growth failure was also significantly more evident in the group with detected mutations.

2. Genotype-phenotype correlations in RD

At a comparable age of observation, patients with truncating mutations had a significantly higher probability of displaying microcephaly, as well as low weight, low height, small foot length and dystonia. Rigidity and oral motor difficulties, albeit not significant, were also more frequent in the group of patients with truncating mutations. Overall, the mean severity score of the patients with truncating mutations was higher, and these patients were more likely to display a severest than a milder or intermediate phenotype.

Interestingly, the distribution of mutation types was similar between the milder and intermediate subgroups, either using this classification of more detailed mutation classification schemes (data not shown).

Regarding the most common *MECP2* mutations in our series, patients with the R294X mutation seemed to have a milder disease, but an extremely unusual hyperactive motor behaviour. They had many different stereotypies, and all had epilepsy. Robertson et al. (16) described also that patients with this mutation were more likely to have mood difficulties, but they did not report hyperactive motor behaviour; they also reported that hand stereotypies were less frequent in these patients, which is in contradiction with our data.

Ambulant RD patients may have a normal gait for decades, mainly those with less severe mutations, as is the case of R133C; however, the majority of ambulant patients have a particular wide-based and rigid gait, unique of this disease. Bradykinesia and rigidity appear at different times of evolution, some patients presenting these neurological signs very early in life. As we showed in previous work, stereotypies other than those with the hands tend to disappear, and even hand stereotypies slowed down with age, which may reflect the neurochemical evolution of this complex disease (21).

In RD patients, motor disability was not necessarily correlated with severity of the behavioural phenotype or mental impairment. In our sample, some girls who never acquired independent gait had a good social contact and seemed to be calmer than others with very good motor function.

3. RD: explaining the symptom clusters

It is not clear why different *MECP2* mutations can produce a particular phenotype, but one hypothesis is that some CNS areas are more susceptible than others to the degree of dysfunction of this protein.

The different timing of disease onset among patients with RD and the different neurological pictures - previously recognized by others, but with a different classification (22) - may be partially explained by the preferential function of *MECP2* in mature, rather than immature neurons (23). One could speculate that a less functional protein may affect more dramatically the brainstem and cerebellar structures, provoking, for instance, such a severe hypotonia that patients can never acquire an independent gait. On the other hand, a more functional (or less disruptive) protein may not affect so severely the cerebellar

structures, but absence of its full functionality may still be critical for the cortical function and, thus, provoke a milder phenotype where mental retardation predominates. In the human brain, although the number of *MECP2*-expressing neurons increases dramatically throughout gestation, the percentage of neurons expressing *MECP2* in the cortex continues to increase from birth to age 10 years. This may also contribute to explain the progressive features of this disorder.

4. Final remarks

Some important questions remain, nevertheless: why are some classical forms of RTT not associated with *MECP2* mutations? How do different gene mutations produce a similar phenotype? In our series, for example, we identified a clinically homogeneous group patients (n=7) who had never been normal since birth, nor had a clear regressive period, but who fulfilled the major criteria for the diagnosis of Rett syndrome by age five years; however, their hand stereotypies were considerably less frequent and tended to disappear with age, and they had less non-manual stereotypies; eye contact was not very good, and they did not present severe growth impairment. None had an ataxic-rigid gait. This particular group of patients may be good candidates for the search of mutations in other genes.

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Video segment 1 – illustration of the severest form of RD: this 12 years old patient has a R168X mutation. She never acquired an independent seating position. She has a severe scoliosis, segmental dystonia of lower limbs and mild to moderate rigidity.

Video segment 2 – illustration of the intermediate form of RD: The patient

Video segment 3 - illustration of the intermediate form of RD

3.1.3. Nutritional and gastrointestinal dysfunction in Rett disorder- importance of an early evaluation and intervention (Article 2)

Nutritional and gastrointestinal dysfunction in Rett disorder: importance of early intervention

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Abstract

Objectives: Feeding difficulties and digestive disturbances are common in patients with neurological disorders, particularly Rett disorder. They may compromise weight and growth, often leading to malnutrition. The aim of the present study was to characterize the nutritional and gastrointestinal status of a group of children with Rett disorder and to evaluate the benefits of clinical intervention.

Patients and methods: Based on a previously designed protocol, the authors performed gastrointestinal and nutritional assessment of 25 girls with Rett disorder (with identified *MECP2* mutation). Intervention was performed individually and subsequent evaluation involved 7 patients.

Results: Feeding problems were present in 11 patients (44%), and only one had partial self-feeding ability. Body mass index (BMI) was under the 5th percentile in 40%. Constipation (75%) and gastroesophageal reflux (32%) were the main gastrointestinal problems. Anemia was present in 16% and iron deficiency/low ferritin in 12%. Hypocalcemia occurred in 44%. After therapeutic intervention all the girls reevaluated revealed improvement in BMI, constipation and gastroesophageal reflux symptoms.

Conclusions: Management of patients with Rett disorder requires a multidisciplinary team that should include Gastroenterologists. Individually tailored feeding strategies are essential to promote adequate nutrition. Early identification of nutritional and gastrointestinal disturbances and their proper management contribute to the improvement of quality of life in these patients.

Key words: Rett disorder, growth, nutrition, gastrointestinal disturbances

Introduction

Classical Rett syndrome is a progressive neurodevelopmental disorder clinically defined by criteria developed in 1988 by the Rett Syndrome Criteria Work Group and revised in 2002 (1,2). Aetiology was established in 1999 by the identification of mutations of the methyl-CpG binding protein 2 (MeCP2) gene (*MECP2*), mapping to Xq28 (1). Since then, patients meeting the revised diagnostic criteria can be divided in two groups: those with a *MECP2* mutation – Rett disorder patients (RD), and those without it – Rett syndrome patients (RTT).

Several studies have been published on nutritional and gastrointestinal dysfunction in patients diagnosed with Rett syndrome regardless of the presence or absence of *MECP2* mutation. In this study patients included in those publications will be designated as RS patients (therefore including both cases with RD and RTT).

Nutritional and gastrointestinal problems are found in about 80% of RS patients. Its severity commonly parallels that of the neurological picture which may lead to malnutrition and failure to thrive, cachexia and death (1,2,3,4,5). Several factors may be at the origin of digestive disturbances, and their pathophysiology has not yet been clearly established. Typically, patients progressively lose their self-feeding ability when hand stereotypies emerge and hand praxic function disappears.

Disturbances of chewing and swallowing are common, possibly related to the reduction of medium and posterior tongue voluntary movements, involuntary tongue movements, fast passage of food from mouth to pharynx and retarded oropharyngeal phase of deglutition, all leading to a severe oropharyngeal dysfunction (5,9). These disturbances seem to be most common with liquid foods, as observed in cerebral palsy patients (4). The chewing and swallowing disorder, associated to an altered oral tonus and gastroesophageal dysfunction, contribute to frequent choking, dysphagia and gastroesophageal reflux (GER), commonly observed in RS. Moreover, especially in presence of the breathing irregularities often present in RS, digestive disturbances pose a substantial risk of aspiration and respiratory infections (5,10).

Constipation has been described as the most common gastrointestinal disorder in RS, occurring in up to 85% of patients (9,11). Again, its aetiology is multifactorial, related to altered muscular tonus, reduced physical activity, dietary factors and ongoing treatments.

Although the prevalence of GER in RS patients has not been clearly established, it is often mentioned as a problem (4,9,12,13).

Failure to thrive and nutritional disturbances have been related to reduced intake, negative energetic balance and gastrointestinal pathology, all common in RS (14,15).

The aim of the present work was to characterize nutritional and gastrointestinal disturbances in a group of patients with Rett disorder and to evaluate the effect of preventive and therapeutic measures.

Patients and Methods

A group of 25 patients with RD underwent gastrointestinal and nutritional assessment.

All patients had been previously observed and videotaped by the same paediatric neurologist (TT), and a clinical checklist was fulfilled.

Blood samples from patients and their parents were analysed: genomic DNA was extracted using the Puregene DNA isolation kit (Gentra, Minneapolis, MN, USA). The coding region and exon-intron boundaries of the *MECP2* gene were amplified by PCR and sequenced. The RD-PCR method was used as previously described for the detection of large rearrangements in the *MECP2* gene.(16) Primers and PCR conditions are available upon request.

MECP2 mutations were categorized and grouped for analysis according to: (i) type of sequence change – missense or truncating (including nonsense and frameshift, but also large deletions); and (ii) location of the mutation (affected domain) – TRD (transcriptional repression domain), MBD (methyl-CpG-binding domain).

Clinical evaluation of the patients was based on a previously designed protocol. Patients were staged in the progression of the disease according to Pineda's scoring system (2,17,18). Signs of malnutrition and digestive dysmotility were detailed in the physical examination. Body mass index (BMI) was determined.

Laboratory investigations included complete blood count, serum iron and lipid profile, liver biochemistry, serum proteins, albumin, renal function, sodium, potassium, chloride, calcium, phosphorus, vitamins D, B₁₂ and folate.

An individually designed therapeutic plan was established for identified digestive and nutritional problems with subsequent assessments in clinic.

Data were collected with Epi Info™ (version 3.4.1 from the CDC, Atlanta, USA) and analysed with Fisher's Exact Test using SPSS 12.0 for Windows (rel. 09/08/2003. Chicago: SPSS Inc.).

Protocol was approved by the Hospital Ethics Committee. Informed consent was obtained from all parents to collect blood and to take and use videos and photographs.

Results

Twenty five female patients with Rett disorder were evaluated (*Table 1*). Median age at the first evaluation was 7.4 years (average 8.4; range: 2 – 17.8).

Feeding difficulties in chewing and deglutition, as well as oral and nasal regurgitation, were identified in 44%. These were noticed at different ages, from the neonatal period up to age 14 years (median 24.0 months; average 38.2 months). Food fractioning, short meal times and avoidance of chewiness, in favour of gummy foods, were advised. Only one patient (case 10) had partial self-feeding ability.

In 56% of the patients weight was in the normal range (P_5 - P_{95}), with 40% being below the 5th percentile and one above the 95th. The Z score for weight ranged between -6.77 and 1.7 (median -1.1). Stature was under the 5th percentile in 32% of the patients, and between P_5 and P_{95} in 64%. The stature of one patient (case 17) was above the 95th percentile, corresponding to +3,15SD. BMI was in the normal range in 68% of the patients, and below P_5 in 28.0% (*Fig. 1*). BMI Z score (*Fig. 2*) ranged between -5.36 and 1.9 (median -0.8; average -0.9).

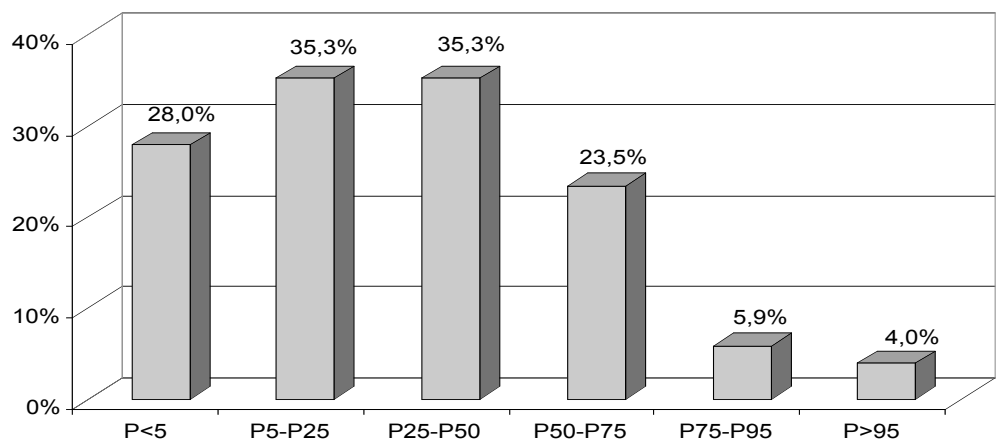
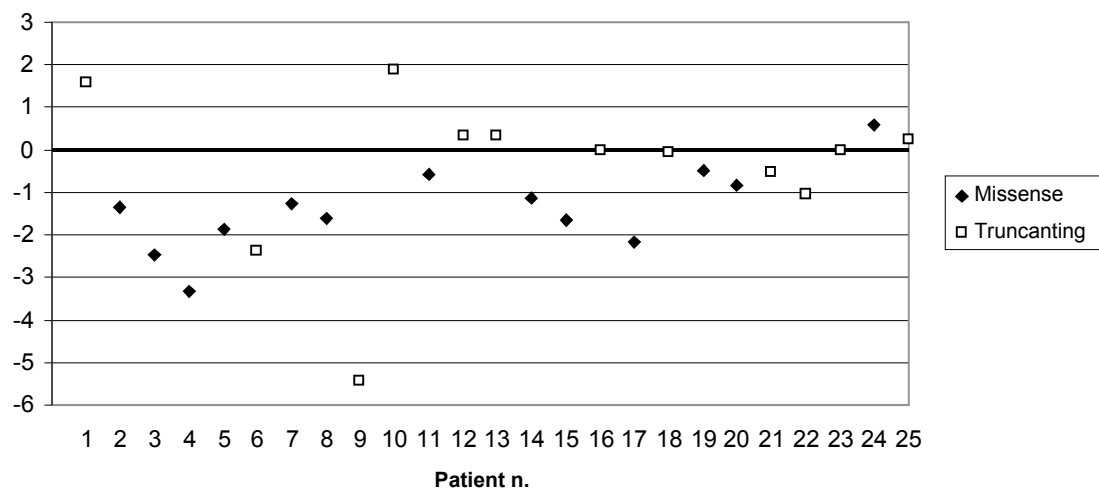


Figure 1 - Percentile distribution of the BMI in 25 patients with Rett disorder.



Fig

Figure 2 - Distribution of the BMI Z score according to mutation type.

Seven patients were evaluated more than once (*Table 2*). In all cases an improvement of nutritional status was noted, as shown by a positive deviation of BMI Z score (range 0.43 [case 21] to 1.96 [case 8]).

There was no correlation between low BMI or its Z score, and the stage or severity of the disease and mutation type (missense vs. truncating mutation).

Constipation was the most common gastrointestinal problem, occurring in 76% of the patients. There was no correlation between constipation and independent gait, scoliosis, epilepsy, disease severity score or mutation type. Most patients with constipation (84.2%) had been previously managed for that problem, with fibre-rich diet, lactulose and enemas, but their compliance was poor. Counselling on this subject was reinforced underlining the need of a liquid and fibre rich diet. Lactulose was prescribed to all patients. In 68.8% of them an initial course of three-day bisacodyl was prescribed due to severity of constipation. At reassessment all patients improved with increased defecation frequency and looser stools.

Symptoms of gastroesophageal reflux (GER) occurred in 32% of the patients. A 24h oesophageal pH monitoring was performed in two patients (cases 8 and 21) because the symptoms were not clear. Conservative management was advised to all, and, those who underwent pH monitoring, were treated with metoclopramide. Both were reevaluated and showed clinical improvement. A statistically significant relation was found between disease severity score and the presence of GER ($p=0.02$).

No correlation was found between feeding difficulties, constipation or GER and mutation type (*Table 3*).

Four patients (16%) (cases 12, 19, 20 and 21) had iron deficient anaemia, and three other (28%) (cases 9, 11 and 16) had low iron/ferritin values. Their BMI percentile ranged from 0.0 to 62.1. Iron was prescribed to all, but control evaluation was not performed yet.

In 92% of the patients, creatinine levels were below normal range. Unbalanced phospho-calcic metabolism occurred in 15 patients (60%) - hypocalcemia was present in eleven girls, and hypophosphatemia or hyperphosphatemia in five patients each. Vitamin D levels were below normal range in seven patients (28%). Of these, five had also hypocalcemia, two had hypophosphatemia, and two had hyperphosphatemia.

Most girls had normal lipid profile. Three patients (cases 3, 16 and 15) had high total cholesterol levels, all associated with elevated LDL-cholesterol (LDL). Another girl (case 6), despite normal total cholesterol, had elevated LDL and low HDL-cholesterol (HDL)

levels. Five other patients (cases 1, 7, 11, 18 and 22) had low HDL, with other lipid parameters being normal. None of the patients had low total cholesterol levels.

Plasmatic folate levels, as well as those of urea, sodium, chloride and potassium were all normal.

Discussion

Several studies have been published on nutritional and gastrointestinal dysfunction in RS but, to our knowledge, this is the first including only patients with identified *MECP2* mutations.

None of the patients had complete self-feeding autonomy. Interestingly, the only patient with some self-feeding ability (case 9), albeit being in stage III as the majority of the patients, had a R133C mutation, and showed the lowest disease severity score. It seems that hand skills are more dependent of severity score than on the stage of the patient disease.

The percentage of patients with BMI under the 5th percentile was smaller than previously described in RS patients (9,7,15,19,20). This may be related to the present tendency towards earlier diagnosis, with prompt adequate management and proper cultural family approach to their child's food intake. A well balanced Mediterranean diet may have also contributed to the better nutritional status of our patients compared to other series.

In all patients with second evaluated, an improvement of the nutritional status was observed. Even though the requirements are still a matter of controversy, nutritional counselling, and correction of feeding posturing, has probably contributed to patients' improvement (7,12,15).

Constipation was the most common gastrointestinal dysfunction, in a proportion similar to previous series (9,19). Its aetiology, however, has not yet been clearly established. Intestinal *MECP2* expression, in girls with RS, was found to be normal by Deguchi et al., therefore it is unlikely that it may contribute to this symptom (21). Counselling and pharmacological treatment were also valuable regarding constipation, with improvement in all girls that were reevaluated.

In the present study the percentage of girls with symptoms suggestive of GER was similar to previous ones (4,13). It is possible that GER may be underestimated due to the inability of patients to complain. We propose that GER must be actively investigated in all

patients, especially in those with failure to thrive. Our 2 patients with demonstrated GER that required pharmacological treatment had improvement on reassessment.

As oral-pharyngeal-esophageal dysmotility has been associated to RS, it is possible that RGE might also occur in all other the patients. Nevertheless, RGE investigations were not performed systematically unless there were suggestive symptoms.

The absence of a statistically significant difference between gastrointestinal pathology, low BMI or its Z score and the type of mutation (missense vs. truncating) could be due to the small sample size. In another study, with a larger sample (n=60, 28 missense vs. 32 truncating) a statistically significant difference was found between weight and height under the 5th percentile, and mutation type (22). Also, in a recent work, Oddy et al. suggested that genotype is likely to have an influence on growth in RS patients (23).

The presence of ferropenic anaemia or isolated low iron/ferritin levels was not correlated with feeding difficulties or with low BMI, underscoring the importance of looking for these disturbances even in the absence of malnutrition or clinical signs of anaemia or iron deficiency.

All but two girls had low creatinine levels for their age, which in some of them might be due to decreased muscle mass.

Abnormal phospho-calcium metabolism was found in more than half of the patients requiring further study. Patients with RS have deficient bone mineralization even when compared to other disabled groups as cerebral palsy patients (24). Osteopenia seems to be independent of nutritional status, being present despite adequate intake of calories, calcium and vitamin D. The results of studies on the effect of antiepileptic drugs and the ambulatory status on bone mineral content have been conflicting (23,25,26). Pathophysiology of osteopenia in RS has not been established, but a metabolic or neuroendocrine abnormality, influencing bone mineralization or increasing its resorption, possibly under *MECP2* influence, could be responsible (23,24,27,28). Whatever the mechanism, previous works are unanimous to point the importance of intervention in this field, aiming at the prevention of osteoporosis and spontaneous fractures, that can be difficult to diagnose in this nonverbal and mentally retarded population (23,27,29). Bone densitometry study is now being adopted.

Lipid profile was abnormal in 36% of the patients. Further studies with serial lipid profile determination on the patients as well as on their parents, are needed to clarify these disturbances.

Several strategies have been proposed for nutritional and digestive management of RS patients, as caloric foods, ketogenic diet, vitamin supplementation, tube feeding, fundoplication, and gastrostomy (4,5,7,9,30,31,32) We believe that management of these issues should be tailored to the individual need to the patient status, considering the progressive nature of the disease.

Conclusions

Rett disorder management requires a multidisciplinary team that should include a pediatric gastroenterologist and a nutritionist. In this study, although only a few patients were reevaluated, all these improved their condition after gastrointestinal and nutritional counseling. Physicians should be aware of this and advise parents to pay attention to possible feeding and digestive disturbances and their alert signs. This approach will allow early diagnosis and individually designed management that will undoubtedly contribute to a better quality of life of RD patients.

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Table 1. Summary of patients' characterization

Patient n.	Age (years)	Stage	Score	Mutation type	Gait	Scoliosis	Epilepsy	Self feeding	Feeding difficulties	P BMI	Z BMI	Constipation	GER
1	7,4	III	9	missense	yes	yes	yes	no	no	94,38	1,59	yes	no
2	5,6	II	9	truncating	yes	yes	yes	no	no	9,06	-1,34	yes	no
3	11,2	IVA	13	truncating	no	yes	yes	no	yes	0,65	-2,48	yes	yes
4	9,9	III	9	truncating	yes	no	no	no	yes	0,04	-3,32	yes	no
5	17,0	IVA	15	truncating	no	yes	yes	no	yes	3	-1,88	yes	yes
6	10,2	III	7	missense	yes	yes	no	no	no	0,82	-2,4	yes	no
7	4,3	III	11	truncating	yes	no	yes	no	no	10,54	-1,25	no	no
8	6,0	III	18	truncating	no	yes	yes	no	yes	5,49	-1,6	yes	yes
9	8,9	III	4	missense	yes	no	no	no	yes	0	-5,36	yes	no
10	7,9	III	11	missense	yes	yes	yes	yes	yes	97,07	1,89	yes	no
11	2,0	II	10	truncating	yes	no	no	no	no	28,13	-0,58	yes	no
12	7,4	III	14	missense	yes	yes	yes	no	yes	62,11	0,31	no	yes
13	7,3	III	15	missense	no	yes	yes	no	yes	62,75	0,33	yes	yes
14	16,3	IVB	17	truncating	no	yes	no	no	no	12,99	-1,13	yes	no
15	7,2	III	14	truncating	yes	yes	yes	no	no	4,82	-1,66	no	yes
16	2,4	III	14	missense	no	yes	yes	no	no	48,7	-0,03	yes	no
17	2,4	III	15	truncating	no	no	no	no	no	1,46	-2,18	yes	no
18	4,2	III	6	missense	yes	no	no	no	no	48,01	-0,05	yes	no
19	17,8	IVB	16	truncating	no	yes	yes	no	no	30,82	-0,5	yes	no
20	6,4	III	13	truncating	yes	no	no	no	no	20,09	-0,84	yes	no
21	13,9	IVB	17	missense	yes	yes	no	no	yes	29,33	-0,54	yes	yes
22	3,1	III	13	missense	no	no	no	no	no	14,53	-1,06	yes	no
23	12,3	III	11	missense	no	yes	yes	no	no	48,73	-0,03	no	no
24	4,5	III	8	truncating	yes	no	yes	no	yes	71,97	0,58	no	no
25	4,8	III	9	missense	no	no	no	no	yes	60,31	0,26	no	yes

Table 2. Revaluated patients' evolution

Patient n.	Age (years)	Stage	Mutation type	Z BMI evolution*	Constipation improvement	GER improvement
2	5,6	II	truncating	1.47	yes	NA
6	10,2	III	missense	0.6	yes	NA
7	4,3	III	truncating	0.67	NA	NA
8	6,0	III	truncating	1.96	yes	yes
9	8,9	III	missense	0.87	yes	NA
21	13,9	IVB	missense	0.43	yes	yes
22	3,1	III	missense	1.05	yes	NA

* - Z BMI on 2nd evaluation *minus* Z BMI on 1st evaluation
 NA – not applicable

Table 3. Digestive pathology and mutation type

	Missense (n=12)	Truncating (n=13)	p value
Feeding difficulties	6	5	0.085
GER	4	4	0.999
Constipation	9	10	0.999

3.1.4. Mutations in the *MECP2* gene are not a major cause of Rett-like phenotype in male patients ([Article 3](#))

Mutations in the *MECP2* gene are not a major cause of Rett syndrome-like phenotype in male patients.

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Running title: *MECP2* and RTT-like phenotypes in males

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Abstract

Rett syndrome is a genetic neurodevelopmental disorder that affects mainly girls, but mutations in the causative *MECP2* gene have also been identified in boys with classic Rett syndrome and Rett syndrome-like phenotypes. We have studied a group of 28 boys with a neurodevelopmental disorder, 13 of which with a Rett syndrome-like phenotype; the patients had diverse clinical presentations that included perturbations of the autistic spectrum, microcephaly, mental retardation, manual stereotypies, and epilepsy.

We analysed the complete coding region of the *MECP2* gene, including the detection of large rearrangements, and we did not detect any pathogenic mutations in the *MECP2* gene in these patients, in whom the genetic basis of disease remained unidentified. Thus, additional genes should be screened in this group of patients.

Introduction

Rett syndrome (RTT; MIM 312750) is a neurodevelopmental disorder affecting mainly girls that combines mental retardation, autism and neurological features (Hagberg et al. 1983). Amir and colleagues (Amir et al. 1999) identified as the genetic cause of RTT mutations in the X-linked methyl CpG-binding protein 2 gene (*MECP2*), which encodes the MeCP2 protein, that binds methylated DNA and is thought to repress transcription of target genes. Classically, patients with this syndrome present an apparently normal initial developmental period (6 to 18 months of age) followed by an arrest and then regression of development, with deceleration in head growth, psychomotor delay and loss of acquired social and motor skills, and appearance of stereotypies (Hagberg et al. 1983). The atypical forms of the disorder may present as either milder or more severe phenotypes. Given the very low frequency of male cases with RTT, the disorder was initially thought of as an X-linked dominant disorder, usually fatal in males. However, the low frequency of male cases could also be due to the fact that most mutations in *MECP2* occur *de novo* in paternal transmissions (Trappe et al. 2001). In the small number of males with RTT described in the literature, classical RTT is associated with (i) a somatic mosaicism of the mutation (ii) a Klinefelter syndrome (47, XXY) or (iii) a hypomorphic *MECP2* allele (Hoffbuhr et al. 2001; Leonard et al. 2001; Topcu et al. 2002; Ravn et al. 2003). As happens with other X-linked dominant disorders, the hemizygous males most often exhibit a more severe phenotype and most of the mutations that cause classical RTT in females originate a very different clinical presentation in males (Trappe et al. 2001; Zeev et al. 2002). The most common of phenotype associated with *MECP2* mutation in males is a severe neonatal encephalopathy (see supplementary review table S1) (Wan et al. 1999; Villard et al. 2000; Hoffbuhr et al. 2001; Imessaoudene et al. 2001; Zeev et al. 2002; Lynch et al. 2003; Leuzzi et al. 2004; Kankirawatana et al. 2006; Venancio et al. 2007). However, mutations in this gene have been identified in patients with X-linked mental retardation associated with neurological features, such as limb spasticity with truncal and facial hypotonia (Meloni et al. 2000; Kleefstra et al. 2002; Van Esch et al. 2005; Moog et al. 2006), with nonspecific mental retardation (Orrico et al. 2000; Couvert et al. 2001; Winnepeninckx et al. 2002; Yntema et al. 2002b) autism (Carney et al. 2003). *MECP2* mutations have also been identified in male patients with language disorder and schizophrenia (Cohen et al. 2002), with an X-linked syndrome including psychosis, pyramidal signs and macro-orchidism (Klauck et al. 2002) and with Prader-Willi and Angelman syndrome-like phenotypes (Watson et al. 2001; Kleefstra et al. 2002; Kleefstra et al. 2004). For this reason, in a research context, the molecular testing of *MECP2* gene in males with a wide spectrum of neurodevelopmental disorders such as unexplained

early neonatal death, neonatal encephalopathy or mental retardation, with or without neurological signs, has been proposed, even in the absence of familial history of similar disease, to further characterize the role of *MECP2* as an etiologic factor in this wide range of diseases.

The aim of the current study was to identify the extent to which mutations in the *MECP2* gene could explain the neurodevelopmental phenotypes presented by a group of 28 male patients of Portuguese origin displaying neurodevelopmental phenotypes compatible with those previously associated with *MECP2* mutations.

Material and Methods

Patients:

The 28 Portuguese male patients selected for this study were sent to our laboratory by clinical geneticists, pediatricians or pediatric neurologists to be tested for *MECP2* gene mutations. A questionnaire asking for clinical, molecular and familial information was filled in by the clinicians requesting the diagnosis; a summary of the clinical presentation for all the patients is presented in figure 1 and table 1. Analysis of this information suggested to us that 13 of the patients had a clinical presentation compatible with a variant form of Rett syndrome (RTT-like), according to the criteria defined by Hagberg and colleagues (Hagberg et al. 2002) (group I, patients 1 to 13 in figure 1); whereas the others did not fulfil the criteria but had clinical manifestations previously seen in *MECP2* mutant males, namely mental retardation of unknown etiology with one or more of the following features: autism, microcephaly, epilepsy, manual apraxia and/or stereotypies (group II, patients 14 to 28 in figure 1). Patients 1, 2, 4, 8, 9, 10, 16, 26 and 28 presented an epileptogenic encephalopathy. Metabolic studies were performed for all patients and did not reveal any alterations. Informed consent for the genetic study was obtained from all patients or their legal representatives.

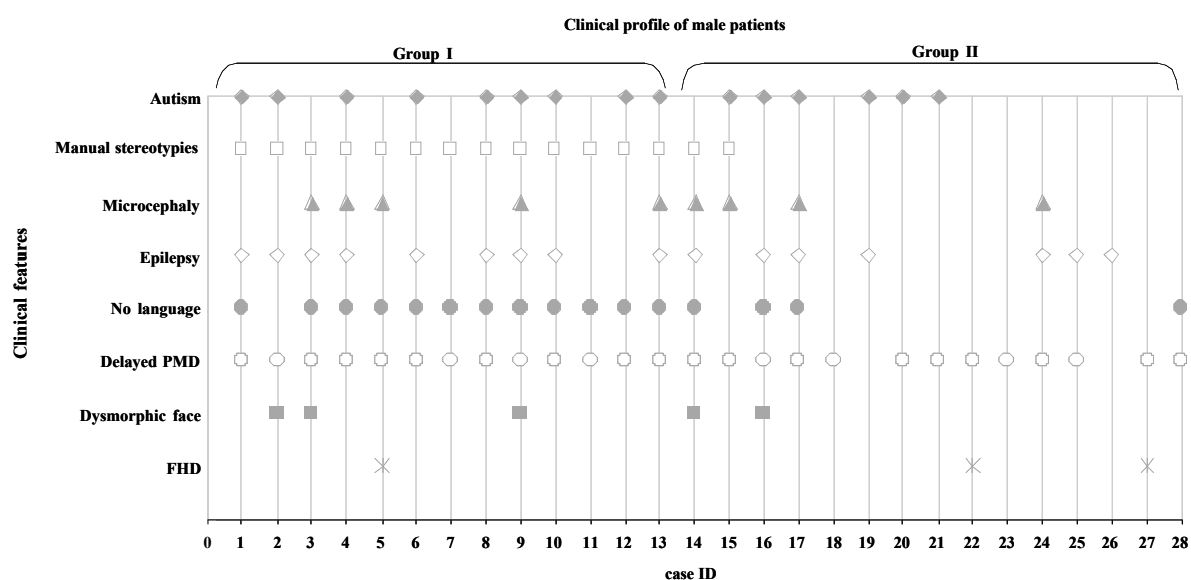
Molecular analysis:

Genomic DNA was extracted from peripheral blood 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; the amplified fragments were automatically sequenced and analysed for point mutations or small rearrangements. Sequence changes

were confirmed by re-amplification with genomic DNA and sequencing in the opposite direction. The RD-PCR method (Shi et al. 2005) was used for the detection of large duplications of the *MECP2* gene. Primers used, PCR mixtures and cyclic conditions are available upon request.

Results

Figure 1 presents a summary of the clinical presentations of the patients we analyzed. The patients' age at the last observation ranged from 4 to 19 years (mean age $10 \pm$ years). Some of the patients had previous molecular exclusion of other clinical conditions and their karyotypes had all been assessed as normal (table 1). Analysis of DNA sequence of the *MECP2* gene in all patients (group I and group II) did not reveal any potentially pathogenic mutation. We found a silent mutation in the *MECP2* gene in one patient (897C>T; T299T) and an intronic deletion of one nucleotide (IVS3-17delT) in another patient, both already described as polymorphisms in the literature (<http://mecp2.chw.edu.au/mecp2/>). We also searched for large duplications or deletions in the *MECP2* gene in both groups of patients but we did not find any alteration in the exons or gene dosage.



Legend Figure 1. Clinical manifestations presented by 28 boys with a neurodevelopmental disorder. Patients 1 to 13 had a clinical presentation compatible with a RTT-like disorder (group I) and patients 14 to 28, did not, but displayed clinical manifestations previously seen in *MECP2* mutation carrier males (group II). FH, family history of mental retardation; PMD, psychomotor development

Table 1. Age, karyotype and molecular exclusions of 13 RTT-like boys (group I).

Case ID	Age	Karyotype	Angelman syndrome	ATR-X	Fragile X	Other
1	8	46, XY	negative		negative	
2	6	46, XY	negative*			
3	19	46, XY	negative			
4	5	46, XY	negative			
5	5	46, XY			negative	FISH (subtelomeric probes)
6	11	46, XY	negative			
7	15	46, XY	negative			
8	5	46, XY			negative	
9	17	46, XY	negative	negative	negative	
10	17	46, XY	negative		negative	
11	12	46, XY	negative			
12	7	46, XY	negative			
13	4	46, XY				FISH (subtelomeric probes)

Discussion

Male patients with neurodevelopmental disorders present a wide spectrum of phenotypes and share a combination of symptoms, which encompass mental retardation, autism, epilepsy and movement disorders. In most cases the genetic basis of the pathology is unknown and *MECP2* is an interesting candidate gene to be analyzed.

Mutations in *MECP2* are found in female patients with heterogeneous clinical presentations; contributing to this fact are the effects of X-chromosome inactivation patterns as well as a potentially significant influence of epistatic, epigenetic or environmental modifiers. Furthermore, mutations known to be RTT-causing in females do not produce similar phenotypes in males, due to the X-linked dominance of the disorder (Ravn et al. 2003) and possibly to differences in the above-mentioned modifier effects. The heterogeneity of phenotypes associated with *MECP2* mutation in males (supplementary table) opened the possibility that mutations in this gene could underlie a variety of X-linked neurodevelopmental phenotypes.

We were not able to identify any mutation in the *MECP2* in our sample of boys with Rett syndrome-overlapping (RTT-like) phenotypes, including the large duplications of this gene, which were described to be frequent in mentally retarded males with progressive neurological symptoms (Van Esch et al. 2005). Mutations in the non-coding regions of *MECP2*, introns and regulatory 5'UTR and 3' UTR, might have been missed since these regions were not covered in this study. However, although Shibayama et al. (Shibayama et al. 2004) reported that 3'UTR variants in the *MECP2* gene seemed to be more frequent in autism patients than in the general population, we have previously searched for

mutations in the 3'UTR region of the *MECP2* gene in a group of Portuguese RTT female patients and we did not find any pathogenic mutation (unpublished data), suggesting that this must be a rare cause of RTT.

Our data suggests that, prior to the indication of systematic molecular testing of *MECP2* in all males with neurodevelopmental pathologies, the study of larger population series should be performed. In fact, the majority of male patients with RTT-like symptoms do not present mutations in the *MECP2* gene, which is in favour of the hypothesis that mutations in other gene(s) may be involved in this disorder (Schanen 2001). Even using a stricter phenotype definition, there are males with a clinical diagnosis of RTT without identified *MECP2* mutation (Leonard et al. 2001).

There are a number of genes in which mutations have been found in patients with pathologies that partially overlap RTT, which it would be interesting to test in patients with RTT or a RTT-like clinical presentation without a *MECP2* mutation: neuroligin 3 (*NLGN3*) and neuroligin 4 (*NLGN4*), mutated in patients with autism, mental retardation or Asperger syndrome (Laumonnier et al. 2004), the aristaless-related homeobox (*ARX*) (Stromme et al. 2002) and the serine/ threonine kinase 9 (*STK9*) genes, mutated in patients with West syndrome (Kalscheuer et al. 2003) and *UBE3A* (involved in Angelman syndrome) (Samaco et al. 2004) are interesting candidate genes. Brain derived neurotrophic factor (*BDNF*) and distal-less homeobox 5 (*DLX5*); two downstream target genes of MeCP2 regulation (Chen et al. 2003; Martinowich et al. 2003; Horike et al. 2005), would also be potentially important candidates for future analysis. Nevertheless the possibility remains that additional novel genes may be identified as the molecular basis of disease in these patients.

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3.2. Movement disorders in Rett syndrome and Rett disorder

The results described in this chapter are included in the following manuscripts:

Article 4. Movement disorders in Rett Syndrome (published)

Article 5. Movement disorders in Rett Disorder: analysis of 60 patients (submitted)

Article 6. Stereotypies in Rett Syndrome: analysis of 83 patients with and without detected MECP2 mutations (published)

Article 7. Stereotypies are present before the regression period in RS – a case study (published)

3.2.1. Introduction

Rett syndrome (RTT) is one of the best human conditions to study movement disorders: patients evolve from a hyperkinetic to a hypokinetic state, and a large series of abnormal movements may be observed along their lives: stereotypies, tremor, chorea, myoclonus, ataxia, dystonia and rigidity.

The aim of this work was to analyze movement disorders in RTT patients with and without a detected MECP2 mutation, as well as their correlation with genotype, in a clinically and genetically well characterized sample of patients, and thus contribute to redefine the clinical profile of this disease.

**3.2.2. Temudo T (2007) “Movement Disorders in Rett Syndrome”. In :
Movement Disorders in Children: a clinical update with video recordings.
Eds: Nardo Nardocci and Emilio Fernandez-Alvarez . Editions John Libbey
Eurotext, Montrouge; pp153-167 ([Article 4](#))**

Chapter 16

Movement disorders in Rett syndrome

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Video 7*

Summary

Rett syndrome is a progressive neurodevelopmental disorder with onset in early childhood, occurring almost exclusively in girls and caused by mutations in methyl-CpG-binding protein 2 (MECP2). It is one of the commonest causes of mental retardation in females. Regression is a defining feature of Rett syndrome, and during the regression period the patients develop autistic behaviour. This is the reason why Rett syndrome has been classified as a pervasive developmental autistic spectrum disorder.

However, movement disorders are so exuberant, characteristic, and unique in Rett syndrome that it is unlikely that an experienced clinician would mistake the two conditions. We can define Rett syndrome as a condition which manifests in the majority of the cases as a hyperkinetic movement disorder and progresses (at varying rates) to a bradykinetic disorder.

My aim in this chapter is to characterize and describe the movement disorders of Rett syndrome, based on a review of published reports and my personal experience.

Introduction

Rett syndrome was discovered by Andreas Rett, a Viennese paediatrician, when he noticed that two girls who were waiting for a consultation with him had the same movement disorder: hand stereotypy. In 1966 he published data on 22 girls with progressive cerebral atrophy, stereotyped hand movements, dementia, alalia, gait apraxia, and a tendency to epileptic fits (Rett, 1966). This disorder was named Rett syndrome in 1983 by Hagberg and his colleagues, who studied 35 female patients from Sweden, France, and Portugal and emphasized autistic behaviour, acquired microcephaly, jerky truncal ataxia, and vasomotor disturbance (Hagberg *et al.*, 1983). Diagnostic criteria for this syndrome were further defined in 1988 (The Rett syndrome Diagnostic Working Group, 1988). It took more than 30 years following Rett's discovery of this syndrome to determine the genetic basis of the condition – mutations in methyl-CpG-binding protein 2 (MeCP2) – largely because the disease is primarily sporadic and familial cases are rare (Amir *et al.*, 1999).

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Among the specific diagnostic criteria of Rett syndrome (Tables 1 and 2) are movement disorders such as stereotyped hand movements, gait apraxia, and dystonia (Hagberg *et al.*, 2002). However, other movement disorders can be present and are less well described. These include additional types of stereotyped movements or behaviours apart from hand stereotypies, tremor, chorea, myoclonus, rigidity, and gait abnormalities. It is apparent that Rett syndrome starts as a hyperkinetic disorder and progresses to a hypokinetic one.

Table 1. Revised diagnostic criteria for Rett syndrome

Necessary criteria 1. Apparently normal prenatal and perinatal history 2. Psychomotor development largely normal through the first 6 months or may be delayed from birth 3. Normal head circumference at birth 4. Postnatal deceleration of head growth in the majority 5. Loss of achieved purposeful hand skill between ages 1/2 -2 1/2 years 6. Stereotypic hand movements such as hand wringing/squeezing, clapping/tapping, mouthing, and washing/rubbing automatisms 7. Emerging social withdrawal, communication dysfunction, loss of learned words, and cognitive impairment 8. Impaired (dyspraxic) or failing locomotion
Supportive criteria 1. Disturbances of breathing while awake (hyperventilation, breath-holding, forced expulsion of air or saliva, air swallowing) 2. Bruxism 3. Impaired sleep pattern from early infancy 4. Abnormal muscle tone successively associated with muscle wasting and dystonia 5. Peripheral vasomotor disturbances 6. Scoliosis/kyphosis progression through childhood 7. Growth retardation 8. Hypotrophic small and cold feet; small, thin hands
Exclusion criteria 1. Organomegaly or other signs of storage disease 2. Retinopathy, optic atrophy, or cataract 3. Evidence of perinatal or postnatal brain damage 4. Existence of identifiable metabolic or other progressive neurological disorder 5. Acquired neurological disorders resulting from severe infections or head trauma

Clinical presentation

Stereotypies

Definition

Stereotypies may be defined as involuntary rhythmic, patterned, coordinated, repetitive, and seemingly purposeless movements or utterances (Jankovick, 2005). Stereotypies are usually continuous, in contrast to mannerisms or tics. However, the differential diagnosis between stereotypies and tics may be difficult or sometimes impossible, and both movement disorders may coexist in the same patient. The term stereotypy should be used to describe a phenomenological and not an aetiological category of hyperkinetic movement disorder, but in rare situations a particular type of stereotypy may help in making a precise diagnosis (such as hand-washing movements in Rett syndrome). Stereotypies can be transient (physiological) or persistent. Transient stereotypies are common in infants. Thelen has identified 49 patterns of stereotypy in normal infants. The time spent in such activities is variable but increases progressively up to the age of 6 months and then decreases, while at the same time the pattern becomes more varied (Thelen, 1979; 1980).

Table 2. Revised delineation of variant phenotypes

Inclusion criteria 1. Must meet at least three of six main criteria 2. Must meet at least five of 11 supportive criteria
Six main criteria 1. Absence or reduction of hand skills 2. Reduction or loss of babble speech 3. Monotonous pattern to hand stereotypies 4. Reduction or loss of communication skills 5. Deceleration of head growth from the first years of life 6. Rett syndrome disease profile: a regression stage followed by a recovery of interaction contrasting with slow neuromotor regression
Eleven supportive criteria 1. Breathing irregularities 2. Bloating/air swallowing 3. Bruxism (harsh sounding type) 4. Abnormal locomotion 5. Scoliosis/ kyphosis 6. Lower limb atrophy 7. Cold, purplish feet, usually growth impaired 8. Sleep disturbances including night screaming outbursts 9. Laughing/screaming spells 10. Diminished response to pain 11. Intense eye contact/eye pointing

Persistent stereotypies are common in children with sensory deprivation (blindness, deafness) or emotional deprivation (institutionalized infants) (Fernandez-Alvarez & Aicardi, 2001), and they often accompany various psychiatric or neurological disorders such as anxiety, obsessive-compulsive disorders, Tourette syndrome, schizophrenia, autism, Rett syndrome, mental retardation, akathisia, restless legs syndrome, and several neurodegenerative disorders including fronto-temporal dementia (Nyatsanza *et al.*, 2003).

In addition to motor and phonic types, stereotypies can be classified as either simple (for example, tapping, mouthing, clapping) or complex (a sequence of different movements always performed in the same way), or they can be classified by the predominant site of involvement (head, trunk, hand, lower limbs, and so on) (Table 3).

The pathophysiology of stereotypies

The neurophysiological basis of stereotypies is unknown. There is no clear anatomical-clinical correlation for stereotypies, although it is believed that both cortical and subcortical structures are involved (basal ganglia dysfunction, mesolimbic system) (Jankovick, 2005). Stereotypies have also been observed in patients with structural lesions of the central nervous system including bilateral areas of the medial frontoparietal cortices (Sato *et al.*, 2001) and cerebellum (Hottinger-Blanc *et al.*, 2002).

The dopaminergic system in the basal ganglia has been implicated in the production of stereotypies. Intrastratial injection of dopamine and the systemic administration of both presynaptically active dopamine agonists (such as amphetamine) and postsynaptically active dopamine agonists (such as apomorphine) in rats produce dose-related repetitive sniffing, gnawing, licking, biting, rearing, head bobbing, and other stereotyped learned activities (Costall *et al.*, 1977). These stereotypies can be blocked by neuroleptic drugs (Tschanz & Rebec, 1988). SKF 38393, a D1 agonist, did not induce stereotypies, but it enhanced stereotypies induced by apomorphine (a mixed D1 and D2 agonist) and by PHNO, a selective D2 agonist (Koller

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Table 3. Classification of stereotypies, modified from the classification of Jankovick (2005) and Fernandez-Alvarez & Aicardi (2001)

Transient (physiological) Normal child development Stress related Self-gratifying behaviour
Persistent (pathological) Sensory deprivation (restraining, blindness, deafness) Mental retardation Autism Rett syndrome Neuroacanthocytosis Schizophrenia Catatonia Obsessive-compulsive disorder Tourette syndrome Tardive and other dyskinesias Akathisia Restless legs syndrome Fronto-temporal dementia Epileptic automatism Psychogenic
Motor (body part localization) Head/neck Face Mouth Eyes Arm Hand (visual behaviour present or absent) Leg Feet Body
Phonic Repetitive sounds Repetitive words or phrases

& Herbster, 1988). This suggests that D2 receptors mediate stereotypies and D1 receptors potentiate this effect.

Neuropeptides may also modulate stereotyped behaviour. Microinjection of cholecystokinin and neurotensin into the medial nucleus accumbens potentiates apomorphine-induced stereotypy (Blumstein *et al.*, 1987). Injection of these peptides into the striatum had no effect on the apomorphine-induced stereotypy, providing additional evidence for the involvement of the limbic system in the pathogenesis of this movement disorder.

Endogenous opiates (for example β -endorphins) may also be involved in the pathogenesis of stereotypies because some autistic children with self-injurious movements improve their behaviour after administration of opiate blockers (naloxone and naltrexone), and raised plasma and cerebrospinal fluid levels of β -endorphins were found in these patients (Sandman, 1988).

More recently the serotonin system has also been implicated, supported by the observation that certain animal behaviours improve with serotonin uptake inhibitors (Hugo *et al.*, 2003).

Clinical presentation of stereotypies in Rett syndrome

The most striking core symptom of Rett syndrome is the stereotyped movement of the hands. These movements are associated with or follow the disappearance of purposeful prehension.

However, a video analysis of 22 cases of Rett syndrome in the first 6 months of life, before the beginning of regression, showed stereotyped hand movements in 42 per cent (Einspieler *et al.*, 2005). The voluntary hand movements seen in Rett syndrome are limited to gross hand manipulation, and fine movements are not possible. A previous report showed that voluntary movements in Rett syndrome reach the level of the 4th month at most (Nomura & Segawa, 1990).

The almost continuous, repetitive, and compulsive automatisms disappear during sleep, and environmental manipulation has been shown to have a relatively limited effect on the frequency of the repetitive hand movements, suggesting that the movements are a manifestation of automatic reinforcement or neurochemical processes (Wales *et al.*, 2004).

The most characteristic hand movement is the wringing, washing-like movement of both hands, usually in the midline, often in front of the body, and often without crossing the midline. There can also be symmetrical movements of both hands of other types, for example clapping, tapping, or putting their hands in their mouths (see the videoclip on the accompanying DVD, track 1).

Hand stereotypies may manifest with the hands apart and most often with each hand carrying out a different movement: hair pulling with one hand, trunk tapping with the other; hair pulling with one hand and putting the other in the mouth; flapping one hand, pill-rolling with the other; raising one hand above the head and making gestures like a Seville dance, making castanet movements with the fingers of one hand to produce a sound; twisting two or three fingers in a complex way, and so on (personal observations) (video 7, track 1). When they put their hands into their mouths, they often bite them and cause hand mutilations; stereotypies therefore have to be inhibited by mechanical methods or by using low doses of risperidone (Nyatsanza *et al.*, 2003).

One of the peculiarities of hand stereotypies in Rett syndrome is that the patients do not look at their hands when they are carrying out the movements, perhaps because they have very poor eye-hand coordination. I believe this feature may help clinicians to differentiate the hand stereotypies of Rett syndrome from those of other conditions such as mental retardation or autism (personal observation).

The second most common stereotypy in Rett syndrome is bruxism. In a series of 65 Rett syndrome patients I found bruxism in 82 per cent (Temudo, 2005). It occurs in the waking state and ceases in sleep (Hagberg *et al.*, 1983; Naidu *et al.*, 1986), is very common in mentally retarded individuals (Blount *et al.*, 1982), and may be associated with dental and psychological disturbances (Glaros & Rao, 1977; Rugh & Harlan, 1988).

FitzGerald *et al.* (1990) considered that bruxism might be a form of focal dystonia, but other investigators – including myself – consider that it is a type of self-injurious stereotypy. Some older Rett syndrome girls with bruxism have a particular maxillary morphology with prognathism, maybe as a result of this particular type of continuous movement (personal observation).

Apart from hand stereotypies and bruxism, there are additional types of stereotypy involving other parts of the body (personal observations): rhythmical head rolling, cervical retropulsion, eye rolling, eye closure, facial grimacing, lip sucking, repetitive sounds or words, trunk rocking, intermittent leg elevation and tapping of the floor, toe walking, feet stereotypies, and swaying movements of the body with the weight shifting from one leg to the other. Stereotypies can also be very complex at the onset of the disease, some girls exhibiting a form of ‘stereotyped dance’ involving many areas of the body (personal observation; video 7, track 2).

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With the progress of the disease, stereotypies diminish and became simpler and slower with age, with the patients becoming hypokinetic and rigid. Generally, however, patients maintain the same types of hand movement throughout their lives. Stereotypies involving other parts of the body tend to disappear and behave like tics, while new movements substitute preceding movements over time (personal observation).

Dystonia

Postural asymmetry is a common finding in Rett syndrome and at late stages of the disease is always present in the lower limbs. A discrete dystonia of the halux is a very common and early sign of Rett syndrome (Temudo *et al.*, 2005) (Figs. 1-2).



Fig. 1. Foot dystonia in Rett syndrome.

FitzGerald *et al.* (1990) reported dystonia in 19 of 32 patients (59 per cent), usually crural or generalized but sometimes focal, involving the upper or lower extremities. In a series of 71 Rett syndrome patients, Temudo *et al.* found dystonia in 69 per cent (unpublished data). Usually the impairments are asymmetrical, the right side of the body being most affected (Nomura *et al.*, 1984; Hagberg & Romell, 2002).

Scoliosis, which is a common feature of Rett syndrome, may be a sign of truncal dystonia and in those severe cases who never achieve ambulation it is present from the earliest stages of the disease. Temudo *et al.* found scoliosis in 70 per cent of 71 Rett syndrome, patients and in 42 per cent of those with scoliosis it was present before the age of 10 (unpublished data).

Rigid-akinetic syndrome

The facial features in Rett syndrome patients are usually animated in the early stages of the disease but the face later becomes expressionless. However, an inexpressive 'mask-like' face may be an early clinical sign, accompanied by a disproportionate ability to communicate with the eyes and by normal eye blinking. In a series of 32 Rett syndrome patients analysed by FitzGerald *et al.* (1990), hypomimia was more severe and more common in the older girls.

Bradykinesia and rigidity are also common in older girls (FitzGerald *et al.*, 1990) and some severe cases who never acquire independent ambulation show rigidity very early in life. In a series of 71 patients with Rett syndrome, Temudo *et al.* found rigidity in 25 per cent at a mean age of 12.5 years (unpublished). Rigidity was not related entirely to the age of the patients.

Rett syndrome gait

The majority of the patients acquire independent ambulation and this feature is correlated with the type of the mutation in the MECP2 gene (Huppke *et al.*, 2002; Schanen *et al.*, 2004). The gait can be normal but purposeless, or have peculiar characteristics which were described as apraxic by Rett (1966). This apraxic gait consists of a wide base with extended knees and rigid legs and contraction of the gluteal and abdominal muscles ([video 7, track 3](#)). When these girls walk, the stereotyped hand movements continue and the normal arm balance, essential to equilibrium, is absent, provoking retropulsion of the body ([video 7, track 4](#)). Some girls have particular stereotypies, with tiptoe gate, shifting of body weight from one leg to the other, or tapping the floor with one leg.

With the progression of the disease (the rate being variable), neuromotor functions decline in most patients and the majority lose their gait and become wheelchair-dependent. However, 15 to 20 per cent of these patients remain ambulatory well into middle age (Witt-Engerström & Hagberg, 1990). In such patients, the gait becomes more rigid and some may have freezing when they try to initiate a movement.

Tremor

FitzGerald *et al.* (1990) did not find rest or postural tremor in 32 Rett syndrome girls, but they found kinetic tremor in those who were predominantly ataxic. Postural tremor may also be present in some rigid and akinetic cases. In a series of 71 patients, 41 per cent had tremor and in 66 per cent of these the tremor was postural (Temudo *et al.*, unpublished data) ([video 7, track 5](#)).

Chorea

Choreoathetoid movements usually involve the hands (FitzGerald *et al.*, 1990) but in my experience this is not a common finding in Rett syndrome patients. In a series of 71 cases I observed only one girl with generalized choreoathetosis and one other with hand athetosis (Temudo *et al.*, unpublished data).

Myoclonus

Myoclonus was reported in 34 per cent of 32 Rett syndrome patients examined by FitzGerald *et al.* (1990), and predominantly involved the head or the trunk. Guerrini *et al.* (1998) studied 10 patients with Rett syndrome and observed myoclonus involving the distal limbs in nine. The

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Fig. 2. Hand dystonia or athetosis in Rett syndrome.

severity of the myoclonus was not correlated with other symptoms or with age. These investigators undertook neurophysiological studies and concluded that their Rett syndrome patients had a distinctive pattern of cortical reflex myoclonus.

I observed one girl with Rett syndrome with generalized continuous myoclonus. During this state she maintained normal interpersonal contact but there was cessation of her usual continuous hand stereotypies. The myoclonic movements did not respond to benzodiazepines,

valproate, lamotrigine, or topiramate, and they disappeared with sleep or spontaneously (video 7, track 6).

Biochemical studies

Assays of biogenic amines and their metabolites have given conflicting results, showing either decreased levels of homovanillic acid (HVA), 3-methoxy-4-hydroxyphenylglycol (MHPG), and 5-hydroxyindoleacetic acid (5-HIAA) (Zoghbi *et al.*, 1989; Ramaekers & Blau, 2001), or no significant changes in these metabolites (Lekman *et al.*, 1990). Decreased CSF levels of 5-HIAA are accompanied by low levels of 5-methyltetrahydrofolate (5-MTHF), in contrast to normal serum folate levels, while oral supplementation with folinic acid restores 5-MTHF values and serotonin turnover (Ramaekers & Blau, 2001). The CSF concentrations of γ -butyric acid (GABA) and other amino-acids are normal (Perry *et al.*, 1988), while glutamate is markedly raised (Lappalainen *et al.*, 1996). Decreased CSF levels of β -phenylalanine – an endogenous amine synthesized by decarboxylation of phenylalanine – may reflect impairment of the nigro-striatal dopaminergic system (Satoi *et al.*, 2000). Plasma levels of β -endorphin and prolactin are mildly decreased (Fanchetti *et al.*, 1986), with increased CSF β -endorphin in some but not in all cases (Budden *et al.*, 1990). CSF levels of substance P are decreased (Matsuishi *et al.*, 1977). Bioppterin, a cofactor in the synthesis of biogenic amines found in blood and urine, is normal, with a mild decrease in the CSF but no deficiency of the critical enzyme dihydropteridine reductase (Zoghbi *et al.*, 1989). Membrane cerebral lipids are decreased in the CSF (Lekman *et al.*, 1991). Necropsy studies showed reduced levels of dopamine, serotonin, and their respective metabolites HVA and 5-HIAA in the substantia nigra of older girls with Rett syndrome, but no generalized deficiency of dopamine (Lekman *et al.*, 1990), while others reported decreased levels of dopamine, norepinephrine, serotonin, and their metabolites in most brain regions (Riederer *et al.*, 1986). An increased striatal dopamine-to-HVA ratio and raised 3,4-dihydroxyphenylacetic acid indicate increased dopamine turnover in the brain, with reduced serotonin and a regional increase in its precursor tryptophan in the striopallidum and substantia nigra (Lekman *et al.*, 1990). A reduction in cortical choline acetyltransferase (ChAT) suggests a dysfunction of the cholinergic forebrain system (Lekman *et al.*, 1990).

Neuropathology

There is a generalized reduction in neuronal size with increased cell-packing density in the cerebral cortex, thalamus, basal ganglia, amygdala, and hippocampus (Bauman *et al.*, 1995). Other studies have revealed a decline in neuronal numbers in the frontal and temporal cortex but no decrease in cortical thickness. The decrease in cell numbers primarily involves the large pyramidal cells, more prominent in layers II and III than in the deep layers, with preservation of the visual cortex (Belichenko *et al.*, 1994). These changes are associated with abnormalities in the orientation pattern of dendrites and axons, decreased dendritic branching (Armstrong, 1997), small neurons with increased neuronal packing (Armstrong, 2002), loss of dendrites of pyramidal cells in the frontal, motor, and subicular areas (Cornford *et al.*, 1994), shortening of the apical and basilar dendritic branches in layers 3 and 5 of the frontal, motor, and inferior temporal cortex (Armstrong *et al.*, 1998), and ‘naked dendrites’ without spines in pyramidal neurons, indicating a reduction in synaptic contacts from afferent neurons (Belichenko *et al.*, 1994). Golgi studies showed a selective alteration in the size of dendrites of pyramidal neurons in the frontal, motor, and temporal cortices, while the dendrites in the pyramidal neurons in

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the hippocampus and visual cortex showed no changes. Similar abnormalities characterize the medial temporal structures in infantile autism (Bauman & Kemper, 1994).

Mild hypochromasia has been observed in the striatum, and hyperchromia of large neurons in the striatum and internal pallidum – here with abnormal dendrites and degeneration of thick myelinated fibres (Belichenko *et al.*, 1994; Leontovich *et al.*, 1999), and mild diffuse gliosis.

Another finding is hypomelanation of the substantia nigra pars compacta without definite neuronal loss – the majority of large nigral neurons containing little or no neuromelanin (Kitt & Wilcox, 1995; Jellinger *et al.*, 1988). In one Rett syndrome patient only, aged 21 years, nigral cells were reduced by about 30 per cent of age-matched controls, and there were free pigment granules in the neuropils, as in Parkinson's disease. These and preliminary data showing labelling of fragmented intranucleosomal DNA using the TUNEL (TdT-mediated dUTP-biotin 3'-OH nick-end labelling) method in substantia nigra neurons suggest that they undergo active degeneration (Kitt & Wilcox, 1995). No apparent abnormalities in other pigmented brain stem nuclei are seen; morphometric studies of the serotonergic dorsal raphe nuclei showed no neuronal decrease, though reduced cell numbers have been reported in the cholinergic nucleus basalis of Meynert (Kitt *et al.*, 1990).

The cerebellum may show gross atrophy of all lobules of the vermis and progressive loss of Purkinje cells with preservation of the basket and stellate cells independent of age (Oldfors *et al.*, 1990), and simplification of the inferior olivary nucleus without cell loss or with gliosis. These findings, which are also seen in infantile autism (Bauman & Kemper, 1994), suggest arrested development beginning before birth (Bauman *et al.*, 1995).

Drug trials

The benefit of dopaminomimetic drugs – for example, L-dopa and dopamine agonists – is controversial but may improve motor abilities (Zappella, 1990), while naltrexone, an opiate antagonist, may help to stabilize breathing irregularities (Percy *et al.*, 1994). Drugs that block postsynaptic dopamine and serotonin receptors – such as risperidone – have been found to be effective in the treatment of tantrums, aggression, and self-injurious behaviours in patients with autistic disorders (Percy, 2002; Research Units on Pediatric Psychopharmacology Autism Network, 2002; Gagliano *et al.*, 2004).

Conclusions

The motor symptoms seen in Rett syndrome are unique. The core motor symptoms of the condition are considered to be an abnormality of muscle tone, posture, locomotion, and voluntary movement. Each symptom may be observed in other diseases, but the prototypical symptoms of Rett syndrome and their combination are seen only in this disorder.

Though Rett syndrome has been studied for almost 40 years, little is known about the pathophysiology of this neurodevelopmental disease. Recent studies point to a deficiency of the dendritic and synaptic apparatus in selected neurons, their neurotransmitters, and possibly some cellular proteins. The cause of these deficiencies is not yet understood.

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Chapter 16 *Movement disorders in Rett syndrome*

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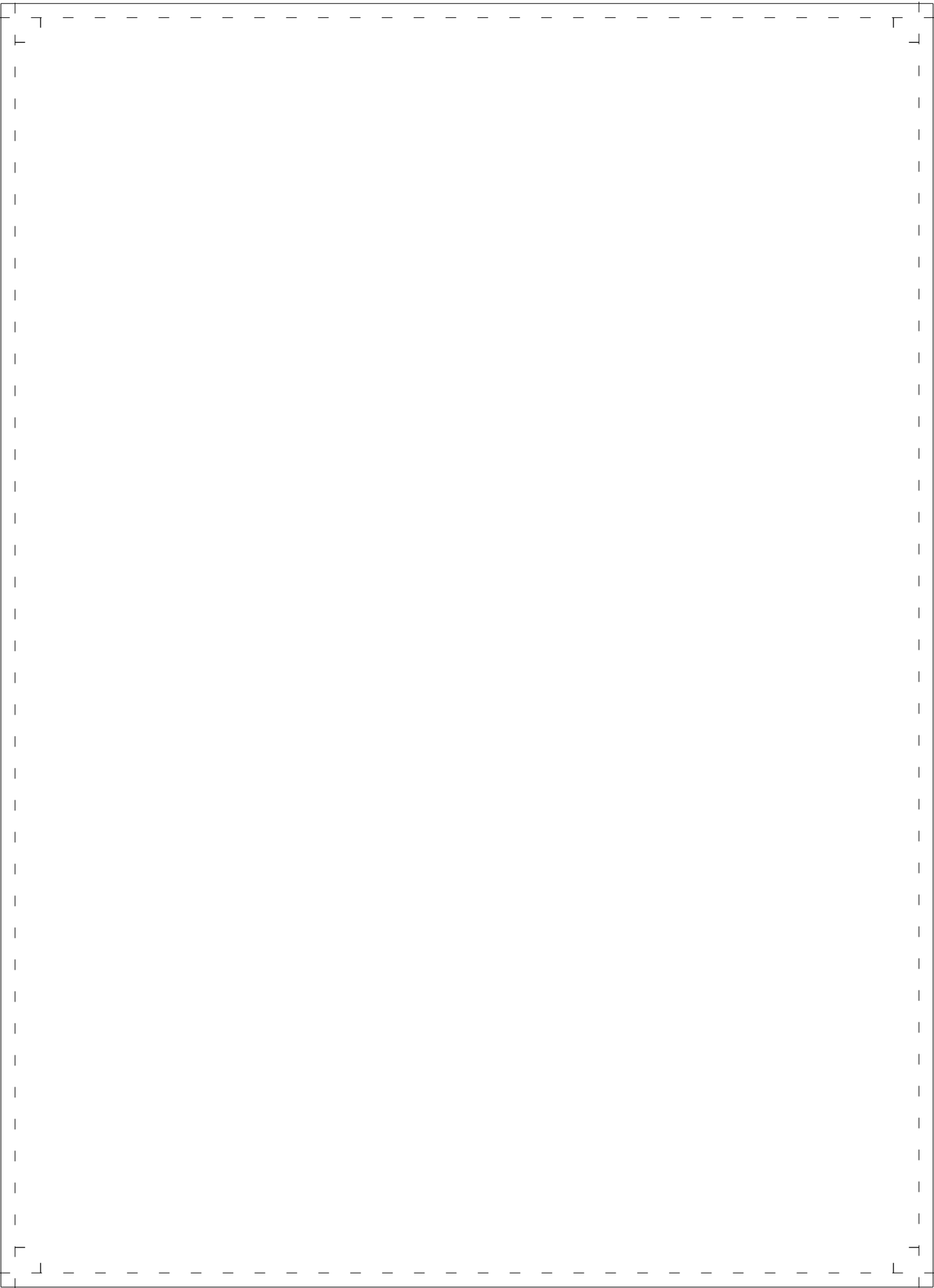
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3.2.3. Movement disorders in Rett Syndrome: analysis of 60 patients with detected *MECP2* mutation ([Article 5](#))

Movement Disorders in Rett syndrome: an analysis of 60 patients with detected *MECP2* mutation

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Abstract

Rett syndrome (RTT) is one of the best human models to study movement disorders: patients evolve from a hyperkinetic to a hypokinetic state, and a large series of abnormal movements may be observed along their lives: stereotypies, tremor, chorea, myoclonus, ataxia, dystonia and rigidity.

The aim of this work was to analyze movement disorders in RTT patients with a detected *MECP2* mutation, as well as their correlation with genotype, in a clinically and genetically well characterized sample of patients, and thus contribute to redefine the clinical profile of this disease.

We included in this study 60 patients with detected *MECP2* mutations. These were categorized and grouped for analysis, according to: (1) type of change (missense or truncating, including nonsense and frameshift but also large deletions); and (2) location of the mutation.

Differences were found concerning the frequency of independent gait, dystonia, type of tremor, and global score severity when comparing the group of patients with missense and truncating mutations.

We also found differences in the presence, distribution, severity or type of movement disorders in the two groups of patients according to the median duration of the disease (less than 60 months; 60 months or more)

We conclude that movement disorders seem to reflect the severity and rate of progression of RD, patients with truncating mutations presenting a higher rate and more severe dystonia and rigid-akinetic syndrome, when comparing groups with similar time of disease evolution.

Introduction

Rett disorder (RD) is caused by mutations in the methyl-CpG binding protein 2 gene (*MECP2*) (1). It is one of the best human conditions to study movement disorders: patients evolve from a hyperkinetic to a hypokinetic state, and a large series of abnormal movements may be observed along their lives: stereotypies, tremor, chorea, myoclonus, ataxia, dystonia and rigidity.

In 1990, FitzGerald et al. (2) published the first analysis of movement anomalies present in 32 patients with Rett syndrome (RTT). However, as this study was performed before the discovery of the *MECP2* mutation, some patients would probably have different disorders, under the larger umbrella of RTT.

The aim of this work was to analyze movement disorders in RD, as well as their correlation with genotype, in a clinically and genetically well characterized sample of patients, as a contribution to redefine the clinical profile of this disease.

Subjects and methods

All Portuguese paediatric neurologists were asked to indicate their patients with possible RTT. Patients thus referred (n=88) were observed and videotaped by the same paediatric neurologist (TT), and a clinical checklist was completed. Blood samples from patients and their parents were obtained, also after informed consent, and genomic DNA was extracted 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. The RD-PCR method was used, as described (3), for the detection of large rearrangements in the *MECP2* gene. Primers and PCR conditions are available upon request.

For this study, we included only patients with detected *MECP2* mutations. These were categorized and grouped for analysis, according to: (1) type of change (missense or truncating, including nonsense and frameshift but also large deletions); and (2) location of the mutation.

Cases were scored using the Pineda general severity scale (4). A classification of stereotypies was used modified from Jankovick (5) and Fernandez-Alvarez & Aicardi (6). A motor-behavioral assessment scale was used to rate the different characteristics on a 0 to 4 scale: 0= normal or never, 1= mild or rare, 2= moderate or occasional, 3= marked or frequent, and 4= very severe or constant. Dystonia was classified according to distribution (focal, segmental, multifocal and generalized).

For statistical analysis, patients were divided into two groups: those carrying missense (group I) and those carrying truncating (group II) mutations. The SPSS (v.14) statistical package was used to analyse the data. Proportions were compared using a Chi-square or the Fisher exact test. Quantitative variables were compared using the Mann-Whitney test and were presented as median (25th percentile – 75th percentile).

Results

In this study we analyzed the entire coding sequence of the *MECP2* gene, in 88 patients that fulfilled the revised clinical criteria for RTT (7). Of those, we further studied the 60 patients with a detected *MECP2* mutation.

Cases were classified as classical (60.7%) and variant (39.3) forms of RTT (seven congenital forms; three preserved speech and one male), according to Hagberg classification (8).

Twenty-nine patients were observed and videotaped at least twice, at intervals of 6 months. Clinical data, partially reported in another work (9), are presented in Table 1.

Median age and median duration of the disease at our observation were not significantly different between group I and II mutation patients.

Comparison between groups I and II showed some important clinical differences: (1) more patients of group II were in stage IV of the disease, which may mean that their rate of evolution was faster; (2) microcephaly was acquired in 67.6% of group II, but only in 19.2% of group I patients; (3) independent gait was acquired by 80.8% of group I, but only in 47.1% group II patients; (4) dystonia and oral motor difficulties were more frequent in patients with truncating mutations; (5) the Pineda's general severity score showed that the median severity was greater in group II patients (Table1).

All but one patient (a male with a severe encephalopathy) displayed hand stereotypies at the age of observation (video 1). Stereotypies with other topographies (video 1) were also present in 95% of the patients, and, among these, the most frequent was bruxism (80% of the patients). A difference was found between the median number of different stereotypies displayed by each patient before (median, 6) or after (median, 3) 60 months of disease evolution ($p=0.046$). Hyperpnea and/or apnea were present in 76.7% of the patients.

Independent gait was acquired by 63.3% of the patients; at age of observation, 43.6% of those who maintained independent gait, this was both ataxic and rigid (video 2). Dystonia was present in 63.3% of the patients, and scoliosis (probably a consequence of truncal dystonia) was seen in 71.7%. Focal dystonia was present in 45.5% of group I and 20% of group II patients; generalized dystonia was present in 46.2% of group II and 18% of group I patients (fig 1). Tremor (kinetic and/or postural) (video 3) was present in 48.3% (predominantly kinetic in group I and postural in group II) of the patients and myoclonus in one patient. During the periods of generalized myoclonia, the patient could not perform their usually continuous hand stereotypies (video 4). The poligraphic register performed

during one of the episodes of generalized myoclonia did not show any EEG paroxysm temporally linked with clinical jerks and its electromyographic correlates.

One 13 years-old patient with a truncating mutation had an episode of generalized choreoathetoid movements, which lasted one month (video 5). She was hospitalized because the involuntary movements increased during a respiratory infection with syncytial virus, accompanied by fever; infections by other agents or other secondary causes of chorea were excluded. She was successfully treated with Haloperidol®, for four months.

Rigidity was present in 48.3% of the patients; 37.9 % of them presented it before 60 months of disease evolution (82% with truncating mutations) (video 6).

We also found differences when analysing the median duration of the disease (less than 60 months; 60 months or more) and the presence, distribution, severity or type of movement disorders in group I and II patients (Table 2).

Frequency of stereotypies was smaller in group II patients (though not significantly), after 60 months of evolution. Rigidity was more frequent (although not significantly) in patients with truncating mutations, with less than 60 months of disease evolution (26.5% of patients).

Finally, patients of group II showed a higher rate of segmental or generalized dystonia, more severe rigidity and a higher rate of postural tremor, at or after 60 months of disease evolution.

Table 1. Clinical data of group I (missense) and II (truncating) mutations in RD patients

	All RD patients (n = 60)	Missense (n = 26)	Truncating (n = 34)	P
MEDIAN AGE (years)*	7.0 (5.0-13.5)	7.0 (4.7-10.5)	10.5 (5.0-14.3)	0.185
MEDIAN DURATION OF DISEASE (months)*	92 (55-140)	72 (26-111)	122 (40-156)	0.325
STAGE (%)				0.010
stage II	10.0	7.7	11.8	
stage III	60.0	80.8	44.1	
stage IV A and IV B	30.0	11.5	44.1	
PSYCHOMOTOR DEVELOPMENT (%)				
normal until 12 months of age	58.3	69.2	50.0	0.188
median age of acquisition of autistic traits	18.0 (12.5-18.0)	18.0 (14.0-21.0)	15.0 (12.0-18.0)	0.175
propositive manipulation	91.5	92.3	90.0	0.999
median age of acquisition of stereotypies	20.0 (14.5-25.0)	24.0 (18.0-28.5)	18.00 (13.3-24.0)	0.086
acquisition of independent gait	63.3	84.6	47.10	0.007
NEUROLOGICAL FEATURES (%)				
microcephaly at time of observation	46.7	19.2	67.6	<0.001
agitation	56.7	50.0	61.8	0.517
hyperpnea/apnea	76.7	69.2	82.4	0.377
vaso-motor disturbances	70.0	53.8	82.4	0.024
neurogenic muscle atrophy	48.3	42.3	52.9	0.446
pyramidal signs	28.3	23.1	32.4	0.566
ataxia	35.0	46.2	26.5	0.190
stereotypies per patient*	4.00 (3.0-6.0)	3.50 (2.8-7.0)	4.00 (3.0-6.0)	0.999
rare	8.3	0	14.7	0.211
occasional	8.3	11.5	5.8	
frequent	25.0	30.8	20.5	
constant	58.3	61.5	55.8	
dystonia	63.3	46.2	76.5	0.029
focal	26.3	41.7	19.2	0.172
segmental	36.8	41.7	34.6	
generalized	36.8	16.7	46.2	
chewing difficulties	76.7	65.4	85.3	0.134
rigidity	48.3	34.6	58.8	0.074
mild	37.9	55.6	30.0	0.323
moderate	34.5	33.3	35.0	
marked	27.6	11.1	35.0	
tremor	48.3	50.0	47.1	0.999
kinetic	58.6	84.6	37.5	0.012
postural	34.5	7.7	56.3	
kinetic and postural	6.9	7.7	6.3	
ataxic-rigid gait	43.6	36.8	50.0	0.523
scoliosis	71.7	69.2	73.5	0.777
Pineda's scale*	13 (9-15)	11 (8-13)	14 (11-16)	0.001

*Data presented as median (25th percentile - 75th percentile)

Table 2. Median duration of disease at observation and movement disorders in group I (missense) and II (truncating) mutations in patients with RD

	DURATION OF DISEASE < 60 months			DURATION OF DISEASE ≥ 60 months		
NEUROLOGICAL SIGNS	missense mutations	truncating mutations	p	missense mutations	truncating mutations	p
n° of different stereotypies*	6.0 (2.0-7.0)	5.5 (3.0-8.0)	0.484	3.0 (3.0-7.0)	3.0 (2.2-4.0)	0.480
frequency of stereotypies						
rare or occasional	11.1	7.1	0.606	5.9	35.0	0.077
frequent	22.2	7.1		35.3	30.0	
constant	66.7	85.7		58.8	35.0	
dystonia	22.2	50.0	0.228	58.8	95.0	0.014
distribution of dystonia						
none	77.8	50.0	0.498	41.2	5.0	0.002
focal	11.1	28.6		23.5	5.0	
segmental or generalized	11.1	21.4		35.3	90.0	
scoliosis	44.4	35.7	0.999	82.4	100.0	0.088
rigidity	22.2	64.3	0.089	41.2	55.0	0.515
severity of rigidity						
none	77.8	35.7	0.165	58.8	45.0	0.039
mild to moderate	22.2	42.9		17.6	0.0	
marked	0.0	21.4		23.5	55.0	
tremor	55.6	42.9	0.680	47.1	50.0	0.999
tremor						
none	44.4	57.1	0.837	52.9	50.0	0.004
kinetic	44.4	35.7		41.2	5.0	
postural or kinetic + postural	11.1	7.1		5.9	45.0	
ataxia	44.4	42.9	0.999	47.1	15.0	0.069
ataxic-rigid gait	40.0	63.6	0.374	35.7	33.3	0.999

*Data presented as median (25th percentile- 75th percentile)



Legend of figure 1.

Several different movement disorders in RD patients

A, B - bruxism and prognathism in a adult patient; C- severe scoliosis in a 14 yrs old patient; D – segmentar dystonia; E, F – the same peculiar dystonic feet posture in two different patients aged 2 and 16 years respectively; G – dystonia of the left inferior limb that interferes with gait; H, I – two different patients with feet dystonia; J – severe fixed feet in an adult patiet; K- dystonia of the hands in a 12 years old patient; hand athetosis in a 14 years old patient

Discussion

1. Stereotypies in Rett disorder

Hand stereotypies are described as the hallmark of Rett disorder. All patients in this series (except for a male with severe encephalopathy) presented hand stereotypies. As we described recently (10), these can be in the midline, with symmetrical movements of both hands (washing, clapping, tapping, wringing, hand mouthing), or with hands apart more frequently each hand performing a different movement (hair pulling with one hand, the other tapping the trunk; hair pulling with one hand, with the other to the mouth; pill-rolling

with one hand; twitching of two fingers; castanets, etc.). These almost continuous, repetitive and compulsive automatisms disappear during sleep and may aggravate with anxiety.

As we underscored before (10, 11), we show that the majority of the patients, in addition to hand stereotypies, also present stereotypies with other topographies: cervical retropulsion, head rolling, lip protusion, eye rolling, trunk rocking, intermittent leg elevation and tapping of the floor, toe walking, swaying movements of all the body with shifting weight from one leg to the other, etc. Stereotypies can also be very complex at the beginning of the disease, some girls exhibiting like a “stereotyped dance” (10).

Bruxism, so common in mentally retarded individuals (12), was almost always present in our RD patients and represented the second most frequent stereotypie. It occurred in the waking state and ceased during sleep. FitzGerald et al. (2) considered that bruxism could be a form of focal dystonia, but other authors consider it to be a type of self-injurious behaviour. Some older RD girls with bruxism present a particular maxilar morphology, with prognatism, possibly as a consequence of this particular continuous movement (figure 2).

Hyperpnea and apnea are considered by most authors as a signal of brainstem dysfunction (13) and deregulation of neurotransmitters (14); however, patients only present this when awake, and in some patients it disappears after some years of evolution, favouring the alternative concept that apnea and hyperpnea may be just another type of stereotypie. In addition, apnea and hyperpnea can also be present in mentally retarded individuals with autism (15), and in other neurological disorders associated with severe mental retardation, as the Pitt-Hopkins (16) and the Joubert syndromes (17).

We showed in a previous work (10) that the number of stereotypies tends to diminish, specially after age ten years. In this work, we analyzed the frequency of stereotypies with disease progression, and can conclude that the number of stereotypies decreases significantly after 60 months of evolution. Each girl tends to have the same stereotypies all along her life, but they tend to decrease and become more simple and slower with age, when patients become hypokinetic and rigid.

2. Dystonia

FitzGerald et al. (2) reported dystonia in 59% of Rett patients, more often crural or generalized, but also focal dystonia involving the upper or lower limbs. In general, our

results are similar, but we also found a difference in the distribution of dystonia after 60 months of disease evolution, suggesting a faster progression in patients with truncating mutations.

Usually, dystonia is asymmetric, the right side being more affected (18, 19); scoliosis, a common feature of RD, mainly at its later stages, is considered to be a consequence of this postural asymmetry. Some of our patients (11.7%), however, presented scoliosis after 48 months of disease evolution, without postural asymmetry. Like other authors (2), we believe that scoliosis in RD may correspond to truncal dystonia.

3. Rigid-akinetic syndrome

As Hagberg described (20), an inexpressive, “mask-like”, face may be an early clinical sign of Rett disorder, when accompanied by disproportionate eye communicating ability and normal eye blinking.

In a series of 32 patients analysed by FitzGerald et al. (2), hypomimia, bradykinesia and rigidity were more severe and common in older girls. Interestingly, we found that some patients with severe motor presentations, who never acquired an independent gait, presented rigidity very early in the disease evolution (less than 5 years). In our series, rigidity was more severe in patients with truncating mutations, especially those with a longer evolution of the disease.

4. Ataxic-rigid gait: Rett’s gait

The majority of the patients acquired independent ambulation. With the progression of the disease (the rate of which is variable), gait became more rigid, with poor arm balance, and some patients exhibited a particular wide-based, rigid, gait with abdominal muscle contraction and hyperextension of the legs (video). They can also present freezing when initiating a movement.

Patients presenting a segmental dystonia of their lower limbs tend to loose independent gait. Interestingly, one them regained the ability of walking, after being treated with botulinum toxin.

5. Tremor

FitzGerald et al. (2) described only kinetic tremor in the patients of their study; we propose that postural tremor also, or both, although less frequently, may also be present in some rigid and akinetic RD girls, especially in patients with truncating mutations, 5 years after of the onset of this disease.

6. Chorea and athetosis

Some hand stereotypies resemble choreoathetoid movements (2); however, in our experience, generalized chorea is very rare in RD patients.

7. Myoclonus

Myoclonus was reported in 34% of the patients examined by FitzGerald et al. (2), involving predominantly the head or trunk, and Guerrini et al. (21) observed myoclonus involving the distal part of the limbs, in 9 of 10 patients. Severity of myoclonus was not correlated with other symptoms or with age. Neurophysiological studies concluded that these patients showed a distinctive pattern of cortical reflex myoclonus.

Our classification of movement disorders in RD was based on clinical observation alone, as we did not perform electrophysiological studies to differentiate tremor from myoclonus. We only identified myoclonus in one of our patients, and the electrophysiologic study performed did not show a cortical myoclonus. Interestingly, during the periods of generalized myoclonia, the patient could not perform her usually continuous hand stereotypies, which may point to the overlap of neurological pathways of stereotypies and myoclonus. Further neurophysiologic studies are needed to clarify this point, and to differentiate tremor from myoclonus in some patients.

Conclusion

Movement disorders seem to reflect the severity and rate of progression of RD, patients with truncating mutations presenting more often a rigid-akinetic syndrome, when comparing groups with similar time of disease evolution.

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Legend for video 1.

Different stereotypies in patients with Rett disorder

Legend for video 2.

Rigidity in an 8 years old patient with Rett disorder

Legend for video 3.

Generalized myoclonia in a 14 years old patient with Rett disorder. During this episode she could not perform her usual hand stereotypies

Legend for video 4.

Generalized chorea in a 12 years old patient with Rett disorder. She was already under treatment with haloperidol.

Legend for video 5.

Postural tremor in two twelve old patients patients with Rett disorder; Kinetic tremor in a 22 old patient with Rett disorder.

Legend for video 6.

Particular ataxic and rigid gait of Rett disorder patients.

3.2.4 Stereotypies in Rett Syndrome: analysis of 83 patients with and without detected MECP2 mutations ([Article 6](#))

NEUROLOGY

Stereotypies in Rett syndrome: Analysis of 83 patients with and without detected MECP2 mutations

T. Temudo, P. Oliveira, M. Santos, K. Dias, J. Vieira, A. Moreira, E. Calado, I. Carrilho, G. Oliveira, A. Levy, C. Barbot, M. Fonseca, A. Cabral, A. Dias, P. Cabral, J. Monteiro, L. Borges, R. Gomes, C. Barbosa, G. Mira, F. Eusébio, M. Santos, J. Sequeiros and P. Maciel

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Stereotypies in Rett syndrome

Analysis of 83 patients with and without detected MECP2 mutations

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Abstract—Background: Hand stereotypies are considered a hallmark of Rett syndrome (RTT) and are usually described as symmetric movements at the midline. However, related pathologies may show the same type of involuntary movement. Furthermore, patients with RTT also have stereotypies with other localizations that are less well characterized. **Methods:** We analyzed stereotypies in 83 patients with RTT, 53 with and 30 without a mutation detected in the *MECP2* gene. Patients were observed and videotaped always by the same pediatric neurologist. Stereotypies were classified, and data were submitted to statistical analysis for comparison of mutation-positive and -negative patients and analysis of their evolution with the disease. **Results:** All the patients showed hand stereotypies that coincided with or preceded the loss of purposeful hand movements in 62% of the patients with *MECP2* mutations. The hair pulling stereotypy was more frequent in the group with detected mutations, whereas hand washing was not. Hand gaze was absent in all RTT patients with *MECP2* mutations. Patients with *MECP2* mutations also had more varied stereotypies, and the number of stereotypies displayed by each patient decreased significantly with age in this group. In all patients, stereotypies other than manual tended to disappear with the evolution of the disease. **Conclusions:** Although symmetric midline hand stereotypies were not specific to patients with an *MECP2* mutation, some of the other stereotypies seemed to be more characteristic of this group. In patients younger than 10 years and meeting the necessary diagnostic criteria of Rett syndrome, the association of hand stereotypies without hand gaze, bruxism, and two or more of the other stereotypies seemed to be highly indicative of the presence of an *MECP2* mutation.

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Rett syndrome (RTT) was discovered by Andreas Rett who noted that two girls waiting for his consultation presented the same movement disorder: hand stereotypies.

In 1966, Rett¹ published data on 22 girls with progressive cerebral atrophy, stereotyped hand

movements, dementia, alalia, gait apraxia, and a tendency toward epileptic attacks. As the disease is primarily sporadic in nature and familial cases are rare,² it took more than 30 years to determine its genetic basis: mutations in the methyl-CpG-binding protein 2 (*MECP2*) gene.

Stereotypies may be defined as involuntary, rhythmic, patterned, coordinated, repetitive, and seemingly purposeless movements or utterances that are usually continuous, in contrast with mannerisms or tics.³ They can be transient (physiologic)^{4,5} or persistent (pathologic).^{6,7}

Additional material related to this article can be found on the *Neurology* Web site. Go to www.neurology.org and scroll down the Table of Contents for the April 10 issue to find the title link for this article.

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In addition to motor and phonic, stereotypies can be classified as either simple (e.g., tapping, mouthing, clapping) or complex (e.g., a sequence of different movements always performed in the same way and sometimes seeming to have a purpose); they can also be described according to the predominant site involved (e.g., head, trunk, hand, inferior limbs).^{3,6}

Stereotyped hand movements are a hallmark of RTT, and one of its necessary diagnostic criteria.⁸ Usually they are associated with or follow the disappearance of purposeful hand movements, but can also be present before developmental regression begins.⁹ These almost continuous, repetitive, compulsive automatisms disappear during sleep. Environmental manipulation was shown to have a limited effect on their frequency, suggesting that these movements are reinforced through neurochemical processes.¹⁰

Other stereotyped movements and behaviors can also be present in RTT, but are much less well described.¹⁰⁻¹³

We describe the stereotypies that can be present in RTT and their lifelong evolution, based on an observational study of 83 patients with a clinical diagnosis of RTT, all studied for the presence or absence of *MECP2* mutations. We also compare the stereotypies present in patients with and without a positive molecular diagnosis.

Methods. All Portuguese pediatric neurologists were asked to indicate their patients with possible RTT. Patients were always observed and videotaped by the same pediatric neurologist, and a clinical checklist for RTT was completed. A classification of stereotypies was used that was modified from Jankovick³ and Fernandez-Alvarez and Aicardi.⁶ Informed consent was obtained from all parents to collect blood, and to take and use video and photographs.

Blood samples from patients and their parents were received at our laboratory, and genomic DNA was extracted 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. The robust dosage PCR method was used, as described,¹⁴ for the detection of large rearrangements in the *MECP2* gene. Primers and PCR conditions are available upon request.

Contingency tables were obtained, and χ^2 and Fisher exact tests were used. Groups were compared with Student's *t* test. The SPSS (v.14) statistical package was used to analyze the data.

Results. We observed 117 cases with possible RTT; 33 were excluded because they did not fulfill the revised diagnostic criteria and one because the patient was a man with a severe encephalopathy who never presented stereotypies.⁸ The mean age at the first observation of the 83 patients who fulfilled the diagnostic criteria was 10.0 years (range, 1 to 31, median, 7 years). Twenty patients were observed and videotaped two or more times at 6-month intervals.

Cases were classified as classic (60.2%) and variant (39.8%) forms of RTT. Mutations were found in 63.9% of all patients (*n* = 53), corresponding to 84.0% of the classic forms and 33.3% of the variants; all were *de novo*. Patients were thus divided into two groups: those with a positive molecular diagnosis (Group I) and those without (Group II).

All cases presented hand stereotypies that appeared

at a mean age of 22.3 months in Group I and 25.4 months in Group II, after decrease or loss of purposeful hand movements (22.2 months in Group I and 17.4 months in Group II), and several months after losing social contact (17.0 months in Group I and 11.1 month in Group II). In 25 cases (23 in Group I and two in Group II), loss of purposeful hand movements and appearance of stereotypies coincided; in 11 cases (10 cases in Group I and one in Group II), stereotypies preceded the loss of purposeful hand movements (mean time, 9.5 months; range, 5.0 to 25).

The most frequent hand movement observed was the compulsive wringing, washing-like movement of both hands, usually at the midline, most often in front of the body (73.26% in Group I and 80.0% in Group II). Other symmetric movements of both hands were also present (table; video E-1 on the *Neurology* Web site at www.neurology.org; figure 1). Stereotypies with separated hands, more often each hand performing a different movement and coexisting or not with midline hand stereotypies, were present in 60.2% of all the patients (60.4% in Group I and, 60.0% in Group II) (table; video E-2; figure 1). Of the Group I patients, 20.4% showed only hands-apart stereotypies. None of the patients in Group I looked at their hands while performing hand stereotypies, whereas 40.0% of those in Group II did (*p* < 0.001).

The second most frequent stereotypy in RTT was bruxism. We found bruxism in 90.4% of 83.0 RTT patients (94.3% in Group I and 83.3% in Group II); in all patients, it occurred while awake and disappeared during sleep.

Stereotypies with other topographies are described in the table (see also videos E-3 and E-4; figure 1). They could be very complex at the beginning of the disease; two girls had a stereotyped dancelike behavior involving many sites of the body, always performed in the same sequence, and seemingly with a purpose (table; video E-5).

Most patients (97.6%) had more than one stereotypy, and 31.7% (*n* = 26) had five or more different ones (38.5% in Group I and 20.0% in Group II). The number of stereotypies per patient was larger in Group I (a mean of 4.8 stereotypies in Group I and 3.7 in Group II), and the patients in this group exhibited a significantly greater number of topographies of stereotypies: 28 different stereotypies were found in Group I, whereas only 19 stereotypies were seen in Group II. Although 54.6% of the patients in Group I performed five stereotypies, only three were performed by 52.7% of the patients in Group II. Patients who acquired independent gait had a significantly larger number of stereotypies, in both groups. Group I (but not Group II) patients with rigidity showed a significantly smaller number of stereotypies.

The most frequent association was that of the hand-washing stereotypy with bruxism (69.1%; 70.6% in Group I, 66.7% in Group II). Among patients with more than one manual stereotypy, mouthing and hand washing were the most frequently associated (43.4%; 39.6% in Group I, 50.0% in Group II).

Significant differences between the mutation-positive (Group I) and mutation-negative groups (Group II) regarding the frequency of specific stereotypies were found (table).

We also found that the number of stereotypies decreased with age (particularly after the age of 10), mainly

Table Stereotypies found in a series of 83 patients with Rett syndrome

	Total, % (n = 83)	Group I, % (n = 53)	Group II, % (n = 30)	χ^2
Motor stereotypy				
Simple				
Head				
Rolling	1.2	1.9	0.0	0.573
Retropulsion	7.2	11.2	0.0	3.661*
Grimacing	8.4	11.2	3.3	1.583
Bruxism	90.4	94.3	83.3	5.956†
Protrusion of the lips	6.0	9.4	0.0	3.012
Repetitive closure of the eyes	7.2	9.4	3.3	1.063
Eye rolling	12.0	15.1	6.7	1.284
Joined hands	80.7	81.1	80.0	0.016
Washing	75.9	73.6	80.0	0.431
Clapping	14.5	15.1	13.3	0.048
Mouthing	21.7	22.6	20.0	0.079
Separated hands	60.2	60.4	60.0	0.001
Mouthing	36.1	37.7	33.3	0.161
Hair pulling	10.8	17.0	0.0	5.714†
Pill rolling	8.4	3.8	16.7	4.124*
One hand behind the neck	4.8	5.7	3.3	0.226
Castanets	1.2	1.9	0.0	0.573
Twisting two or three fingers	3.6	3.8	3.3	0.011
Flapping	2.4	1.9	3.3	0.170
Tapping	30.1	28.3	33.3	0.230
"Sevillana"	2.4	3.8	0.0	1.160
Hand twirling	8.4	9.4	6.7	0.190
Hand gaze	14.5	0.0	40.0	24.783‡
Arms				
Repetitive and rhythmic flexion of the arms	4.8	5.7	3.3	0.226
Legs				
Intermittent leg elevation and tapping of the floor	10.8	11.3	10.0	0.035
Toe walking	15.7	17.0	13.3	0.193
Jumping	1.2	1.9	0.0	0.573
Feet				
Feet twirling	3.6	5.7	0.0	1.762
Whole body				
Trunk rocking	21.7	22.6	20.0	0.079
Shifting weight from one leg to the other	20.5	24.5	13.3	1.474
Complex	2.4	3.8	0.0	1.160
Phonic stereotypy				
Repetitive sounds	4.8	5.7	3.3	0.226
Repetitive words or phrases	1.2	1.9	0.0	0.573

* $p < 0.1$.† $p < 0.05$.‡ $p < 0.010$.

in Group I (figure 2): a difference was observed between patients younger and older than 10 years of age ($t = 3.749$, $p = 0.001$) in Group I.

In the 20 patients observed various times (15 in Group I and five in Group II), the mean length of follow-up was 3.75 years (range, 1 to 6 years; median, 4

years). All maintained the pattern of manual stereotypies, but there was a change in the pattern of stereotypies with other localizations: new stereotypies might be added to or replace one of previous ones. Stereotypies other than manual ones tended to disappear with evolution of the disease.



Figure 1. Different topographies of stereotypies in Group I Rett syndrome patients. Typical hand washing stereotypies (A to C); mouthing with hands apart (D to G); mouthing with joined hands (H); hair pulling (I, J); cervical retropulsion (K to M) (K and L with simultaneous closure of the eyes); twisting of the fingers with hands apart (N); shifting weight from one leg to the other (O, P).

Discussion. Stereotyped hand movements are considered a hallmark of RTT, and it has been assumed that they are symmetric and at midline.^{10-13,15-19} The aim of this study was to analyze the specificity of the stereotypies in patients with a positive molecular diagnosis of RTT. There are a number of limitations to this study, including the fact that it was a single-rater, cross-sectional, and observational, and, therefore, state-dependent study; however, 24% of the patients were observed several times, and complete video records allowed reanalysis and increased objectivity.

Although washing-like movements of both hands are considered an indicator of RTT, this finding is not specific of the syndrome; remarkably, in this study, this stereotypy was more frequent in the group with no mutation in the *MECP2* gene. In addition,

a significant proportion of the mutation-positive patients showed only hands-apart stereotypies. One of the singularities of *MECP2* mutation-positive RTT patients was that they tended not to look at their hands when performing hand stereotypies, possibly because they have very poor ocular-manual coordination.

Differences were found in the frequency of four stereotypies, and of these, hair pulling, bruxism, and cervical retropulsion were more frequent in the mutation-positive group.

In general, mutation-positive patients had more diverse stereotypies that diminished after the age of 10. These patients also had a larger number of stereotypies per patient. In patients younger than 10 years and with the necessary diagnostic criteria of RTT, the association of hand stereotypies without

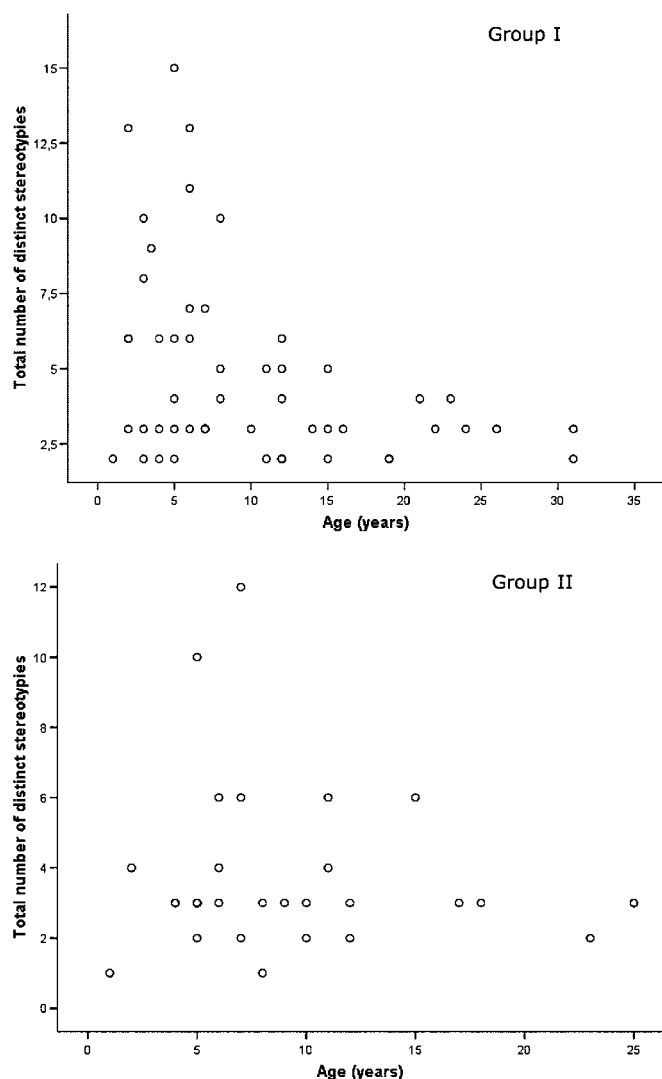


Figure 2. Total number of distinct stereotypies by age (some points in the graph may correspond to several patients). The number of stereotypies decreased with age (particularly after age 10 years), especially in Group I, in which a significant difference was observed between patients younger and older than 10 years ($t = 3.749$, $p = 0.001$).

hand gaze, bruxism, and two or more of the other stereotypies seemed to be highly indicative of the presence of an *MECP2* mutation.

The pathophysiologic basis of stereotypies in RTT remains elusive, and we do not know whether these involuntary movements interfere with motor learning. Most studies stress that hand stereotyped movements coincide with or follow the disappearance of purposeful prehension in RTT; however, a video analysis of 22 patients in the first 6 months of life, before the beginning of regression, showed stereotyped hand movements in 42% of cases.⁹ In our series, in the group with *MECP2* mutations, stereotypies were also described by the parents as coinciding with the loss of purposeful hand movements in 43.3% and preceding it in 18.8%. We thus

believe that the particularly compulsive behavior of stereotypies in patients with RTT may have a role in the complex process of loss or reduction of hand use.

Like other authors,^{12,13} we found that manual stereotypies became simpler and slower with the progress of the disease, as patients become hypokinetic and rigid; however, each patient maintained the same type of hand movements throughout the period of observation. Other stereotypies tended to disappear and behaved like tics, with one stereotyped movement replacing another. This allows us to speculate that the physiopathology of hand stereotypies may be different from that of other topographies.

We conclude that stereotypies in RTT can be pleomorphic, mainly in the first decade of life. Nevertheless, the pattern of some repetitive movements associated with this disorder suggests that they have underlying monotonous motor programming, the basis of which should be investigated.

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3.2.5 Abnormal movements in Rett syndrome are present before the regression period: a case study ([Article 7](#))

how the deep gray matter is involved in the control of movement, behavior, and sleep.

VIDEO LEGEND TO FULL VIDEO CLIP

Segment 1 (0:00–0:36). Case 1 demonstrates repetitive stereotypical self-injurious behavior, pushing his finger into his eye. He exhibits echolalia and palilalia. The hyperkinetic movements are flailing and stereotypical.

Segment 2 (0:37–1:26). After weeks of sedation and ventilation, the patient was lightened for reassessment. Stereotypical cycling movements remained. There were frequent episodes of upward eye deviation plus rabbit-like oro-buccal dyskinesias.

Segment 3 (1:27–1:55). As the patient improved, he began talking again, although still with echolalia and palilalia. He had on-going dyskinesias (chorea), which was thought to be partly L-dopa induced.

Segment 4 (1:56–2:21). Before discharge, the patient is now walking, although still with occasional extra intrusive movements. He is nearly back to his premorbid function.

VIDEO LEGEND TO EARLYVIEW CLIP

Case 1 exhibits repetitive stereotypical self-injurious behavior, pushing his finger in his eye. He has hyperkinetic movements that are flailing and stereotypical. Half way into his illness, the stereotypical cycling movements remain. There were frequent episodes of upward eye deviation plus rabbit-like oro-buccal dyskinesias.

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Abnormal Movements in Rett Syndrome are Present Before the Regression Period: A Case Study

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Video



Abstract: The suspicion of a diagnosis of Rett syndrome (RTT) is based on clinical criteria that are often not present in the first two stages of the disease, as many of its symptoms will appear at a later age. This sometimes postpones the genetic diagnosis and an early clinical intervention. We present the case of 19-months-old girl who came to the consultation be-

This article includes supplementary video clips, available online at <http://www.interscience.wiley.com/jpages/0885-3185/suppmat>

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cause of an arrest of psychomotor development noticed 5 months earlier without change in sleep pattern, behavior, or social communication. In the observation of 1 hour videotape, she presented subtle stereotypic movements of the face and hands as well as repetitive dystonic posturing of her limbs. A genetic test confirmed the diagnosis of RTT, showing a truncating mutation in the *MECP2* gene (*R270X*). This case confirms that stereotypic movement anomalies, albeit infrequent and subtle, are already present before the regression stage and while maintaining prehension and that, in addition, repetitive dystonic postures may occur. Recognition of these early movement disorders will improve clinicians' ability to perform an earlier diagnosis of RTT. © 2007 Movement Disorder Society

Key words: stereotypies; transient dystonia; paroxysmal dystonia.

Stereotypies are the most constant finding in Rett syndrome (RTT) patients in all stages of the disease. Apart from the severe and fatal forms of conatal encephalopathy in RTT males, there are no RTT patients without these abnormal movements.

As we have shown recently,¹ the clinical spectrum of stereotypies in RTT is very wide, and they may have a rather variable topography. In this work, we showed also, as others have done before,² that hand stereotypies coincided with or preceded the loss of purposeful hand movements, in most patients with *MECP2* mutations. Furthermore, studies based on the observation of domestic videos of RTT girls, before the period of regression, demonstrated that other subtle stereotypies, although usually unnoticed by the patient's family, are already present before the appearance of the exuberant hand stereotypies characteristic of the disorder.² However, to our knowledge, these videos were never published.

Herein, we present the case of a 19-months-old girl with RTT showing very subtle stereotypies and repetitive dystonic movements, before a clear regressive period and while maintaining prehension.

CLINICAL PRESENTATION

We saw this 19-months-old girl for the first time due to an arrest of psychomotor development noticed at 15 months of age. She was the first child of a young, healthy, unrelated couple, and had a normal obstetrical and neonatal history.

At birth, her occipital frontal circumference (OFC), weight and height were at the 5th percentile, but reached the 25th percentile by the age of 6 months. Then, OFC growth slowed down, progressively, until reaching the 5th percentile at 19 months of age.

She controlled her head at 3 months, grasped an object at 4 months, and had propositive prehension when she was 5 months old, without hand preference. By 8 months of age, she reached the unsupported seated position.

When she was aged 15 months, the parents became preoccupied because she did not crawl in an usual way or pass from the seated to the supine position. They did not notice any change in her sleep pattern, behavior, or social communication.

At a first glance, she was a normal, happy, and well nourished 19-months-old girl. She had no dysmorphic features and her eye contact was normal. She was interested in toys, but had no interest in their properties: she manipulated them in a very simple and stereotyped manner, and took them systematically to the mouth. She repeatedly interrupted her playing to do repetitive, dystonic movements with her right limbs (Fig. 1; Video, Segment 1). Intermittently, she also had stereotyped facial movements, such as closing of her eyes, protrusion of her tongue or lips, and grimacing (Fig. 1).

During the observation of a one-hour videotape, she twisted two fingers of the left hand, five or six times, had two instantaneous washing-like movements of her hands (Fig. 1), clapped hands three times, and twirled the hands for several times. She also had intermittent hand-to-mouth movements (Video). She seated well, without support, but could not crawl and propelled her body in a very peculiar way (Video, Segment 1). Sometimes, she tried to crawl on the four limbs, and then adopted, on occasion, a dystonic posturing of the right hand (turning it inward) (Fig. 1).

Her neurological examination, apart from the abnormal movements described earlier, was normal, as was her general examination.

Cerebral MRI was normal, but the EEG showed bilateral centroparietal spikes. Genetic testing confirmed the diagnosis of RTT, showing a truncating mutation in the *MECP2* gene (*R270X*).



FIG. 1. (A) Maintenance of good social contact; (B) eye closure stereotypie; (C) protrusion of the lips; (D) hand-washing stereotypie; (E) stereotypie with hands separated; (F–H) repetitive dystonic movements of her right limbs; (I) dystonic posture of her right hand while crawling.

She was reobserved at 22 months of age. She acquired independent gait at 20 months of age. The same hand and face stereotypies were present, and more frequent, but repetitive dystonic movements were no longer observed. Manipulation was more frequently interrupted by hand stereotypies. Social contact remained good but she showed mood disturbance with irritability and laughing spells (Video, Segment 2). Head size was now below the five percentile.

DISCUSSION

Clinical suspicion of a diagnosis of RTT, an X-linked disorder, is based on well-defined criteria,³ which, nevertheless, are often not present in the early stages of the disease. This sometimes delays a genetic diagnosis and the clinical intervention. There is growing evidence, however, that development is not normal in RTT patients since infancy. Observational studies of home videos have shown that some movement abnormalities may already be present before developmental regression occurs.^{2,4-6}

This case confirms that movement abnormalities, although infrequent, are present before the regression stage of RTT, including washing-like, twirling, clapping, or twisting hand movements, as well as repetitive closure of the eyes, grimacing, and tongue protrusion.¹ Differential diagnosis between stereotypies and tics is sometimes difficult, mainly because both can coexist in the same patient. Stereotypies have an earlier age of onset, usually appearing before the age of 2 years, and their topography is usually more distal, tics affecting proximal muscles of the limbs and face. However, both can affect all the body parts.⁷ As recently described,¹ stereotypies in RTT can be very pleomorphic, mainly in the first decade of life, and stereotypies other than manual ones (like lip protrusion and eye closure) behave like tics, one replacing the other, and tend to disappear with time. Furthermore, there are an overlap of symptoms between RTT and autism and in the former condition tics and stereotypies frequently coexist.⁸ We hypothesize that some of the abnormal movements named as stereotypies in RTT may correspond to tics.

Besides these stereotyped movements, we demonstrate for the first time that repetitive dystonic movements may also occur, preceding exuberant stereotypies. Dystonia is a common symptom in RTT but, usually, it presents as a fixed posture and appears after some years of the evolution of the disease.⁹⁻¹¹ Some of the repetitive dystonic limb movements of the presented patient also resemble dystonic tics. On the other hand, the hand dystonic posture when crawling (Fig. 1), resemble those described by Willemse in the syndrome of transient idiopathic dystonia of infancy; however, in that entity abnormal

movements appear at rest and cease when the infant carries out propositive movements with the affected extremity¹² and, in addition, the hemidystonic character of the movements favored a symptomatic etiology.¹³

Although transient abnormal movements in the pre-regression stage of RTT were also referred by other authors when analyzing domestic videos of these children,^{2,5} to our knowledge it is the first time that these movements are described with detail and videos and photographs published. In most cases, these abnormal movements are not noticed by the parents. Often, only a long observation of the patient by a clinician, particularly if a video review procedure is included, may allow their identification.

We believe this case description and video may contribute to the early recognition of movement disorders in RTT, and potentially allow an earlier diagnosis of this disease.

LEGENDS TO THE VIDEO

Segment 1. Videotape of the patient when she was 19 months of age. She maintained prehension and was attracted by objects, but had no interest in their properties. She only manipulated them in a very simple and stereotyped manner, and repeatedly took them to the mouth. When playing with toys, she repeatedly interrupted this playing to do repetitive dystonic movements with the limbs of the right side of her body. She tried to crawl, but could not alternate her legs, and propelled her body in a very particular way.

Segment 2. Videotape of the patient when she had 22 months of age. She acquired independent gait at 20 months of age. Hand stereotypies were more frequent but repetitive dystonic movements were no more observed. Manipulation was more frequently interrupted by hand stereotypies. Social contact remained good but she showed laughing spells.

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improvement was bilateral. Our case illustrates that stereotactic thalamotomy may be considered as an option in treating severe tremor in selected patients of Wilson's disease and merit further trials. © 2007 Movement Disorder Society

Key words: Wilson's disease; thalamotomy; tremor.

The common neurologic manifestations in Wilson's disease (WD) are dysarthria, tremor, dystonia, Parkinsonism, and gait disturbances.^{1,2} The characteristic "wing-beating" tremor is often resistant to decoppering therapy and antitremor drugs, leading to severe morbidity. Though the usefulness of thalamotomy and thalamic deep brain stimulation for drug-resistant essential tremor and tremor predominant Parkinson's disease has been well established, there is lack of experience in surgical treatment of tremor associated with other neurologic disorders, in particular WD. We report a young man with WD and uncontrollable bilateral upper limb tremor, who had an excellent response to unilateral thalamotomy.

CASE REPORT

A 30-year-old man presented with disabling tremor of right hand, which was severe on action and in certain postures for the past 3 years. Six months after the onset of tremor in right hand, he developed tremor in left hand, and after another 2 years, tremor of head and slurring of speech. He had jaundice 15 years and 18 months prior to his presentation to us. For the past 15 years, he was also having behavioral disturbances in the form of irritability and outbursts of anger.

He had Kayser–Fleischer rings, reduced facial expression, dysarthria, and mild restriction of upward gaze. Examination of other cranial nerves, muscle tone and power, sensory examination, stretch reflexes, and plantar responses were normal. Other findings were dystonia of left great toe, a slightly broad-based gait, and Grade 1 postural instability on pull test. Video segment 1 shows the type and severity of tremor, which was of wing beating type. Head tremor was present most of the time. When the hands were at rest, tremor was minimally present intermittently. On attempting to raise hands, an escalating tremor appeared bilaterally resulting in wide flinging movements and the limbs needed to be restrained to prevent injury. He was unable to do finger-to-nose test or hold a glass or pen.

A diagnosis of WD was confirmed by low total serum copper (42.5 µg/dl; reference range: 70–150 µg/dl), low serum ceruloplasmin (<0.072 g/L; reference range: 0.2–0.6 g/L), and high 24 hours urinary copper (700 µg/24 hours; reference range: up to 70 µg/24 hours). Ultrasound showed diffuse heterogeneous echo pattern of

Successful Treatment of Tremor in Wilson's Disease by Thalamotomy: A Case Report

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Video



Abstract: Little information is available on the surgical treatment of movement disorders in Wilson's disease. We report a successful outcome of left-sided stereotactic thalamotomy in a 30-year-old man with Wilson's disease, who had severe postural-kinetic tremor of both hands. The

This article includes supplementary video clips, available online at <http://www.interscience.wiley.com/jpages/0885-3185/suppmat>

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3.3. Biochemical studies in Rett syndrome and Rett disorder

The results described in this chapter are included in the following manuscripts:

Article 8. Is it worth to evaluate CSF neurotransmitters and folate in Rett disorder? (submitted to Neurology)

Article 9. Folate Receptor Autoantibodies and Cerebral Folate Deficiency in Rett Syndrome (published)

3.3.1. Introduction

In his initial neuropathological examinations, Andreas Rett described a delay in the development of melanin pigmentation in the brainstem neurons. This was the very first description implicating the involvement of the monoaminergic system in this disorder.

Nomura *et al.* were also among the first to suggest that neurotransmitter anomalies could form the chemical basis for RTT, postulating that the movement disorders and altered sleep pattern could be related to a deficiency in brainstem dopamine and serotonin, respectively, but conflicting reports regarding the levels of biogenic amine metabolites in CSF have appeared since, from several laboratories. The hypothesis that a neurotransmitter deficiency could affect brain function and/or development in RTT may be appropriate. Rett syndrome manifests itself in infancy, a critical period for brain development, when many neurotransmitters change their pattern of expression and act as growth factors.

More recently, Raemaekers *et al.* 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, as well as serotonin and dopamine biosynthesis. Nevertheless, a larger study concluded that folate deficiency, or a disorder in folate transport across the blood-brain barrier was not contributing to the pathogenesis of RTT.

The aim of our work was to re-evaluate these hypotheses and, thus, the role of neurotransmitters and folate in the pathophysiology of RTT.

3.3.2. Is it worth to evaluate CSF neurotransmitters and folate in Rett disorder? (Article 8)

Is it worth to evaluate CSF neurotransmitters and folate in Rett disorder?

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“Disclosure: The authors have reported no conflicts of interest”

Key words: Rett syndrome, neurotransmitters, folate, movement disorders

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 folate deficit is not contributing to the pathogenesis of RD and that folinic acid supplementation has no significant effects on the course of the disease. In contrast, 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.

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 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 (5MTHF) and normal serum folate levels. After oral folinic acid supplementation, clinical improvement and normalization of CSF 5MTHF levels were reported. Two years later, a Spanish study of 16 RTT patients (8 with a detected *MECP2* mutation) described low CSF 5MTHF in 50% (3 with a *MECP2* mutation). All patients with low 5MTHF had epilepsy; thus, a positive correlation between low CSF 5MTHF 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 5MTHF 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 5MTHF levels; we also aimed at (2) evaluating the clinical response to folinic acid supplementation in patients with low levels of 5MTHF, in terms of social contact, behaviour, motor function and epilepsy control.

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.

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 5MTHF, neopterin, biopterin, 3-O-methyldopa, homovanillic acid (HVA), 3-methoxy-4-hydroxy phenylglycol (MHPG), 5-hydroxytryptophan, 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 and plasma were compared with the reference ranges obtained in controls of similar age.

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 to 1.5 mg/Kg/day and 1mg/Kg of B12, 3 days in a week, after observation of low 5MTHF 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 analyse the data.

Results

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

1. RD and CSF folate

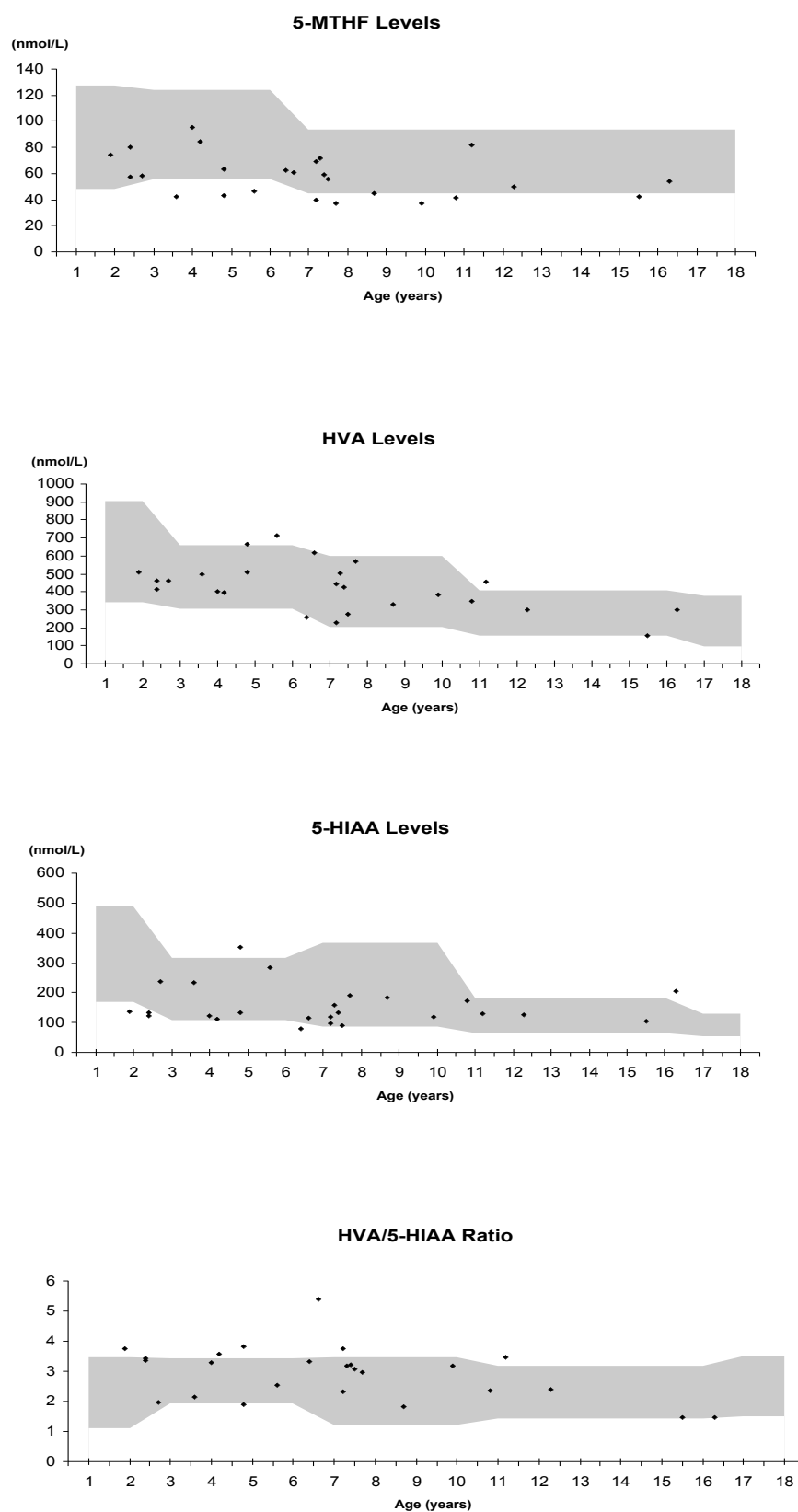
All patients had normal plasma folate values, but in 8 (32%), CSF folate was below the reference values (mean 41, median 41.5, range 37-46 nmol/L). Only two of the patients with low CSF 5-MTHF levels had epilepsy, both being resistant to the prescribed antiepileptic drugs (Valproate and Carbamazepine). Supplementation with folinic acid and B12 vitamin was applied to all patients with low CSF folate. After six months of treatment, no improvement in seizure control, global motor function, or reduction of stereotypies was observed.

2. RD and CSF neurotransmitters

Three of the four patients with the R294X mutation (cases 2, 3, 8 – see Table) 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 (video). Moreover, four patients with a median age of 7 years (range 2-7) had high HVA/ 5-HIAA (patients 10, 11, 14, 25).

Three patients (two under age 3 years and one aged 6) had low levels of 5 HIAA (patients 11, 18, 21). One (patients 21) had also low levels of HVA; this patient had a segmental dystonia of the right inferior limb, which had developed in the last 18 months (Figure 2). In two girls with low levels of 5 HIAA (pts 11 and 18), 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.



Legend of Figure 1 - CSF folate, biogenic amine metabolites and pterines in 25 patients with RD



Legend of Figure 2: Patient n° 21 (table), who had low CSF levels of 5HIA and HVA. We can observe a segmentar dystonia of the right inferior limb.

Discussion

Amongst the unique combination of symptoms in RD, motor symptoms stand out as its hallmark when analyzing the essential pathophysiology. Neuropathology, so far (13), has not provided capable answers as to why RD patients develop such complex clinical features.

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 folinic 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 folinic acid supplementation, lumbar puncture was not repeated for ethical reasons. Our results support that folate deficit is not contributing to the pathogenesis of RD and that folinic acid supplementation has no significant effects on the course of the disease. More studies with RD patients may be needed to clarify this further.

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 5MTHF 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 three 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 (14-20). Serotonergic receptor-binding was found to be increased in the brainstem nuclei, in RTT, which may reflect decreased serotonergic input, with a compensatory upregulation of receptor binding and/or binding affinity (21).

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, 22, 23), 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 an-age related abnormality was considered (24-25). 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 (26). Intriguingly, in our study, three of the four patients with the R294X mutation (patients 2, 3, 8) 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 (video) (27). Robertson et al. (28) 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.

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

“Online Supplemental Data”

Patient	Age (years)	Stage	MECP2 mutation	Indepen dent 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	7,5	III	R306C	+	-	-	-	NA	56	5,6	14	17	90	277	3,08	NA	NA	NA
2	5,6	III	R294X	+	+++	+	-	NA-	46()	8,9	24	16	283	714()	2,52	Yes	NA	Yes
3	11,2	IVA	R294X	+	+++	+	+	LVT, CMZ	82	6,5	10()	20	130	453()	3,48()	NA	NA	NA
4	9,9	III	R168X	+	+	-	-		37()	5,9	13	21	120	382	3,18	Yes	NA	No
5	15,5	IVB	K39fsX43	-	-	+	+	CMZ, VPA	42()	6,4	11	8()	105	154()	1,47	Yes	No	No
6	7,7	III	R133C	+	-	-	-	NA	37()	6,5	13	18	192	570	2,97	Yes	NA	No
7	10,8	III	T158M	+	+	-	-	NA	41()	9.1	12	18	174	347	2,36	Yes	NA	Yes
8	4,8	III	R294X	+	+++	+	-	NA	43()	10,1	31	21	352()	666()	1,89()	Yes	NA	Yes
9	7,2	III	R168X	-	+	+	+	CMZ, VPA	40()	7,6	11	10	98	226	2,31	Yes	No	No
10	6,6	III	T158M	+	+	+	+	CMZ, VPA	61	11,3	25	19	114	614	5,39()	NA	NA	NA
11	1,9	III	R270X	+	+	-	-	NA	74	12,6	9	25	135()	506	3,75()	NA	NA	NA
12	7,4	III	T158M	+	-	-	+	CMZ	59	12,9	17	21	132	424	3,21	NA	NA	NA
13	7,3	III	R106W	-	-	+	+	CMZ	72	16,1	14	29	158	502	3,18	NA	NA	NA
14	7,2	III	Del (exon 3 and 4)	+	-	-	+	CMZ	69	7,1	14	22	119	445	3,74()	NA	NA	NA
15	2,4	II-III	S113P;P251P	-	-	+	+	CMZ, VPA	80	15.6	13	20	122()	411	3,37	NA	NA	NA
16	4,0	III	R270fsX288	+	+	-	+	VPA	95	16,3	12	28	122()	401	3,29	NA	NA	NA
17	8,7	III	K305R	+	-	+	-	NA	45	12.1	14	12	182	331	1,82	NA	NA	NA
18	2,4	III	R168X	-	+	-	-	NA	57	9,4	8	24	134()	459	3,43	NA	NA	NA
19	4,2	III	R306H	+	+	-	-	NA	84	4,6	16	17	110	393	3,57()	NA	NA	NA
20	16,3	IVB	R168X	-	-	+	+	CMZ	54	9	12	13	203()	299	1,47()	NA	NA	NA
21	6,4	III	Del (7pb) V300frX318	+	++	++	-	NA	62	12.7	14	17	78()	259()	3,32	NA	NA	NA
22	2,7	II-III	T158M	-	+	-	-	NA	58	8,7	22	17	236	460	1,95	NA	NA	NA
23	12,3	III	R133C	+	-	-	-	NA	50	3,2	11	23	125	300	2,4	NA	NA	NA
24	3,6	III	R294 X	+	+++	+	+	CMZ	42()	17,9	22	14	234	500	2,14	NA	NA	NA
25	4,8	+	R133C	+	-	-	-	NA	63	9,7	13	26	133	510	3,83()	NA	NA	NA
Controls	0.5-2								48-127	2,6-20	8-43	8-54	170-490	344-906	1,11-3,48			
	56-124								7-55		10-52	106-316	304-658	1,92-3,44				
	45-94								10-46		8,2-68	87-366	202-596	1,20-3,45				
	45-94								10-24		13-36	63-185	156-410	1,44-3,17				
	45-94								9-27		11-30	55-130	98-376	1,50-3,50				

LVT – levetiracetam; CMZ – carbamazepine; VPA – valproic acid; NA - not applicable

3.3.3. Folate Receptor Autoantibodies and Cerebral Folate Deficiency in Rett Syndrome ([Article 9](#))

Folate Receptor Autoantibodies and Spinal Fluid 5-Methyltetrahydrofolate Deficiency in Rett Syndrome

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Key words

- Rett Syndrome
- folate metabolism
- folate receptor antibodies

Abstract

▼
Rett syndrome was associated with low cerebrospinal fluid (CSF) 5-methyltetrahydrofolate (5MTHF) in 42–50% of European patients whereas approximately 93% of the patients from North-America had a normal CSF 5MTHF status. We determined the CSF folate status in Rett patients living in North- and South-Western Europe and measured serum folate receptor (FR) autoantibodies of the blocking type to explain the reduced folate transport across the choroid plexus. Irrespective of their MECP2 genotype and despite normal plasma folate values, 14 of 33 Rett patients (42%) had low CSF folate lev-

els. Blocking FR autoantibodies were found in 8 of the Rett patients (24%), 6 of whom had low CSF folate levels. FR autoimmunity was primarily found within the group of Rett patients with low CSF folate status with a higher incidence in North-Western Europe. In Rett patients from North-America 74 of 76 girls had higher folate values in both serum and CSF than European patients. The food folate fortification in North-America may account for the higher folate levels and may prevent CFD in these Rett patients. FR autoimmunity occurred predominantly in Rett patients from North-Western Europe and may contribute to cerebral folate deficiency (CFD).

Introduction

▼
After intestinal absorption of food-derived folates, intestinal cells will convert folate compounds to 5MTHF, which represents the predominant and only reduced folate form in plasma which is able to cross the blood-brain barrier. Plasma 5MTHF is actively transported into the spinal fluid compartment by folate receptor (FR) endocytosis at choroid epithelial cells. Approximately 42–50% of Rett patients from Europe and Israel were previously reported to have a reduced concentration of 5-methyltetrahydrofolate (5MTHF) in the cerebrospinal fluid (CSF) [1–3]. Oral folinic acid supplementation restored 5MTHF levels in the spinal fluid with partial clinical improvement and better seizure control [1,3]. The reduced folate in the CSF could not be attributed to abnormalities of the FR1 or FR2 genes and was independent of methyl-CpG binding protein (MECP2) status [1]. A recent study from North America reported that only 2 of 76 female Rett patients had a low spinal fluid 5MTHF level [4].

Serum FR autoantibodies of the blocking type have been associated with the infantile-onset CFD syndrome whereas in age-matched controls and in serum from subjects with central nervous system disease unrelated to cerebral folate deficiency these FR autoantibodies were absent. In vitro studies substantiated the blocking action of these FR autoantibodies that form a complex with the FR at its folate-binding site and thus impairs the binding of 5-methyltetrahydrofolate (5MTHF) to the FR [5]. Recent studies now have confirmed an inverse correlation between the titer of the serum FR autoantibodies and the CSF 5MTHF levels (unpublished results). In order to explain the low CSF folate in 42–50% of Rett patients due to reduced folate transport into the CNS, serum samples from all study participants were analysed for blocking autoantibodies against the folate receptors (FRs).

Methods

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According to the internationally defined clinical criteria for Rett syndrome, thirty-three girls with

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Table 1 Age, genotype and laboratory findings in European Rett girls

Patient	Age years	MECP2 genotype	Serum folate nmol/L*	CSF 5MTHF nmol/L §	Folate Ratio CSF/serum	Autoantibodies pmol FRs/ml	AED
1	13.5	normal	47	15	0.32	1.22	none
2	4.3	normal	16.5	12	0.72	1.5	E
3	5.7	normal	22.5	30.9	1.3	1.63	none
4	3.8	R106W	ND	14.1	–	2.04	none
5	13.1	P152R	33.22	12.5	0.37	0.3	none
6	13	dup 763–775	13.2	17	1.28	1.78	none
7	4	del exon 2–3	19	45	2.36	negative	V
8	6	del G 483	29.24	68	2.32	negative	none
9	6	normal	ND	92	–	negative	none
10	16	R294X	ND	68	–	negative	C
11	19	normal	8.8	38	4.31	negative	C + R
12	11	normal	ND	73	–	negative	V + E
13	3	S229L	34.7	40	1.15	negative	V
14	5	R306C	45	64	1.45	negative	C
15	2	R255X	15.1	47	3.13	0.29	none
16	2	normal	62.2	65	1.04	negative	V
17	2	Y141X	40	69	1.72	negative	none
18	4	P302H	9.4	34	3.6	negative	V
19	10	R306C	9.9	33	3.3	negative	C + R
20	5.6	R294X	20.15	46	2.28	negative	none
21	15.5	K39fsX43	14.49	42	2.89	negative	V + C
22	10.9	R133C	ND	41	–	negative	none
23	4.9	R294X	22.8	43	1.88	negative	none
24	7.1	R168X	17.21	40	2.32	negative	V + C
25	3.4	normal	14.94	57	3.81	negative	none
26	9.3	normal	27.18	48	1.76	0.34	none
27	6.4	normal	19.93	42	2.10	negative	none
28	5.6	normal	21.06	45	2.13	negative	none
29	16.3	R168X	20.38	54	2.64	negative	V + C
30	2.8	T158M	19.70	58	2.93	negative	none
31	15.7	normal	12.45	46	3.69	negative	V + C
32	3.7	R294X	40.54	42	1.03	negative	none
33	6.6	T158M	25.59	61	2.38	negative	V

Patient 1–8 from Northern Europe and patient 9–33 from Southern Europe. ND = not determined

*Serum folate for healthy controls with a mean at 24.72 and range 8.3–45 nmol/L

§ CSF folate values below the reference range have been indicated by bold numbers. Reference range of CSF 5MTHF for healthy controls from Northern Europe, 0–1 years 64–182 nmol/L, 2–4 years 63–111 nmol/L, 5–10 years 41–117 nmol/L, 11–16 years 41–117 nmol/L, > 16 years 41–117 nmol/L

Reference range of CSF 5MTHF for healthy controls from South-Europe, aged 0–1 years 63–129 nmol/L, 2–3 years 44–122 nmol/L, 4–18 years 42–81 nmol/L

Abbreviation for anticonvulsant drugs: V: Valproate; C: Carbamazepine; R: Clonazepam; E: Ethosuximide

a classical Rett phenotype were selected for this study [6]. Complete genetic analysis of the MECP2 gene revealed abnormalities in 21 of the 33 patients. Epileptic seizures were found in 15 of 33 Rett patients, for which anticonvulsant drugs were administered. All patients whose serum and red blood cell folate levels were normal, underwent a spinal tap to determine the concentration of 5MTHF in the CSF. Eight girls with Rett syndrome lived in North-Western Europe (patient 1–6 in Germany; patient 7,8 in Belgium), while 25 patients resided in Southern Europe (patients 9–19 in Spain; patients 20–33 in Portugal) (Table 1). Age-related references for 5MTHF in the CSF have been established for both populations from North-Western and South-Western Europe [5,7].

To determine the cause of reduced 5MTHF in the CSF, serum samples were analyzed for the presence of blocking autoantibodies against the FRs using previously reported methods [5]. Statistical analysis used the Fisher exact test to compare the incidence of low CSF folate and FR autoimmunity among the two groups from North-Western and South-Western Europe, and to study the association of FR autoimmunity with CSF folate status.

The Fisher exact test was also applied to compare the incidence of cerebral folate deficiency (CFD) between Rett patients from Europe and North-America, and to determine the incidence of FR autoantibodies in Rett patients in Europe and healthy controls. Statistical analysis used the analysis of variance for independent samples to compare the serum and CSF folate values between the European Rett population, North-American Rett population and healthy controls. The analysis of variance was also used to compare the CSF folate levels between Rett girls residing in North-Western, South-Western parts of Europe and healthy controls. Data have been used from a study on North-American Rett girls and from the available data on serum folate extracted from the American National Health and Nutrition Examination Survey 1999–2000 [4,8].

Results



Compared to North-American Rett patients and healthy European controls, the 33 Rett girls from Europe had a statistically

Table 2 Comparison of folate levels in serum, 5MTHF levels in CSF and serum FR autoantibodies among Rett patients from Europe, North America and healthy controls

Analyte	European Rett (S1)	American Rett (S2)	European Controls (S3)	American Controls	Statistical test	Significance
number of patients	33	70	28	1586		
age (years)	2–19	2.7–20.2	1.9–19	3–19		
serum folate						
mean	24.36	38.14	24.72	39.38	F-value = 7.62	p = 0.00080
range	8.8–62.2	5.4–103.1	8.3–45	10.2–68.6	S1 vs. S2 S1 vs. S3 S2 vs. S3 t-test (&) = 3.71	p < 0.01 NS p < 0.01 p < 0.01
CSF folate						
mean	45.53	72.6	82 *	75.7	F-value = 21.879	p < 0.0001
range	12–92	27–136	44–181	40–150	S1 vs. S2 S1 vs. S3 S2 vs. S3	p < 0.01 p < 0.01 NS
number of patients with CFD	14	2			Fisher test (§) p = 0.0000	significant
FR antibodies						
number	8	unknown	0		Fisher test (†) p = 0.011	significant
mean titer	1.25					
range	0.29–2.04					
incidence of FR antibodies for low vs normal CSF folate	6/14 vs 2/19	unknown			Fisher test p = 0.037	significant
anticonvulsant drugs in CFD vs normal CSF folate	7/14 vs 8/19				Fisher test p = 0.250	NS

* CSF folate data derived from 99 healthy controls;

S1: Sample representing European Rett patients

S2: Sample representing American Rett patients

S3: Sample representing European healthy controls

§: Fisher test between European and American Rett patients

†: Fisher test between European Rett patients and healthy controls

&: t-test for comparison between healthy European and American controls

lower 5MTHF value in their CSF (analysis of variance F-value = 21.879 and $p < 0.0001$; ● **Table 2**).

In 14 European Rett patients (10 subjects with MECP2 gene abnormalities and 4 with a normal genotype), the concentration of 5MTHF in the CSF was below the lower reference limit of healthy controls (● **Table 1**). However, in 19 patients (11 subjects with an abnormal MECP2 genotype and 8 with a normal genotype), the 5MTHF concentration in the CSF was within the normal control range.

Compared to 28 healthy control subjects with no FR autoantibodies, the serum of 8 out of 33 Rett patients from Europe contained blocking FR autoantibodies (a mean of 1.25 pmol FR blocked/ml of serum; range 0.29–2.04; significant Fisher exact test with probability $p = 0.011$). The serum of 6 of the 14 patients with a low folate concentration in the CSF contained autoantibodies against FRs. In contrast, the serum of only 2 of the 19 patients with normal CSF folate levels contained low titers of the FR autoantibodies (patients 15, 26). Comparison of the groups of Rett patients with low CSF folate and those with normal CSF folate, showed a significantly higher incidence of FR autoantibodies for the Rett patients with low CSF folate (● **Table 2**; significant Fisher exact test $p = 0.037$). It should be stressed that the serum of 8 out of 14 Rett patients with low CSF folate, did not contain FR autoantibodies (patients 7, 11, 13, 18, 19, 22, 24, 32;

● **Table 1**). The use of anticonvulsant drugs in 15 of 33 Rett patients was not found to exert a statistically significant effect on their folate status (● **Table 2**).

The 8 Rett patients from Northern Europe (patient 1–8 from Germany and Belgium) showed a higher incidence of CSF 5MTHF deficiency and FR autoantibodies than the 25 Rett patients from Southern Europe (patients 9–33 from Spain and Portugal; ● **Table 3**) with a calculated Fisher exact probability at $p = 0.005$ and $p = 0.037$ respectively. The serum folate values between patients from Northern and Southern Europe showed no statistical differences, but comparison of the CSF folate values between Northern Europe, Southern Europe and healthy controls showed the lowest CSF folate values for Rett patients from Northern Europe, followed by moderately lowered CSF folate values for Rett patients from Southern Europe (analysis of variance $F = 20.92$ and $p < 0.0001$; ● **Table 3**).

The serum folate levels in Rett patients from North-America (mean value: 38.14 nmol/Liter; range 5.4–103.1) were significantly higher than the levels in European Rett patients (mean value: 24.36 nmol/Liter; range 8.8–62.2), and healthy European controls (mean value: 24.72; range 8.3–45) using the analysis of variance for three independent samples ($F = 7.62$ and $p = 0.000802$). Serum folate levels from healthy North-American girls were also significantly higher than healthy European controls (● **Table 2**).

Table 3 Comparison of folate levels in serum, 5MTHF levels in CSF and FR autoantibodies between Rett patients from Northern and Southern Europe

Analyte	Northern Europe (S1)	Southern Europe (S2)	Controls (S3)	Statistical test	Significance
number of patients	8	25	99		
serum folate					
mean	25.8	23.88		t-test t = 0.33	NS
range	13.2–47	8.8–62.2			
CSF folate					
mean	26.8	51.52	82	F-value = 20.92	p < 0.0001
range	12–68	33–92	44–181	S1 vs. S2	p < 0.05
				S1 vs. S3	p < 0.01
				S2 vs. S3	p < 0.01
incidence of low CSF folate	7/8	7/25		Fisher test p = 0.005	significant
incidence of positive FR antibodies	6/8	2/25		Fisher test p = 0.037	significant

S1: Sample representing European Rett patients

S2: Sample representing American Rett patients

S3: Sample representing European healthy controls

An interesting finding was that among the 12 European Rett patients with a normal MECP2 genotype, four patients had low CSF folate levels with FR autoimmunity as contributing factors in 3 of these 4 patients (patients 1–3).

Discussion

We have identified autoantibodies against the FRs in 24% of 33 Rett patients from Europe. This finding suggests a mechanism for the low CSF concentration of 5MTHF and the accompanying CFD-like features in these Rett patients. In contrast, a subgroup of Rett patients with low CSF folate and most Rett patients with normal CSF folate levels lack these autoantibodies. Furthermore, two Rett patients with low titers of autoantibodies had normal CSF folates (patients 15,26). Our study also showed a higher incidence of CFD and FR autoimmunity among the group of Rett patients residing in North-Western Europe, when compared to those living in Southern Europe. The lowest CSF folate values associated with FR autoantibodies were found in the Rett patients living in Northern Europe.

It is likely that Rett syndrome patients with these autoantibodies in their serum cannot transfer the 5MTHF into the CSF because the autoantibodies block the binding of folate to the FR expressed on the choroid plexus and thereby prevent the transfer of folate to the CNS. Our previous study showed that the CSF of Rett patients with low folate contained a normal concentration of immunoreactive folate receptor protein that did not bind radiolabelled folate [1,2]. These soluble non-functional FR proteins in CSF could represent autoantibody- FR complexes released into the spinal fluid after cleavage from their GPI-anchor attachment to the choroid plexus epithelial membranes. Our observation of circulating FR autoantibodies of the blocking type in three patients with a Rett phenotype, low CSF folate and a normal MECP2 genotype may point to a variant of the classical Rett phenotype that presents as a CFD-like syndrome. However, the finding of low CSF folate in 1 of the Rett patients with a normal MECP2 genotype and absence of FR autoimmunity suggests that other unknown aetiologies can contribute to the low CSF folate status in the Rett phenotype.

The geographical differences for FR autoimmunity and CFD between North-Western and South-Western Europe may be explained by nutritional differences with the higher consumption of milk and milk products in Northern Europe. Bovine FR

has high amino acid sequence homology with the human FR [9] and may be the source of the antigen. Cross-reactivity of the antibodies generated against the soluble bovine FR with the human FR antigen may provide a basis of our hypothesis on FR autoimmunity, which was proposed as a possible mechanism for the observed FR autoimmunity in children with the infantile-onset CFD syndrome [5]. The lower incidence of cerebral folate deficiency in the Rett population of North America may be explained by the folate food fortification and the folate/betaine trial, where the participants stopped folate at least 30 days or 1 week prior to lumbar puncture [4]. After folate is incorporated into cells, it takes more than 90 days before these folate reserves decrease [10]. Therefore, if folate supplementation is interrupted for less than 100 days before a lumbar puncture, the results have to be interpreted with caution. Comparison of the data from America and Europe has confirmed the significantly higher serum and CSF folate levels present in the American Rett population. A comparison of the incidence of the FR autoantibodies in the two patient populations from both continents would be an interesting issue to address in order to explain these conflicting results.

Our finding of low CSF folate in 42% of Rett patients suggests that folate transport across the choroid plexus may be blocked. The presence of serum FR autoantibodies in some of these patients provides an explanation for their low CSF 5MTHF. However, in Rett patients where the CSF/plasma folate ratio remains normal other mechanisms such as an increased folate turnover due to an increased utilization or catabolism of 5MTHF or other metabolic abnormalities have to be considered [10].

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3.4. Rett syndrome and autism

The results described in this chapter are included in the following manuscripts:

Article 10 - Atypical stereotypies and vocal tics in Rett syndrome: an illustrative case (in press)

Article 11 - MECP2 coding sequence and 3'UTR variation in 172 unrelated autistic patients (published)

3.4.1. Introduction

Autism is a behavioural disorder characterized by impaired social interaction and communication, and a restricted range of activities and interests, which emerges during infancy or early childhood. Although most individuals with autism have mental retardation (75%), its behavioural changes vary in type and frequency.

Although the aetiology of autism is currently unknown, there is considerable evidence to suggest an organic involvement. MRI data support cerebellar anomalies, particularly in the vermis and increased ventricular size. Genes play a greater role in the risk for autism than in any other common neuropsychiatric disorder: the concordance ratio in MZ twins is 25 times that in DZ twin pairs, and heritability is above 90%. Unspecified autistic, Asperger, desintegrative and pervasive developmental disorders are not linked to any particular genetic or non-genetic cause. There is convincing evidence for multiple interacting genetic factors as the main determinants of autism.

Autistic spectrum disorders and RTT share many characteristics. Both may be characterized by a period of normal-appearing development, followed by a period of regression, with loss of social, cognitive, and language skills during the first 2-4 years of life. In addition, repetitive stereotypic behaviours are characteristic of both. Certain neurophatological features are also common, as deficiency of dendrites in frontal, motor and temporal cortex in RTT and similar anomalies in the medial temporal lobe in infantile autism.

Though there are clear differences between clinical and genetic aspects of RTT and autism, common pathways may underlie regression in these disorders and the phenotypic overlap between RTT and autism is well recognized. Timing and symptoms of regression are similar. Sleep anomalies and mood fluctuations may also appear in both.

There are distinct differences, however, between RTT and autism: deceleration of head growth, growth failure, vasomotor disturbances, dystonia, rigidity and ataxia are defining features of RTT; hand stereotypies are usually characteristic of RTT, but many of those with other topographies are also seen in autism; other repetitive behaviours, as compulsions, rituals, sameness, restriction and other movements, as tics and akathisia, are typically observed in autism but, only exceptionally in RTT.

Patients with autism spectrum disorders may have larger head size, in contrast with RTT, beginning age 6 to 14 months.

The regression that characterizes all cases of RTT, but only some cases of autism, may be a consequence of similar processes. Onset of the clinical phenotype and regression phase, in RTT and autism, occur at a time when synaptic development and maturation are taking place. Studies of the expression of *MECP2* suggest that the protein is important in the maturation and maintenance of neurons and synapses. Mutated *MECP2* may be unable to suppress expression of genes that inhibit synapse proliferation and stimulate synapse pruning.

The RTT brain is small, but normally formed, without anomalies of migration or evidence of a degenerative process. Neurons are small, but densely packed, consistent with a reduction in the development of axons and synaptic connections. Similar neuropathological findings, including small neuronal size, increased packing density and decreased dendritic branching have been reported in autism.

MECP2 expression may be abnormal in neurodevelopmental disorders without *MECP2* mutations, as well as in RD. A significant decrease in *MECP2* expression was also seen in three of four autism samples, a patient with Angelman syndrome and four with Prader-Willi. In addition, increase in *MECP2* expression was observed in one autism sample. These findings suggest that both decrease and increase expression of *MECP2* may be associated with abnormal development phenotypes. Although mutations in the *MECP2* gene are rarely identified in autism, other mutations or defective pathways may act to modify *MECP2* expression in these disorders.

Characterization of regression in RTT may provide insight into other developmental disorders. Accumulating information suggests that abnormalities in synaptic development play an important role in the pathophysiology of RTT. Similar processes may be fundamental to autism, although its primary aetiological factors and genetic abnormalities may be different. Discovering how *MECP2* mutations cause RTT will undoubtedly further our understanding of other neurodevelopmental disorders, such as autism.

3.4.2. Clinical overlap between Rett Syndrome and autism: an illustrative case (Article 10)

Article 10 is partially published as: Temudo T, Freitas P, Sequeiros J, Maciel P, Oliveira G. Atypical stereotypies and vocal tics in Rett syndrome: an illustrative case. 2008. Movement Disorders (in press)

Clinical overlap between Rett Syndrome and autism: an illustrative case

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Abstract

We present a mildly affected Rett Syndrome girl with a *MECP2* mutation illustrating the overlap of symptoms (autistic behaviour, obsessions, compulsions, stereotypies and vocal tics) between autism and Rett syndrome and highlighting the phenotypic variability of the former.

We conclude that though there are clear differences in clinical and genetic aspects of RTT and autism, common pathways may underlie developmental regression in these disorders. The phenotypic similarities of the two entities suggest that understanding the characteristics and degree of symptom overlap may contribute to the discussion of models for neuropathological development in both disorders.

Key words: stereotypies, tics, obsession, compulsion

1. Introduction

After the identification of the *MECP2* gene mutations as a cause of Rett syndrome (RTT) (1) there is an increasing tendency to exclude this entity from the ICD-10 group of pervasive developmental autistic spectrum disorders (2). However, there are striking similarities between autism and Rett syndrome, especially during its regressive stage. The timing of disease onset is similar, both entities manifest abnormal social and communication behaviour and movement disorders. The onset of both disorders after neurogenesis, neuronal migration and maturation have occurred suggests that they may affect synaptic maturation, connectivity, or stabilization (3).

Hand stereotypies are the signature sign of Rett syndrome girls and their presence plays a key role in diagnosis (4). We have found that hand movements, which may involve the hands together or apart, are very standardized in individual girls. We also found that there are also stereotypies involving other parts of the body, especially during the first decade of life (4).

The pathophysiological basis of stereotypies in RTT remains elusive and we do not know whether these involuntary movements interfere with motor learning. Most studies stress that stereotyped hand movements coincide with or follow the disappearance of purposeful prehension in RTT; however, a video analysis of RTT patients in the first 6 months of life, prior to the onset of regression, showed stereotyped hand movements in 42% of cases (5); in our series, most parents of girls with *MECP2* mutations described stereotypies as coinciding with or preceding the loss of purposeful hand movements. Thus we believe that the particularly compulsive stereotypies in patients with RTT are likely to contribute to the complex process of loss or reduction of hand use.

We present the case of a girl with RTT and a very atypical presentation. We think that this case illustrates the compulsive behaviour in RTT and its clinical overlap with autism.

2. Case report

In a centre for autistic children a child psychiatrist noticed that one 6 year old girl stood out from the majority of the other children in that she had intense eye contact.

This girl was the second child of an unrelated and healthy couple and her perinatal history was normal. Family history was unremarkable for tics, obsessive-compulsive symptoms, and neurologic or psychiatric problems. When she was born her occipital frontal

circumference (OFC) was below the 5th percentile and weight and height were at the 50th percentile. OFC increased progressively to the 50 percentile by 6 months of age. She achieved head control at 3 months, grasped an object at 4 months, and had intentional prehension at 5 months, without hand preference. She sat unsupported at 8 months, walked unsupported and uttered her first words at 13 months. She never crawled.

She acquired language, but had a high pitched voice and echolalia. She spoke complex frequently coprolalic phrases and her vocabulary was notable for unusual words. She had some phobias and repetitive compulsive behaviours such as switching lights on and off and opening and closing drawers. She never acquired sphincter control.

At 4 years old she met DSM-IV (6) diagnostic criteria for autistic disorder and Autism diagnostic interview revised (ADI-R) (7) was positive for autism in three domains (social interaction, communication and repetitive behaviour). Developmental quotient (QD) evaluation with Griffiths Scale (8) revealed mild mental retardation (global QD of 55%). Her clinical report indicates that by that time she already had hand stereotypies.

She attended a special class for mentally retarded children where she drew and sang rhymes. We evaluated her for the first time when she was 6 years and 9 months because of an increase in hand stereotypies accompanied by deterioration in manual abilities. She was a thin girl with bizarre face expression, intense eye contact and complex rhythmical movements with separate hands (figure and video) and even more complex hand stereotypies with only one hand against her mouth or other people mouths (figure and video). She was able to manipulate objects but this was constantly interrupted by compulsive hand stereotypies.

She also closed her eyes repetitively, protruded her lips or grimaced (figure).

She answered simple questions in a high pitched voice and had echolalia only for particular complex or unusual words.

Neurological examination, except for the mental retardation, autistic traits and stereotypies, showed no abnormalities. OFC was in the 75th centile, height at the 50th and weight below the 5th centile.

Cerebral MRI and EEG were normal. Genetic test confirmed the diagnosis of Rett syndrome with a missense mutation in the *MECP2* gene (R133C).

At age 8 years she presented frequent episodes of hyperpnoea and apnoea which started one year earlier. She maintained the ability of speak, although spontaneous language has decreased. She also continued to have a particular predilection for coprolalia or repeating

complex words. She maintained the same manual stereotypies and showed new stereotypies with other topographies. She had no epilepsy but had occasional screaming or laughing spells. A resting and postural tremor of the upper limbs was now evident.



Figure legend : Different stereotypies performed by the patient

A- Grimacing; B- Lip protusion and closure of the eyes; C- Eye closure; D- Tongue rolling; E – Complex self-directed hand stereotypies; F- Same hand stereotypies directed to the mouth of the examiner; G-I – complex hand stereotypies with separated hands

Discussion

It has long been recognized that there is phenotypic overlap between RTT and autism (9). The majority of RTT patients have a typical neurodevelopmental course of regression, loss of hand skills and appearance of stereotypies, deceleration of head growth and progressive neuromotor disabilities which make this entity relatively easy to diagnose what can then be confirmed with a molecular genetic test. However, behavioural changes that are not currently part of the diagnostic criteria have also been reported in the literature (10) including a number of autistic-like behaviors, sleep abnormalities, and features of anxiety and low mood. Zappella et al (11) reported that all but one of 30 cases with the preserved speech variant of RTT met DSM-IV criteria for autism.

Autistic individuals have an obsession for repetition and sameness. These behavioural traits are not described in RTT, perhaps because these girls are so profoundly retarded. Stereotypes in RTT are highly compulsive and interfere with hand usage (4). We think that the more mildly affected girl we just described illustrates the overlap of symptoms between the two conditions and highlights the phenotypic variability of RTT. She was diagnosed as classically autistic until the age of five and her hand use was considered “normal”. She had repetitive behaviours, disturbed language, echolalia and coprolalia, and stereotypic movements. Like autistic children, but unlike RTT, her OFC never slowed down but progressively accelerated after birth until it reached the 75th centile. It was only after age five years, following a progressive increase in manual stereotypies, that she partially lost prehension. Some studies of autism report that stereotypies are correlated with its severity (12,13) and with cognitive deficiency (14). In our patient, stereotypies also increased with the cognitive regression she experienced at five years.

Her hand stereotypies are very complex and the movements, albeit in the midline, are quite different from those we described in a series of RTT patients (4). Some of her other stereotypic movements such as grimacing, closing of the eyes, mouth opening, rolling of the tongue, and echolalia are described in children with autism. She seems to have an unusually intense interest in the mouth. One of her hand stereotypies is obsessively self-directed at her mouth or directed to the mouths of people or her dolls, which may be analogous to autistic interest in parts of objects. She has the peculiar tone of voice of many individuals with autism.

Tourette syndrome, a neurobehavioral disorder characterized by multiple chronic vocal and motor tics, is not rare in autism. (15, 16). In a recent study, (4) we observed other stereotypies (including vocal ones) besides hand stereotypies, and behaviours, like tics, that changed over time, not previously reported in RTT; we hypothesised that the physiopathology of hand stereotypies differs from that of stereotypies with other topographies. The present case reinforces that hypothesis.

The timing of the period of regression in RTT parallels the period of intense synaptic development. Neuropathological findings in RTT include the selective reduction of dendritic spines in the pyramidal cells of the brain; this feature has also been reported in autism. Abnormalities in synapse maintenance and modulation may contribute to regression in RTT and autism.

The phenotypic overlap between RTT and autism has led to some debate about whether RTT is correctly classified as a pervasive developmental disorder in the diagnostic systems but the recent advances in the genetic understanding of RTT moved these

debates forward. Whilst we can now recognize Rett Syndrome as a genetically distinct disorder, autism is recognized as a behavioural phenotypic endpoint of a wide variety of aetiological pathways.

Elucidation of the nature and degree of symptom overlap might contribute to models of the neurophatological development of both disorders. The regression that characterizes the majority of cases with RTT but only some cases of autism is not well understood in relation to either disorder. It may be that, in at least some cases of autism, the pathological processes that lead to loss of skills are similar to those operating in RTT. It might be this partial phenotypic overlap between RTT and autism is indicative of pathological development of a particular brain system at the structural, connectivity or neurotransmitter level. Once the pathogenic processes in one disorder become better understood they may shed light on those in the other (17).

We conclude that though there are clear differences in clinical and genetic aspects of RTT and autism, common pathways may underlie regression in both disorders. The phenotypic similarities of the two entities suggest that understanding the characteristics and degree of symptom overlap may contribute to models of neuropathological development of both disorders.

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Video legend

Complex hand self-directed hand stereotypies and complex hand stereotypies with separated hands

3.4.3. MECP2 coding sequence and 3'UTR variation in 172 unrelated autistic patients ([Article 11](#))

MECP2 Coding Sequence and 3'UTR Variation in 172 Unrelated Autistic Patients

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Mutations in the coding sequence of the methyl-CpG-binding protein 2 gene (*MECP2*), which cause Rett syndrome (RTT), have been found in male and female autistic subjects without, however, a causal relation having unequivocally been established. In this study, the *MECP2* gene was scanned in a Portuguese autistic population, hypothesizing that the phenotypic spectrum of mutations extends beyond the traditional diagnosis of RTT and X-linked mental retardation, leading to a non-lethal phenotype in male autistic patients. The coding region, exon–intron boundaries, and the whole 3'UTR were scanned in 172 patients and 143 controls, by *Detection of Virtually All Mutations-SSCP* (DOVAM-S). Exon 1 was sequenced in 103 patients. We report 15 novel variants, not found in controls: one missense, two intronic, and 12 in the 3'UTR (seven in conserved nucleotides). The novel missense change, c.617G > C (p.G206A), was present in one autistic male with severe mental retardation and absence of language, and segregates in his maternal family. This change is located in a highly conserved residue within a region involved in an alternative transcriptional repression pathway, and likely alters the secondary structure of the MeCP2 protein. It is therefore plausible that it leads to a functional modification of MeCP2. *MECP2* mRNA levels measured in four patients with 3'UTR conserved changes were below the control range, suggesting

an alteration in the stability of the transcripts. Our results suggest that *MECP2* can play a role in autism etiology, although very rarely, supporting the notion that *MECP2* mutations underlie several neurodevelopmental disorders.

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KEY WORDS: autism; *MECP2*; 3'UTR; exon 1; *Detection of Virtually All Mutations-SSCP*

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INTRODUCTION

Mutations in the coding region of the methyl-CpG-binding protein 2 gene (*MECP2*) are responsible for about 80% of Rett Syndrome (RTT, OMIM #312750) cases [Amir et al., 1999]. Most RTT patients develop microcephaly, seizures, and autism. Autism (OMIM #209850) is a neurodevelopmental disorder characterized by deficits in social interaction and communication, and by restricted and stereotyped patterns of behavior. It affects more males than females, in a ratio of 3–4:1, which led to the hypothesis of the involvement of an X-linked gene. As autism and RTT share a range of symptoms, it was speculated that specific mutations in the *MECP2* coding region could also be involved in autism etiology. Evidence for linkage to autism was recently found at chromosome Xq27–q28, in the region where *MECP2* maps, supporting it as a candidate gene for this disorder [Vincent et al., 2005]. So far, a number of mutations previously found in RTT have been reported in autism studies [van Karnebeek et al., 2002; Carney et al., 2003; Lobo-Menendez et al., 2003; Zappella et al., 2003]. In two studies novel alterations were reported, one missense change in one autistic male [Beyer et al., 2002] and a de novo intronic variation in an autistic female with mental retardation [Lam et al., 2000], although their functional significance was not demonstrated. Mutations in *MECP2* have also been found in other syndromes, including non-specific X-linked mental retardation in males (reviewed in Bienvenu and Chelly [2006]). Although severe *MECP2* mutations leading to RTT are thought to be lethal in hemizygous males, these studies

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show the existence of sequence changes not found in RTT that segregate in the families of males with autism, mental retardation, and occasionally language problems. Hemizygosity for some *MECP2* mutations, leading to a less severe functional alteration of the protein, may therefore be compatible with life, with heterozygosity for these same mutations insufficient to cause disease in the female carriers.

The *MECP2* gene originates two protein isoforms: MeCP2_e2, encoded by exons 2–4, and MeCP2_e1, encoded by exons 1, 3, and 4, which is more abundantly expressed in the brain [Kriaucionis and Bird, 2004; Mnatzakanian et al., 2004]. MeCP2 acts as a transcriptional repressor and is mainly expressed in the central nervous system (CNS), indicating a role in the regulation of brain gene expression. Although its target genes are not fully known, microarray studies have found several genes with altered expression in RTT, some showing a direct regulation by MeCP2 (reviewed in Bienvenu and Chelly [2006]). In addition, it has been demonstrated that it regulates the expression of the brain-derived neurotrophic factor gene (*BDNF*), which encodes a molecule essential in neurodevelopment and neuronal plasticity, learning, and memory [Chen et al., 2003; Martinowich et al., 2003]. The 3'UTR in exon 4 is unusually long (8.5 kb) and well conserved between human and mouse, with at least eight blocks of strong sequence similarity that can represent important functional domains [Coy et al., 1999]. The size and conservation of this region, as well as expression studies in *post-mortem* brain, indicate an important role in the transcriptional regulation of *MECP2*. Samaco et al. [2004] found altered levels of *MECP2* expression in the brain of four out of five autistic individuals, none of which had mutations in the coding region of *MECP2*, suggesting that variations in the 5'UTR or 3'UTR could be responsible for these changes. These findings support the hypothesis that variants in the 3'UTR, and not only in the coding region of *MECP2*, may be involved in some clinical manifestations.

A previous report has shown a higher frequency of missense and 3'UTR variants in a sample of 24 autistic patients, as compared to other psychiatric diseases [Shibayama et al., 2004]. In the present study, we extend the search for *MECP2* mutations in the coding region, exon–intron boundaries, and in the whole 3'UTR in 172 patients with autism. Our goal was to determine if the phenotypic spectrum of mutations extends beyond the traditional diagnosis in RTT and mental retardation, leading to a less severe phenotype in autistic male and female patients.

MATERIALS AND METHODS

Subjects and Clinical Assessments

One hundred seventy two caucasian unrelated autistic children (141 males and 31 females; age range of 2–14 years old), originating from mainland Portugal and the Azorean islands, were recruited at the Autism Clinic from Hospital Pediátrico de Coimbra (HP). Diagnosis and assessment of the children followed a comprehensive evaluation protocol by a clinical team including a developmental pediatrician, two psychologists, one special education teacher, and a social worker. Observation of the children entailed extensive interaction and semi-structured activities in a clinical setting. ASD was diagnosed using DSM-IV (American Psychiatric Association 1994) criteria, the Autism Diagnostic Interview-Revised (ADI-R) [Lord et al., 1994], and the Childhood Autism Rating Scale (CARS) [Schopler et al., 1988]. Diagnosis required fulfillment of DSM-IV criteria and meeting the ADI-R algorithm cutoff for autistic disorder, and a functional level of 12 months or above. Consensus diagnosis among the clinical team was obtained for all patients. About 90% of these patients

are routinely followed by the same clinical team at HP and therefore are monitored over several years until age 18.

Developmental or intellectual quotients were determined using the Ruth Griffiths Mental Development Scale II [Griffiths, 1984] or the Wechsler Intelligence Scale for Children (WISC 1974). Functional level was assessed using the Vineland Scales for Adaptive Behavior [Carter et al., 1998]. Idiopathic subjects were included after clinical assessment and screening for known medical and genetic conditions associated with autism, including testing for Fragile X mutations (FRAXA and FRAXE), chromosomal abnormalities (karyotype study), neurocutaneous syndromes, endocrine, and metabolic disorders. Control Portuguese unrelated adult individuals consisted of 143 caucasian healthy blood donors (113 males and 30 females), with no family history of neuropsychiatric diseases. Control Portuguese unrelated children consisted of 36 healthy individuals (21 males and 15 females; age range of 2–15 years old), recruited at the surgery service at the HP where they were undergoing minor surgery procedures, requiring blood sample collection for pre-surgery baseline evaluation. The study was approved by the HP ethical committee, and all participants or legal representatives signed an informed consent.

MECP2 Mutation Detection

The coding region of *MECP2* comprising exons 2–4, exon–intron boundaries, and its whole 3'UTR, were scanned for mutations with *Detection Of Virtually All Mutations-SSCP* (DOVAM-S) [Liu et al., 1999]. DOVAM-S is a robotically enhanced and highly redundant form of *Single Strand Conformational Polymorphism* (SSCP) with virtually 100% sensitivity of mutation detection. The gene was first amplified robotically in 42 segments ranging in size from 150 to 476 bp, pooled, denatured, and electrophoresed under five non-denaturing conditions varying in gel matrix, buffer, temperature, and additive. PCR products with mobility shifts were sequenced with the ABI model 377 (Perkin-Elmer Model 377, Norwalk, CT) and nucleotide alterations were analyzed with Sequencher 4.1 software (Gene Codes, Ann Arbor, MI). Sequence changes were confirmed by reamplification with genomic DNA and sequencing in the opposite direction. Exon 1 was analyzed by direct sequencing of the corresponding region. Information on the primers used for amplification of the coding region and the 3'UTR of *MECP2*, as well as the PCR conditions used, is provided on the Supplementary Information section.

Bioinformatic Analysis of the *MECP2* Sequence

In order to assess if the novel intronic changes were affecting normal splicing by altering a consensus sequence, the program GenScan version 1.0 was used, which performs predictions of exon/intron splice sites based on local nucleotide sequence properties of the genomic DNA. Protein secondary structure predictions were performed for the novel missense changes found in *MECP2* using the programs PeptideStructure (GCG package, version 10.0), Garnier (Emboss, version 2.8.0), and SSpro8, which use several algorithms for prediction based on the primary structure and properties of the amino acid residues. Searching for sequence patterns in the 3'UTR of *MECP2* was performed using UTRscan database, which looks for known UTR functional elements, and the program FindPatterns (GCG package, version 10.0), which locates short sequence patterns specified by the user.

RNA Isolation and Quantification of the *MECP2* mRNA

Total RNA was extracted from 5×10^6 peripheral blood mononuclear cells (PBMCs) using the RNeasy Mini Kit (Qiagen, Valencia, CA), and used for amplification of the first

strand of cDNA by reverse transcription with oligo-dT primer (Invitrogen, Carlsbad, CA). Total *MECP2* mRNA (*MECP2_e1* and *MECP2_e2* transcripts) levels were quantified by quantitative PCR with LightCycler Fast Start SYBR Green I (Roche Molecular Biochemicals, Mannheim, Germany). For each sample, 50 ng of first-stranded cDNA were amplified in duplicate by PCR and real-time fluorimetric intensity of SYBR green I was monitored. The levels of *MECP2* mRNA for each sample were normalized by the amount of mRNA of the housekeeping gene *HPRT1*. Details for the quantitative PCR reaction, including primers used for *MECP2* and *HPRT1* are provided in the Supplementary Information section.

Quantitative X-chromosome Inactivation Assay

X-chromosome inactivation (XCI) assays were performed in DNA isolated from peripheral blood leukocytes, to assess the pattern of XCI in the carrier mother and maternal grandmother of the autistic male who presented the novel p.G206A missense change. It was also performed in the proband, in order to determine which allele was inherited from his mother. The assay was based on a previously described method [Allen et al., 1992], which allows the determination of the X-inactivation status using a trinucleotide repeat polymorphism in the androgen receptor gene (*AR*) flanked by two methylation-sensitive restriction enzyme sites. These sites are methylated on the inactive X chromosome, and are unmethylated on the active X chromosome. This allowed the development of an assay that distinguishes between the maternal and paternal alleles (through the repeat number) and identifies their methylation status (through enzymatic restriction). Details for the assay are provided in the Supplementary Information section.

Quantification of BDNF Levels in Plasma

Levels of BDNF were quantified in plasma using BDNF Emax Immunoassay System kit (Promega Corp., Madison, WI), according to the manufacturer's instructions. The assays were performed in duplicate for each sample.

RESULTS

Variation of the *MECP2* Coding Region and Exon-Intron Boundaries in Autistic Patients and Controls

Scanning of *MECP2* sequence changes in exons 2–4 and exon–intron boundaries was performed in 172 patients (141 males and 31 females; 203 X-chromosomes total), revealing that 12 patients (7.0%), 10 males and 2 females, have sequence changes corresponding to 11 different variants (Table I). Of these, four are missense changes observed in males, of which one is novel. In addition, we found four silent and three intronic changes (two novel); of these, two silent and one intronic change were also present in the controls, and have been previously reported [Trappe et al., 2001; Kleefstra et al., 2004]. The novel missense change is a c.617G > C transition found in one autistic male, resulting in a p.G206A amino acid replacement in the inter-domain region of MeCP2, and was not found in 143 controls. Protein secondary structure predictions revealed that this alteration can disturb a α -helix in the inter-domain region of the protein, due to the alteration in amino acid properties from polar to hydrophobic. This amino acid position is included in a region involved in a histone deacetylase-independent pathway of transcriptional repression by MeCP2 [Yu et al., 2000], and thus this sequence change is likely to lead to an alteration in protein function. Predictions of exon/intron splice sites were performed for the novel intronic changes found, and do not alter any putative consensus

sequence in the genomic DNA, therefore it is not likely that they affect normal splicing of *MECP2*. None of the sequence variants found in our autistic children sample have been reported as pathogenic mutations in Rett syndrome studies. Sequencing of exon 1 was performed in 103 autistic patients (88 males and 15 females; 118 X-chromosomes total), and no sequence changes were found, indicating that alterations in this region in autistic patients are likely very rare.

In 143 Portuguese healthy controls (113 males and 30 females; 173 X-chromosomes total), we found 11 individuals (7.7%), 5 males and 6 females, with sequence changes in exons 2–4 and exon–intron boundaries, corresponding to six distinct variants (Table II). Of these, one is a novel missense change (p.K82R) occurring in one male, one intronic change, and four silent changes. The p.K82R missense change does not lead to a change in amino acid properties and does not alter the secondary structure of the protein, so it likely represents a rare polymorphism. None of the sequence variants found in the control samples have been reported as pathogenic mutations in previous studies. Of the sequence changes observed in this study, three were common to both patients and controls (c.378-19delT, p.A131A, and p.T445T), suggesting that they are polymorphisms with no pathogenic effect, as reported before [Trappe et al., 2001; Kleefstra et al., 2004].

Variation of the *MECP2* 3'UTR in Autistic Patients and Controls

Scanning of mutations was performed in the whole 3'UTR of *MECP2*. We found 46 patients out of 172 (26.7%) with 3'UTR variations, some having more than one change. In total, 24 unique sequence changes were found (13 in conserved nucleotides), of which 21 are novel (Table III). In 96 Portuguese controls (76 males and 20 females; 116 X-chromosomes total) we observed 26 individuals (27.1%) with 3'UTR variations, in a total of 20 unique sequence changes (12 in conserved nucleotides) (Table IV). Again, some of the individuals had more than one sequence change, indicating that there is a high variability in the 3'UTR. All of the variations encountered were novel, except the c.9964insC [Bourdon et al., 2001]. Ten of the changes were common to both patients and controls, suggesting that they do not have any pathogenic effect. A high degree of sequence variability in the 3'UTR was found, comparable in patients and controls, even in the most conserved regions between human and mouse. However, of the 21 novel changes found in patients, 12 were not present in the controls. Of these, seven were located in conserved nucleotides in seven autistic males, and only one was found in one autistic female: c.4167G > A (heterozygous). One of these novel changes, c.1655G > A, was localized in a region of strong sequence identity with mouse, suggesting that it may alter the regulation of *MECP2* expression.

Little is known about the functionality of the long 3'UTR of *MECP2*. In order to understand the meaning of the alterations found in this study we performed scans for known sequence patterns in 3'UTR regions. We found eight matches for 15-lipoxygenase differentiation control elements (15-LOX-DICE), which are CU-rich sequences involved in mRNA stabilization and translation inhibition, and at least two matches for AU-rich elements (AREs) and several C-rich regions, involved in the regulation of mRNA stability. However, none of the 3'UTR alterations found exclusively in the patients were localized within any of these regions. *MECP2* mRNA levels were measured in PBMCs from four autistic males presenting changes in conserved nucleotides: c.1832G > C, c.2015G > A, c.4017T > A, and c.4417G > A (Fig. 1). *MECP2* mRNA levels measured in four autistic males in the same age range, but without any detected *MECP2* alteration, were used as controls for specificity of these *MECP2* changes within the autistic

TABLE I. *MECP2* Sequence Changes Identified in the Coding Region and Exon–Intron Boundaries in 172 Unrelated Autistic Patients

Nucleotide change ^a	Amino acid change ^b	Domain	Type of sequence change	Amino acid conservation ^b	Number of samples	Mut/PM ^c	References
c.27-55G > A		Intron	Intronic variation		1		This report
c.377 + 18C > G		Intron	Intronic variation		1		This report
c.378-19delT		Intron	Intronic variation		1	PM	Trappe et al. [2001]
c.393C > G	p.A131A	MBD	Silent	<i>Macaca fascicularis</i> , <i>Mus musculus</i> , <i>Rattus norvegicus</i> , <i>Danio rerio</i> , <i>Gallus gallus</i> , <i>Xenopus laevis</i>	1	PM	Kleefstra et al. [2004]
c.602C > T	p.A201V	Inter-domain region	Missense	<i>Macaca fascicularis</i> , <i>Mus musculus</i> , <i>Rattus norvegicus</i>	1	PM	Amano et al. [2000], Lam et al. [2000]
c.617G > C	p.G206A	Inter-domain region	Missense	<i>Macaca fascicularis</i> , <i>Mus musculus</i> , <i>Rattus norvegicus</i> , <i>Danio rerio</i> , <i>Gallus gallus</i> , <i>Xenopus laevis</i>	1		This report
c.1189G > A	p.E397K	C-term	Missense	<i>Mus musculus</i> , <i>Rattus norvegicus</i>	1	PM	Wan et al. [1999]
c.1197C > T	p.P399P	C-term	Silent	<i>Mus musculus</i> , <i>Rattus norvegicus</i>	2	PM	Cheadle et al. [2000]
c.1233C > T	p.S411S	C-term	Silent	<i>Macaca fascicularis</i> , <i>Mus musculus</i> , <i>Rattus norvegicus</i>	1	PM	Amir et al. [1999]
c.1330G > A	p.A444T	C-term	Missense		1	PM	Buyse et al. [2000]
c.1335G > A	p.T445T	C-term	Silent	<i>Mus musculus</i> , <i>Rattus norvegicus</i>	1	PM	Directly submitted to RettBASE ^d

^aRefSeq ID: NM_004992.2 (mRNA).^bProtein ID: NP_004983.1 (human), AAK97131.1 (*M. fascicularis*), NP_034918.1 (*M. musculus*), NP_073164.1 (*R. norvegicus*), NP_997901.1 (*D. rerio*), CAA74577.1 (*G. gallus*), AAD03736.1 (*X. laevis*).^cMut, mutation; PM, polymorphism.^dTRSA (International Rett Syndrome Association) *MECP2* Gene Variation Database (RettBASE), <http://mecp2.chw.edu.au/>.TABLE II. *MECP2* Sequence Changes Identified in the Coding Region and Exon–Intron Boundaries in 143 Healthy Control Individuals

Nucleotide change ^a	Amino acid change ^b	Domain	Type of sequence change	Amino acid conservation ^b	Number of samples	Mut/PM ^c	References
c.245A > G	p.K82R	MBD	Missense	<i>Macaca fascicularis</i> , <i>Mus musculus</i> , <i>Rattus norvegicus</i> , <i>Danio rerio</i> , <i>Gallus gallus</i> , <i>Xenopus laevis</i>	1		This report
c.378-19delT		Intron	Intronic variation		5		Trappe et al. [2001]
c.393C > G	p.A131A	MBD	Silent	<i>Macaca fascicularis</i> , <i>Mus musculus</i> , <i>Rattus norvegicus</i> , <i>Danio rerio</i> , <i>Gallus gallus</i> , <i>Xenopus laevis</i>	1	PM	Kleefstra et al. [2004]
c.834C > T	p.A278A	TRD	Silent	<i>Mus musculus</i> , <i>Rattus norvegicus</i> , <i>Xenopus laevis</i>	1	PM	Hoffbuhr et al. [2001]
c.948C > G	p.V316V	C-term	Silent	<i>Macaca fascicularis</i> , <i>Mus musculus</i> , <i>Rattus norvegicus</i> , <i>Xenopus laevis</i>	1	PM	Directly submitted to RettBASE ^d
c.1335G > A	p.T445T	C-term	Silent	<i>Mus musculus</i> , <i>Rattus norvegicus</i>	2	PM	Directly submitted to RettBASE ^d

^aRefSeq ID: NM_004992.2 (mRNA).^bProtein ID: NP_004983.1 (human), AAK97131.1 (*M. fascicularis*), NP_034918.1 (*M. musculus*), NP_073164.1 (*R. norvegicus*), NP_997901.1 (*D. rerio*), CAA74577.1 (*G. gallus*), AAD03736.1 (*X. laevis*).^cMut, mutation; PM, polymorphism.^dTRSA (International Rett Syndrome Association) *MECP2* Gene Variation Database (RettBASE), <http://mecp2.chw.edu.au/>.

TABLE III. MECP2 3'UTR Variants Identified in 172 Unrelated Autistic Patients

Nucleotide change ^a	Nucleotide conservation ^a	Number of samples	Mut/PM ^b	References
c.1470G > A		1	PM	Lam et al. [2000]
c.1554G > A	<i>Mus musculus</i>	1	PM	Ylisaukko-Oja et al. [2005]
c.1655G > A	<i>Mus musculus</i>	1		This report
c.1832G > C	<i>Mus musculus</i>	1		This report
c.2005G > A		1		This report
c.2015G > A	<i>Mus musculus</i>	1		This report
c.2228G > T		1		This report
c.2322T > G	<i>Mus musculus</i>	1		This report
c.2339C > G		15		This report
c.2829C > A	<i>Mus musculus</i>	1		This report
c.3198G > A	<i>Mus musculus</i>	8		This report
c.4017T > A	<i>Mus musculus</i>	1		This report
c.4118G > A		1		This report
c.4167G > A		1		This report
c.4417G > A	<i>Mus musculus</i>	1		This report
c.4938G > A		2		This report
c.5119C > T		1		This report
c.5339G > C		1		This report
c.6037A > C	<i>Mus musculus</i>	1		This report
c.6948ins(AT)	<i>Mus musculus</i>	1		This report
c.9209C > T	<i>Mus musculus</i>	1		This report
c.9317A > C	<i>Mus musculus</i>	13		This report
c.9964delC		2		This report
c.9964insC		3	PM	Bourdon et al. [2001]

^aRefSeq ID: NM_004992.2 (human), AF158181.1 (*M. musculus*).^bMut, mutation; PM, polymorphism.

phenotype. *MECP2* mRNA levels of the patients with 3'UTR changes were significantly lower when compared with these controls (Kruskal–Wallis test, $P = 0.021$). Although mRNA levels were measured in very few individuals, all four patients with 3'UTR alterations had lower levels than any of the patients with no *MECP2* sequence changes (Fig. 1), suggesting that at least these alterations in 3'UTR conserved nucleotides may render the *MECP2* transcripts more unstable and subject to degradation.

Analysis of the Novel p.G206A Missense Change

Segregation analysis was carried out in the family of the autistic male in which the novel p.G206A missense change was found (Fig. 2A). Clinical, neuropsychological, and behavioral data on this patient is shown in Table V. This patient was 10 years old at the time of collection, and had severe autism (positive ADI-R and DSM-IV with a CARS score of 50.5), severe mental retardation (Global Developmental Quotient of

TABLE IV. MECP2 3'UTR Variants Identified in 96 Healthy Control Individuals

Nucleotide change ^a	Nucleotide conservation ^a	Number of samples	Mut/PM ^b	Reference
c.1854G > A		1		This report
c.1950G > C	<i>Mus musculus</i>	1		This report
c.1990G > T	<i>Mus musculus</i>	1		This report
c.2267G > A	<i>Mus musculus</i>	1		This report
c.2292G > C	<i>Mus musculus</i>	1		This report
c.2336insA		1		This report
c.2339C > G		8		This report
c.2698T > C	<i>Mus musculus</i>	1		This report
c.3198G > A	<i>Mus musculus</i>	5		This report
c.4938G > A		3		This report
c.5123A > G	<i>Mus musculus</i>	1		This report
c.5339G > C		1		This report
c.5547del(GT)		1		This report
c.6037A > C	<i>Mus musculus</i>	2		This report
c.6948ins(AT)	<i>Mus musculus</i>	1		This report
c.7300C > T	<i>Mus musculus</i>	1		This report
c.9209C > T	<i>Mus musculus</i>	1		This report
c.9317A > C	<i>Mus musculus</i>	5		This report
c.9964delC		5		This report
c.9964insC		2	PM	Bourdon et al. [2001]

^aRefSeq ID: NM_004992.2 (human), AF158181.1 (*M. musculus*).^bMut, mutation; PM, polymorphism.

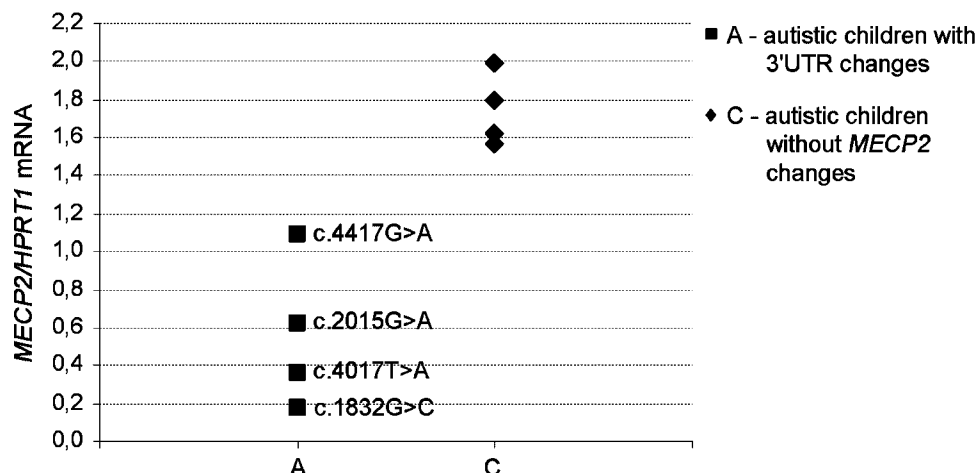


Fig. 1. *MECP2* mRNA quantification in PBMCs from four autistic males with different 3'UTR alterations in conserved nucleotides: c.1832G > C, c.2015G > A, c.4017T > A, and c.4417G > A, compared with four autistic males without *MECP2* changes. Results were normalized for *HPRT1* mRNA levels.

25), and absence of language. He had purposeful hand manipulation of objects and hand stereotypes not characteristic of Rett syndrome. Breathing irregularities were never noticed by the family and he never had epilepsy. In the first year of life he had developmental delay without history of

regression, the weight and height were in the percentile 5, and the cephalic perimeter was in the percentile 50 (without posterior deceleration of head growth). Presently 15 years old, he shows the same symptomatology and his neurologic examination does not show any abnormality besides mental retardation and autistic behavior. This patient has a younger male sibling who is 3 years of age and has a normal development to this day. A maternal aunt died at age 7 and was reported to have mental retardation, abnormal motor development, and uncontrolled seizures of unknown etiology. The available relatives from the proband were sequenced for the p.G206A alteration: his parents, the maternal grandmother, and a maternal uncle (see Fig. 2A). The mother and maternal grandmother are heterozygous asymptomatic carriers of this sequence change. Quantitative XCI assays were then performed in the female relatives; the proband was also tested, to determine which of the alleles was inherited from his mother. A pattern of moderately skewed XCI ratio of $\pm 30\%$:70% was found in the mother, and a normal random pattern of $\pm 40\%$:60% was found in the grandmother (Fig. 2B). Although the three individuals (proband, mother, and maternal grandmother) share the same alteration, they do not share one common androgen receptor gene (*AR*) allele. Because *AR* (Xq12) and *MECP2* (Xq28) are located far apart in the X chromosome, this suggests that a crossing-over event has occurred between generations. If this is correct, the mutation must be segregating with the low molecular weight allele from the grandmother to the mother (allele c in Fig. 2B), and then passed to the proband together with the intermediate molecular weight allele (allele b in Fig. 2B), due to recombination in the mother's germline. Densitometry analysis shows that in the mother, who inherited the mutated *MECP2* allele from the grandmother (associated with allele c in Fig. 2B), the X chromosome carrying the wild type *MECP2* allele (inherited

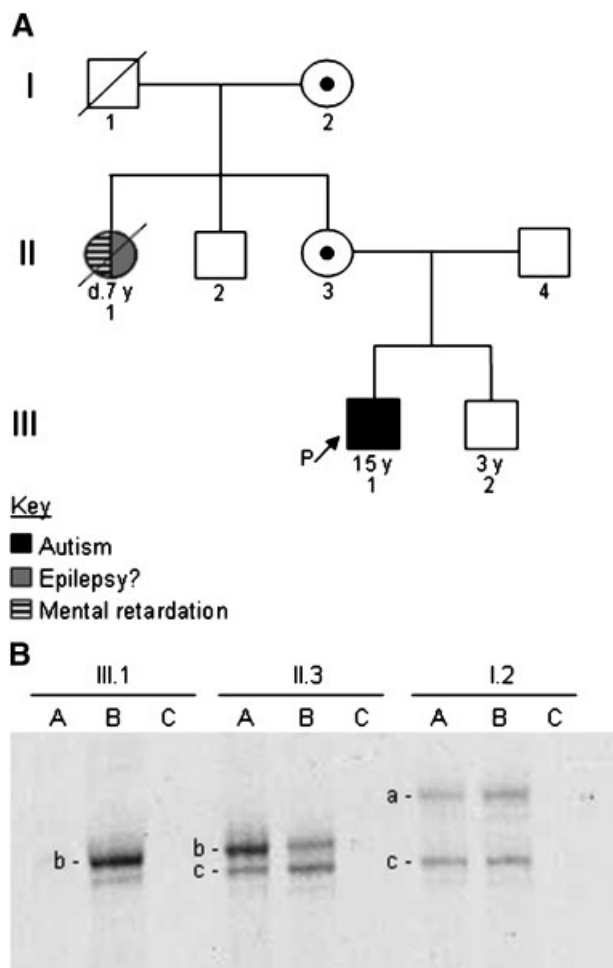


Fig. 2. Pedigree and X-chromosome inactivation (XCI) assay results in the family of the autistic proband who presents the novel p.G206A missense change in *MECP2*. **Panel A:** pedigree showing the structure of the family of the autistic patient in whom the novel p.G206A missense change was found. **Panel B:** XCI assay results, performed in leukocytes of the autistic proband with the p.G206A alteration and the family female carriers; the autoradiography shows the *AR* allelic band pattern after PCR amplification of the DNA samples. III.1—proband; II.3—proband's mother; I.2—proband's maternal grandmother; A—PCR amplification after restriction of genomic DNA with *HhaI* (only methylated, inactivated alleles are amplified); B—PCR amplification of intact genomic DNA; C—PCR amplification with no DNA (negative control); a, b, c—*AR* trinucleotide repeat alleles.

TABLE V. Clinical, Neuropsychological, and Behavioral Data of the Patient Presenting the p.G206A Missense Change in *MECP2*

Variable	Clinical data
Age at examination (years)	10
Physical measurements	
Height	5th percentile
Occipital-frontal circumference (OFC)	50th percentile
Weight	5th percentile
Neurological symptoms	
Seizures	No
Brisk tendon reflexes	No
Tremor	No
Developmental history	
First remarkable signs of autism (months)	<12
Walked independently (months)	12
Motor skills	
Poor-motor coordination	No
Slow	No
Clumsy	No
Regression	No
Sleep problems	No
Developmental quotient (Ruth Griffiths Mental Development Scale II)	
Global developmental quotient (GDQ)	25
Motor developmental quotient (MDQ)	30
Performance developmental quotient (PDQ)	23
Language developmental quotient (LDQ)	13
Vineland adaptive behavior scales domain scores	
Communication (percentile rank ^a)	40
Daily living (percentile rank ^a)	50
Socialization (percentile rank ^a)	40
Adaptive behavior composite (percentile rank ^a)	50
DSM-IV positive criteria	
Qualitative impairment in social interaction	3 out of 4
Qualitative impairments in communication	2 out of 4
Restricted repetitive and stereotyped patterns of behavior, interests, and activities	4 out of 4
Delays or abnormal functioning, with onset prior to age 3 years, in social interaction, language and symbolic or imaginative play	Yes
Disturbance not better accounted for by Rett's disorder or CDD	No
ADI-R domain scores at final diagnosis	
Social interaction (cutoff = 10; max. = 30)	29 out of 30
Communication: nonverbal (cutoff = 7; max. = 14)	13 out of 14
Repetitive behavior (cutoff = 3; max. = 12)	4 out of 12

^aSupplemental Norm Group percentiles ranks—Autism Special Population [Carter et al., 1998].

from her father, associated with allele b in Fig. 2B), is preferentially inactivated. Because both the mother and maternal grandmother show a normal phenotype, any pathogenic effect of the p.G206A missense change can likely be compensated by the expression of the normal allele in the carrier females.

In view of these results, we evaluated the role of the p.G206A missense change in the functionality of MeCP2. Because *BDNF* is one of the few genes known to be directly regulated by MeCP2 [Chen et al., 2003; Martinowich et al., 2003], we quantified plasma BDNF levels in this autistic patient (19.5 ng/ml), which were found to be within normal levels when compared to 36 age-matched control children (range: 8.9–69.8 ng/ml; mean: 28.6 ng/ml $\pm$ 13.9 SD). This result suggests that the potential pathogenic mechanism of this missense change does not involve BDNF level changes in this patient, or that they are too mild in plasma to be detected at this level.

DISCUSSION

In the present study we hypothesized that sequence changes in specific locations within the *MECP2* gene that are less deleterious for MeCP2 function might be involved in a pathway

leading to a milder, non-lethal phenotype in autistic male and female patients, extending beyond the traditional RTT diagnosis.

We found low sequence variability in the coding region and exon–intron boundaries of *MECP2*, comparable in autistic patients (7.0%) and controls (7.7%), in agreement with previous studies in which *MECP2* sequence changes were found only rarely in subjects with autism [Lam et al., 2000; Beyer et al., 2002; van Karnebeek et al., 2002; Carney et al., 2003; Lobo-Menendez et al., 2003; Zappella et al., 2003; Shibayama et al., 2004]. We report two intronic variations and one missense change that were not present in the control sample. The novel missense change (p.G206A) was found in one autistic male, and segregates in his family. It is localized in a highly conserved amino acid, within a region implicated in an alternative transcriptional repression pathway independent of histone-deacetylation [Yu et al., 2000], and likely leads to a change in protein structure, implying that it may alter the function of MeCP2. These findings are compatible with an association of this change with autism in the proband. In the proband's mother and grandmother, who have a normal phenotype, either the *MECP2* mutated allele has a low penetrance or its deleterious effect is compensated by the

expression of the normal allele, which may be sufficient to avoid the appearance of symptoms. It is possible that the maternal aunt, affected with mental retardation, and who died early in life, had the p.G206A alteration with a skewed XCI favoring the expression of the mutated allele. Plasma BDNF levels of the autistic proband were found to be within normality, suggesting that the pathogenic mechanism does not involve BDNF level changes in this patient. However, we cannot exclude that an alteration in BDNF levels in the CNS mediated by the altered MeCP2 is not accompanied by a change in plasma levels. The expression of other target genes which may be involved in the clinical phenotype of this patient was not investigated.

We sequenced exon 1 of *MECP2* in 103 autistic patients, and found no sequence changes in this region. Mutations in exon 1, and thus in MeCP2_e1, either do not play a role in autism etiology or occur very rarely associated with this disorder, possibly with a very severe effect.

To our knowledge, this is the first report in which the whole 3'UTR of *MECP2* was scanned for sequence variations. We found a high degree of sequence variability in the 3'UTR, comparable between patients (26.7%) and controls (27.1%). In 172 patients, 12 novel changes were found, of which 7 were located in conserved nucleotides. In the patients carrying four of these conserved 3'UTR alterations *MECP2* mRNA levels were always lower than in autistic patients without any *MECP2* sequence changes. While the use of autistic individuals with no *MECP2* alterations as controls does not provide us with the normal range of mRNA levels, it suggests that lower expression, and a consequent abnormal overexpression of target genes, may be a specific cause of autism for the individuals bearing these *MECP2* sequence alterations, among autistic individuals. Given the etiological heterogeneity of autism, with multiple genetic alterations known to lead to an autistic phenotype, this hypothesis is plausible. These results are however to be considered preliminary, as the sample size is very small. If confirmed in a larger sample, these observations would indicate the existence of important regulatory regions within the 3'UTR of *MECP2*.

Taken together, our results suggest that mutations in the *MECP2* coding region and 3'UTR alterations in conserved regions may play only a minor role in autism etiology. We report a novel missense change, which may have a non-lethal but severe pathogenic effect in males. Functional studies in order to demonstrate its pathogenicity are under way. Mutations in the coding region, however, are probably restricted to a very small subgroup of subjects with autism, as we found only one patient out of 172 (0.58%) with a potential pathogenic mutation; if we consider exclusively the autistic male sample (n = 141), this frequency increases to 0.71%. Additionally, we report seven novel 3'UTR alterations in conserved nucleotides, present in seven autistic males out of 172 patients (4.07%; 4.96% in the male sample), of which at least four possibly alter the stability of the *MECP2* transcripts. The frequency of *MECP2* alterations in autism might be higher, as we did not scan the promoter region and the method used cannot detect large deletions or duplications that could be missed in heterozygous females (and duplications in males). Our patient sample included only idiopathic cases, which were screened for common medical conditions associated with autism including tuberous sclerosis, Fragile X syndrome, and chromosomal abnormalities, as a standard procedure. The prevalence of these conditions in autism spectrum disorders is estimated to be 1–3%, 2–3%, and 5%, respectively [Rutter, 2005], with full mutations of the gene causing the Fragile X syndrome (*FMR1*) observed in less than 1% of children with autism [Lord et al., 2000]. Similarly, rare mutations in *MECP2* may increase the susceptibility to develop autism in a minority of cases. Screening for *MECP2* mutations in cases of autism

associated with mental retardation, particularly in males who may have a variable phenotype, may be useful for research purposes, to decrease genetic heterogeneity in the study samples and thus facilitate the identification of other genes predisposing to autism.

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4. General discussion

4.1. The clinical picture and differential diagnoses

Rett syndrome was initially described as a progressive syndrome of autism, dementia, ataxia and loss of purposeful hand use in girls (1). Since 1983, these features have been re-evaluated, and there is currently much doubt about the progressive nature of the disorder and whether “dementia” is an appropriate interpretation of the mental handicap. Because of the absence of a recognizable disease process, and because of various functional, physical, anatomical and chemical features, it has been hypothesized that RTT could be a disorder of development.

Several differential diagnoses are considered through the four clinical stages of this disease. At the beginning (stagnation stage), the most common differential diagnosis is benign hypotonia. Some months later, when the child loses her social acquisitions (regression stage), autism is frequently misdiagnosed; and, when motor regression occurs neurodegenerative and metabolic disorders are considered as probable diagnoses. After that stage, a period of pseudo-stagnation of the disease can last for decades, and the clinical picture may resemble, depending of the predominant neurological signs, a cerebral palsy, a mitochondrial cytopathy, a spinocerebellar degeneration or an epileptogenic encephalopathy. The less severely affected patients may resemble severe mental retardation with autistic features. Finally, after the loss of independent gait, or after the age of ten years for those in a wheelchair, a clinical picture with combined upper and lower motor neuron signs may be misdiagnosed as the end stage of a neurodegenerative disorder.

4.2. Explaining the symptoms

There is a temporal correlation between the time at which the clinical deficits are observed in RD and the time of normal maturation for cortical layers III and V. The motor delays and emotional instability are usually observed between 1 and 3 years of age, and it is at this time that the projection and association circuits of cortical layers III and V are organized (2).

Thus, it is possible that the failure of the dendritic arborisation in layers III and V contribute to the decreased brain size and the apparent failure of cortical processing, which results in the functional deficits. The first measurable change in RD patients is a decelerated head growth rate, with a decrease of OFC during the first months of life. Head size seems to be correlated with the degree of motor handicap, as we have observed in our series ([article](#)

1). This is followed by a decrease in height and body weight. This deceleration in growth is correlated with the type of mutation ([article 1](#)), but can also be partly due to the decreased caloric intake, related to lack of hand use and uncoordinated swallowing mechanisms; perseverance of the parents in feeding them can partially correct it. Small feet and hand size were correlated with the type of mutation ([article 1](#)); these features are considered a manifestation of autonomic dysfunction.

Regional abnormalities give rise to speculation about anatomical correlates of clinical findings: frontal cortex involvement and non specific personality changes, social withdrawal, loss of expressive language, irritability, impaired social interaction; temporal cortex involvement and absence of receptive language; motor cortex, basal ganglia and cerebellum involvement and hypotonia/dystonia, poor hand use, hand stereotypies, gait apraxia, gait ataxia; substantia nigra involvement and muscle rigidity and decreased mobility.

Movement disorders are the most prominent neurological signs in RD. Brain regions involved in classical movement disorders have been studied. Mild hypochromasia was observed in the striatum, and hyperchromia of large neurons in the striatum and internal pallidum, here with abnormal dendrites and degeneration of thick myelinated fibres (3, 4), and mild diffuse gliosis. Another finding is hypomelanation of the pars compacta of substantia nigra, without definite neuronal loss, a majority of large nigral neurons containing little or no neuromelanin and less tyrosine hydroxylase immunoreactivity than in controls (5, 6). Only in one Rett syndrome patient, aged 21 years, nigral cells were reduced by about 30% of age-matched controls, and free pigment granules in the neuropil, as seen in Parkinson disease, were demonstrated. These and preliminary data showing labelling of fragmented intranucleosomal DNA, using the TUNEL method in neurons from the substantia nigra, suggested that they were undergoing active degeneration (6). No apparent abnormalities in other pigmented brainstem nuclei have been seen, and morphometric studies of the serotonergic dorsal raphe nuclei showed no neuronal decrease, while reduced cell numbers were reported in the cholinergic nucleus basalis of Meynert (7). In MRI, Reiss observed that the caudate, putamen and thalamus were smaller in RTT than in control cases (8).

Ataxia is present in the majority of patients with RTT, at a variable degree of intensity and with varying age of onset. In our series of patients ([article 1](#)), we found (1) a severe phenotype, in which axial hypotonia (truncal ataxia) predominates at the beginning of the disease, and which evolved, at a variable period of evolution of the disease, to a Parkinsonian clinical picture; and, (2) a less severe form, in which the gait is ataxic and rigid since the beginning of the disease. We hypothesize that the different timing of

disease onset among patients with RD and the different neurological picture may be partially explained by the preferential function of MeCP2 in mature, rather than immature neurons (9).

A less functional protein could affect dramatically the function of the brainstem and cerebellar structures, provoking, for instance, such a severe hypotonia that patients would never acquire an independent gait. On the other hand, a more functional protein could not affect severely the cerebellum structures, but would be critical for cortical function and could provoke a phenotype where mental retardation predominates.

The cerebellum may show gross atrophy of all lobules of the vermis and progressive loss of Purkinje cells, with preservation of the basket and stellate cells, independent of age (10), with simplification of the inferior olivary nucleus without cell loss or gliosis. These findings, also seen in infantile autism (11), suggest arrested development beginning before birth (12). In some studies the cerebellum has been described as showing progressive atrophy by MRI (13).

Certain symptoms and signs in RTT suggest a dysfunction of the autonomic nervous system: small, cold, blue feet; breathing irregularities; sleep disturbances; prolonged QT intervals; altered heart rate variability; constipation.

Julu (14) has documented abnormalities of breathing patterns and vagal tone, related to an immaturity of the integrative processes in the brainstem autonomic centers. These include the nucleus of the solitary tract, the nucleus ambiguus, and the bulbar reticular formation. Several chemo-architectonic studies of these regions suggest that the brain stem is immature; a study of substance P immunoreactivity revealed an infant-like pattern of staining in the spinal cord and brain stem, with a consistent reduction of substance P immunoreactivity in the nucleus of the solitary tract and the reticular formation (15). Its deficiency possibly contributes to the alterations in pain perception, gastrointestinal symptoms, and other autonomic abnormalities in RD.

In another study, serotonin receptor binding was increased in many brain stem nuclei involved in motor control, including autonomic centres (16). Some CSF neurotransmitter studies (17, 18), including ours ([article 8](#)) (19), also found low levels of serotonin metabolites. Serotonin may therefore be involved in producing some of the serious alterations in Rett syndrome physiology, mainly in the first stages of the disease; its role in Rett symptomatology requires further study.

4.3. Neurodegenerative or neurodevelopmental disorder?

Neurodegenerative disorders are progressive illnesses that usually have a free interval and a loss of previously acquired skills. Some have a prenatal origin and manifest clinically at birth (as some peroxisomal diseases, neonatal leukoystrophies, pontocerebellar hypoplasias, the infantile type of neuroaxonal dystrophies, etc.). The recognition of the progressive character of such conditions may be difficult in the absence of a free interval, especially as many are very slowly progressive.

On the other side, neurodevelopmental disorders result from an injury to the developing brain, which can be of genetic, environmental or multifactorial origin. Children with developmental disabilities frequently arrest their maturation at a given stage, from which they do not proceed to a higher level.

Rett disorder is a particular neurological disease, sharing several characteristics with some neurodegenerative and some neurodevelopmental diseases. After an initial period of regression, with loss of previously acquired skills, the majority of the patients stay, for years, at the same neurological state, or acquire new abilities, as independent gait, hand prehension, or even some language. However, at the same time, and more or less insidiously, new neurological signs appear, as dystonia, pyramidal tract and Parkinsonian signs. Although not classically thought as a neurodegenerative disorder, age-related changes in the cerebellum were found in RTT (13, 20), and set in the clinical context of worsening gait disturbance. Reduction in area of the corpus callosum has also been reported (13, 21).

Rett disorder has a characteristic temporal profile that differs from that seen in neurodegenerative disorders. The early features resemble those of other gray-matter degenerative disorders of childhood, but the early decline in function and brain growth plateaus after the first decade.

Lack of evidence of cortical neuronal dysgenesis and dendritic microdysgenesis suggests the absence of a prenatal neurodevelopment disorder (3), while lack of severe neuronal loss and age-related neuronal abnormalities precludes the neurodegenerative hypothesis.

The RTT brain is small, but normally formed without migration abnormalities or evidence of a degenerative process. Neurons are small, but densely packed, consistent with a reduction in the development of axons and synaptic connections (22, 23). Similar neuropathological findings were described in some neurodevelopmental disorders: small neuronal size, increased packing density, and decreased dendritic branching, all have

been reported to occur in autism (24), and reduced dendritic arborisation and spines in Down, Angelman and the fragile X syndromes (25).

MeCP2 expression in brain was shown to be significantly reduced, not only in Rett disorder, but also in Angelman syndrome and autism spectrum disorders (26), which may explain the overlap of neuropathological findings and clinical phenotypes.

Rett disorder is an age-related regressive disease of neuronal development, but it is not degenerative, as some other classical neurodegenerative disorders. The studies of Matarazzo et al. (27) support the hypothesis that MeCP2 allows synapses to be formed, refined and stabilized, or pruned by suppressing genes that antagonize this process. A rupture in the synapse formation could explain the characteristic deceleration of head growth and the initial psychomotor arrest in RD.

We have yet to explain why the disorder progresses and what is the basis for the age-dependent appearance of the symptoms. Our results of CSF neurotransmitters study in RD support the hypothesis, defended for the first time by Nomura et al. (28), that dysfunction of noradrenergic, serotonergic and dopaminergic systems can be responsible for abnormal modulation of ontogeny and function of the higher and lower CNS.

The content of the monoamine levels show age-dependent changes, important to modulate the organization and function of certain neurons. Anomalies of neurotransmitters levels or receptors could explain the age dependent manifestation of each symptom. One hypothesis is that MeCP2 controls gene expression of neurotransmitter receptors. As these receptors probably vary with age, the disease is not degenerative but progressive, because the same dysfunctional MECP2 will cause different consequences at different ages. On the other side, different levels of function of MeCP2 could also explain why some clinical forms are so severe since the beginning of the disease or progress faster.

4.4. Does a genotype predict the phenotype?

Numerous reports provide insights into genotype-phenotype correlation, and the larger studies point to a positive correlation between mutation type and location with RD phenotype (29). Overall, patients with missense *MECP2* mutations may have a milder phenotype than those with truncating mutations, specially those with a C terminal. Phenotype in RTT is also influenced by the location of the mutation: cases with mutations leading to a partial or complete truncation of the NLS were more severe than those with mutations downstream of the TRD (29).

Another major source of the phenotypic variability associated with different MECP2 mutations in females seems to be the pattern of X chromosome inactivation (30).

In general, in our series of 60 RD, patients with truncating presented a more severe phenotype and a higher severity-score than those with missense mutations ([articles 1 and 5](#)).

As previously reported by others (31-34), we also found that patients with the R168X mutation have the most severe phenotype, while those with the R133C mutation have the mildest one.

Mood difficulties (not specific) associated with low number of stereotypies were previously reported in patients with R294X (35). Interestingly, we identified a particular hyperactive behaviour in patients with the R294X mutation, who had simultaneously higher number of stereotypies and increased levels of CSF dopamine metabolites.

We also identified an association of ataxia and rigidity with the T158M mutation, but, unlike other authors (34), we think that patients carrying this type of mutation do not have a phenotype as severe as those carrying the R168X mutation. Patients with T158M mutation are peculiar and have an intermediate form of the disease.

X inactivation studies in our patients only demonstrated an unbalanced pattern in two of them (1). One patient with a R168X mutation has a less severe form (and a lower severity score) compared with the group of patients with the same mutation: she acquired and maintains independent gait, has neither epilepsy nor microcephaly. (2) The other patient carried the T158M mutation, and had a phenotype similar to those carrying the same mutation. Surprisingly, a very atypical patient with a R133C mutation and the less severe phenotype of all patients in the series, had a balanced pattern of X inactivation in blood lymphocytes. We conclude that factors likely to affect the phenotypic expression may include the whole genetic background and regional differences in mutant MECP2 expression in the brain.

4.5. Diagnostic criteria of Rett syndrome: are they useful?

Classical Rett syndrome (RTT) is a clinical diagnosis, based on a set of criteria that describe a progressive neurodevelopmental and behavioural profile, irrespective of mutation status (36, 37). We have performed a detailed clinical and genetic study of patients with a clinical diagnosis of RTT (38), in whom we reassessed these criteria.

The revised diagnostic criteria proposed by the Rett Study Group, in 2002 (36), are, in our opinion, still too vague and inclusive; many children with mental retardation and motor delay or autistic behaviour may fulfil the criteria of variant RTT.

Within the clinical spectrum of Rett disorder (RD) - Rett syndrome patients with a detected MECP2 mutation - there are gradients in terms of age of onset (in some cases the disease may start in uterus) and symptom severity (that may be related to age of regression); however, all RD cases in our series displayed, during the pseudo-stationary stage, a rather similar phenotype and accomplished the majority of necessary diagnostic criteria, reinforcing the idea that strict adherence to these may be a good strategy for a higher rate of molecular diagnoses.

On the other hand, there are patients with a Rett-like phenotype who do not fulfil all major diagnostic criteria of classical phenotypes, but meet enough inclusion criteria of variant phenotypes to be classified as RTT (the majority of whom never had an initial period of normal development - the so-called conatal forms - or did not have a clear regression period) (39). Within this group, one may find patients of both sexes with a particular clinical picture of autistic traits, severe mental retardation, manual apraxia and stereotypies, who do not have detectable mutations in the *MECP2* gene (38).

One of the problems of the diagnostic criteria of variant phenotypes is the non-definition of some obligatory neurological signs. A patient may meet three of the six main, and five of the supportive criteria, and not have hand stereotypies or never had suffered a psychomotor regression.

One of the conclusions of our work ([articles 5](#) and [6](#)), (40) is that hand stereotypies are constant in all the stages of RD, except in the rare cases of males with lethal encephalopathy. The same is true for psychomotor regression: all the patients in our series with a detected MECP2 mutation (38) had psychomotor regression, even those who had never been considered normal since birth.

We thus propose the abolishment of the criteria for Rett variant phenotypes, because these are confusing and not well delineated. As an example, inside the supportive criteria, there are repetitions of the same criteria as “breathing irregularities” and “bloating / air swallowing”, whereas the latter are just a consequence of the first.

Additionally, some of the supportive criteria of variant RTT (albeit frequent in classical RTT) are not mentioned in the necessary criteria of classical RTT (as laughing/screaming spells, diminished response to pain and intense eye contact), or have a different nomen-

clature (as lower limb amyotrophy and abnormal muscle tone, successively associated with muscle wasting and dystonia).

We think that the revised diagnostic criteria for classical Rett syndrome must be applied to all suspected cases (including variants). Cases that do not accomplish these criteria, but have some particularities that resemble RTT patients, should be considered atypical RTT patients.

Within the necessary revised diagnostic criteria for classical RTT, some points should be modified, as is the case of the limit of age for loss of achieved purposeful hand skill, and to have a normal head circumference at birth. In our series of 60 patients with RD (i.e., the clinical diagnosis of Rett syndrome and a confirmed mutation in the *MeCP2* gene), 16.7% lost purposeful hand skills after the age of 30 months, and 10% presented microcephaly at birth (Table 3). To the necessary criterion of a “normal head circumference at birth”, it must be added “in the majority”; and the upper limit of 30 months for the loss of achieved purposeful hand skill must be abolished, because 8.3% of our cases lost that ability after 30 months of age. We underlie that regression of psychomotor milestones should also be considered a necessary diagnostic criteria.

Another important point is the definition of stage-dependent criteria. Rett disorder is progressive, and patients do have different symptoms and signs along their lives. Thus, it would be useful to establish new stage-dependent diagnostic criteria, based on the natural history of this disease.

The number of our patients with and without detected mutations was not sufficient to analyze the rate RD of patients who accomplished the diagnostic criteria in each stage, thus we only analyzed stage III (Table 3). Of our 36 RD patients at stage III, all had hand stereotypies, 91.7% presented bruxism, 72.2% presented hyperventilation or breath-holding, 66.7% showed scoliosis or kyphosis, and dyspraxic or failing locomotion, 63.9% had peripheral vasomotor disturbances. We can thus conclude that, at stage III, hand stereotypies are an obligatory criterium and bruxism should be considered a necessary criterium, not a supportive one.

We conclude that RD is a clinically homogeneous disease, if we observe patients at their pseudo-stationary stage, when most accomplish all major criteria. We suggest that the international diagnostic criteria be revised and defined in a stage-dependent manner, based on the analysis of clinical signs and symptoms present at the different stages of the disease, in large series of patients with *MECP2* mutations. After those modifications, the

rate of *MECP2* mutations detected may improve considerably, independently of the clinical evolution of the disease.

We also suggest that the only variant form that may need to be considered is the male form, as the others do not differ from the classical forms during the pseudo-stationary stage. Otherwise, we must reserve the term “variant” only to those forms where the symptoms and signs do not progress in a typical sequence and have a different quality, as they emerge and do not fulfil most major criteria at the pseudo-stationary stage. These cases probably represent other disorders.

Table 3: Stage-dependent criteria in 36 stage III RD patients.

	Group I - RD (n=36)
Necessary criteria	
4. Postnatal deceleration of head growth	36.1 (13)
5. Loss of achieved purposeful hand skill	72.2 (26)
6. Stereotypic hand movements	100
7. Communication dysfunction or loss of learned words	86.1
8. Dispraxic or failing locomotion	66.7 (24)
Supportive criteria	
1. Hyperventilation or breath-holding	72.2 (26)
2. Bruxism	91.7 (33)
3. Impaired sleep pattern from early infancy	50.0 (18)
4. Muscle wasting and dystonia	69.4 (25)
5. Peripheral vasomotor disturbances	63.9 (23)
6. Scoliosis/kyphosis	66.7 (24)
7. Growth retardation	50.0 (18)
8. Hypotrophic small and cool feet	53.1 (17)

4.6. Movement: the signature of Rett disorder?

The results of this work point to movement disorders being the most specific features of patients with Rett disorder in the sense that they contribute to differentiate them from those with Rett syndrome, or from other related neurodevelopmental disorders, as autism or Angelman syndrome ([articles 5, 6, 7](#)).

Also, in the group of patients with Rett disorder, movement disorders seem to indicate the disease severity, as with the timing of appearance of rigidity, or the number and frequency of stereotypies for disease progression. Movement disorders can also help clinicians to classify patients in a specific sub-group, as we did in our work. More importantly, subtle movement disorders are the clue for the diagnosis in the pre-regression phase of Rett

disorder, as was demonstrated through videotapes of Rett disorder patients in the first months of life, and in a patient with subtle stereotypies and repetitive hemidystonic movements during the stagnation period of the disease.

The CSF neurotransmitter studies performed in a group of RD patients point to a deregulation of the dopamine metabolism. We hypothesize that one of the *MeCP2* targets are cerebral dopamine receptors ([article 8](#)). It remains to explain why some patients have higher levels of dopamine metabolites and other have lower than normal. We hope that animal studies may help answering these questions in the future.

4.7. What were the major contributions of this work?

- We identified a large number of Portuguese RTT patients and contributed for the creation of a Portuguese Rett Syndrome Association.
- We performed, for the first time in Portugal, the genetic study of Rett syndrome, and acquired knowledge about the most prevalent *MECP2* mutations in the country.
- We contributed to a better knowledge of the differences between Rett syndrome and Rett disorder.
- We identified three different clinical subtypes among Rett disorder patients: (1) a severe form in which initial hypotonia evolves to a neurological picture of ataxia and rigidity, (2) an intermediary form in which ataxia predominates, and (3) a less severe form in which mental retardation predominates .
- We described, for the first time, an association between the genotype *R294X* and a particular phenotype, and CSF neurotransmitter abnormalities.
- We described (and showed as videotapes), for the first time, some of the movement disorders that can be present in Rett patients, and how these can contribute to differentiate Rett disorder from Rett syndrome.
- With the publication of the movement disorders present before the regression period in one Rett disorder patient, we believe that we contributed to help clinicians to perform an earlier diagnosis of this disorder.
- We proposed a revision of the clinical criteria for RTT.

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5. Ongoing work and perspectives for future research

5.1. Ongoing work

5.1.1. Stereotypies in Rett syndrome and cognitively impaired children with autism

(In preparation)

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Objective: Rett syndrome (RTT) and autism disorders (AD) share many phenotypical features, one of them being the stereotypies. The neurological and neurotransmitter basis of these repetitive behaviours is poorly understood. While the genetic basis of RTT has been clearly demonstrated, the aetiology of AD remains unclear. This study will describe and compare motor stereotypies, defined as repetitive purposeless patterned movements like hand-flapping, in two groups of children: (1) children with RTT and (2) cognitively impaired children with autism disorders, matched on chronological age and NVIQ. Our previous research showed that, in addition to their well known hand stereotypies, girls with RTT have others as well, some of which overlap those of some of the most severely affected children with autism. As a first goal toward advancing in the understanding of the pathophysiology of stereotypies, we will identify similarities and differences between these two groups of children, in terms of their topographical distribution, frequency, and variety. The second goal will be to determine whether some types or characteristics of stereotypies, besides hand stereotypies, characterize one or the other disorder, and therefore might be helpful for early diagnosis. In contrast to the stereotypies of RTT, which are unequivocally accepted as a neurological movement disorder, the basis for those in

children with AD is controversial, as they are not universally acknowledged as signs of a movement disorder. Detailed comparison with the stereotypies of RTT and AD is a step toward future investigation of their neurological basis, in the hope that better understanding will help developing a treatment for a symptom that interferes significantly with the children's function and development.

Methods:

Subjects:

_ RTT group: 20 girls with RTT were recruited among child neurologists in Portugal and were videotaped during a neurological examination. All children had MECP2 mutation and NVIQ<70, and 9 were able to walk independently. Their mean chronological age was 60 months (range 36-96).

_ AD group: 20 children (11 boys, 9 girls¹) with autistic disorder, based on DSM-III-R criteria, were recruited from a large nosological study in the USA, between 1985 and 1988, in which preschool children with autism were compared to other non-autistic developmentally impaired children and re-evaluated at school age. All children underwent comprehensive behavioural and neurological evaluations and were videotaped during a standardized play session. The children for the present study were randomly selected from the subgroup of children with autism presenting stereotypies, and were matched for chronological age and NVIQ with those with RTT. The children's mean age was 68 months (range 33-98) and their NVIQ was <70 (mean=31). All children were ambulatory and none had a frank neurological disorder.

Informed consent was obtained from all parents in both groups.

Scoring:

For each child, we scored a 5 minute segment most representative of their overall behaviour. Stereotypies were counted and each was tallied according to the body parts involved, including several subtypes of hands and finger stereotypies (unilateral, bilateral, hands together, different independent movement of each hand, hand to mouth, hand to object). Also coded was the frequency of stereotypies (rare, frequent, continuous), their variety (number of different types of stereotypies observed during the 5 minutes), and the most prevalent type of stereotypy (counting each one during the 5 minute clip was not reliable, because some children had very rapid and almost continuous stereotypies).

Analysis: The groups will be compared using Student's *t* test and chi-square test based on contingency tables.

Expected results:

The results of the study should provide new information about the characteristics of the movement phenotype of genetic disorders, such as RTT and AD. This information may help identifying the neuropathogenic processes that lead from the gene to the brain and to behaviour.

¹Autism is much more common in boys than in girls. As preliminary analyses showed no difference in stereotypies between boys and girls, both sexes were included, in order to have an adequate number of children with autism for the comparison with the RS girls.

5.1.2. CNS magnetic resonance volumetric studies in patients with Rett disorder and idiopathic autism

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Background and purpose:

Previous studies have examined volumetric abnormalities in Rett syndrome (RTT), using MR imaging and focusing on selective changes. These studies, however, preceded the identification of *MECP2* as the gene mutated in most RTT patients. We studied regional brain volume changes, as noted by MR imaging, in 15 girls with RTT with an identified *MECP2* mutation (Rett disorder, RD), with different age and clinical severity. We will compare these results with MR imaging of 15 idiopathic autistic-age matched patients. We will also perform longitudinal MR imaging studies in the younger patients with both conditions. We hope this study will contribute to a better characterization of the neuroanatomy of RD and its correlation with different phenotypes, and to clarify the progressive character of the disorder. The comparison of MRI volumetric studies in RD and autism may help elucidating the similarities and differences between both conditions.

Subjects and methods:

Participants

Study participants included 15 females diagnosed with Rett disorder (RD) (patients with an identified *MECP2* mutation) and 15 age-matched idiopathic autistic patients with low

IQ. Each participant gave written informed consent for their participation, after a complete description of the study.

Neurocognitive assessment

To assess general cognitive functioning of RD patients, we used the Pineda's severity scale.

To assess general cognitive functioning of autistic patients, we used the Griffiths' Scale for participants younger than 8 years of age, while those aged 8-16 years were administered the Wechsler Intelligence Scale for Children - Third Edition (WISC-III) (Wechsler, 1991).

MRI acquisition and processing

MRI images were obtained on a 1.5-T General Electric system (GE Medical Systems). The scan acquisition protocol consisted of contiguous 1.5 mm coronal T1 (spoiled gradient - SPGR) slices of the whole brain and an axial PD/T2 sequence (proton density and T2-weighted). The parameters used were: echo time - 5.0 msec, repetition time - 35 msec, flip angle - 45°, acquisition matrix - 256x192, voxel dimensions - 9375 x 0.9374 x 1.5mm). Images were aligned by using the line between the anterior and posterior commissures and the sagittal sulcus, to correct head tilt, and were also resampled to make isotropic voxels (0.9375 mm³, cubic interpolation). Then, an Atlas Based Expectation Maximization (EM) segmentation program separated raw MR data into cerebrospinal fluid, gray matter (including cortical and cerebellar cortices, basal ganglia and the hippocampal-amygdala complex) and white matter (Pohl et al., 2004).

Regions of Interest:

- Frontal, parietal, temporal and occipital regions
- Basal ganglia
- Cerebellar and vermis area
- Cortical superior temporal gyros
- Grey matter volume relative to the white matter volume

▪ Expected results

The results of the study should provide new information about the differences between autism and Rett disorder.

- We expect to find more severe selective reductions of frontal and parietal cortex in RD patients and in cerebellar hemispheres and vermis in autistic patients.

5.2. Perspectives for future research

5.2.1 National reference centre for Rett syndrome

I intend to improve the multidisciplinary consultation of Rett syndrome (RTT) at Hospital Geral de Santo António, in order to be the national reference centre for RTT. This centre will allow all RTT patients the opportunity to have a specialized approach. It will also be a training centre for medical professionals (physicians, therapists) and of scientific research.

5.2.2 Motion analysis and quantification of hand stereotypies in Rett syndrome patients with and without detected mutations in the *MECP2* gene

Background: Rett syndrome is a progressive neurodevelopmental disorder, clinically defined by criteria revised in 2002. In 1999, its aetiology was first established: mutations of the methyl-CpG binding protein 2 (MeCP2) gene (*MECP2*), which maps to Xq28. Since then, patients meeting the revised diagnostic criteria can be divided in two groups: those with a detected *MECP2* mutation (Rett disorder, RD) and those without it (Rett syndrome, RTT).

Stereotyped hand movements are a striking feature of both conditions. The aim of the present study is to analyse hand stereotypies in patients with and without a detected *MECP2* mutation and subject these movements to objective analysis, comparing the results in the two groups.

Patients and methods: The hand movements of five girls with Rett syndrome will be subjected to accurate, three-dimensional (3D) computerized motion analysis, and compared to 3D video analysis of 5 girls with Rett disorder.

Expected results: We believe the 3D computerized analysis will reveal different frequency of the hand movements in the two groups of patients. We hope in the future to use this method of automated video analysis, for early diagnosis and selection of patients for genetic study.

6. Attachments

6.1. Clinical database and scales

Base de dados S. Rett

Data do exame: __/__/__

Nome: Data nascimento: dia mês ano País: Médico que segue o doente: Idade Diag. S. Rett Consanguinidade Não ☐ Sim ☐ 1º Grau ☐ 2º Grau ☐

Outros membros da família

com Rett S., encefalopatia epileptog. ou atraso mental : Não ☐, Sim ☐, NS ☐GRAVIDEZ: Normal ☐ Anormal ☐ Não sabe ☐PARTO: Normal ☐ Anormal ☐ Não sabe ☐Nascido Termo: Sim ☐ Não ☐ Não sabe ☐Peso ao nascer: gr. Índice Apgar: Perímetro cefálico ao nascer cm Não sabe ☐Estagnação crescimento cefálico (PC): Sim ☐ Não ☐ Não sabe ☐Idade: anos mesesDesenvolvimento psicomotor: Normal ☐ Anormal ☐

(desde o nascimento até 12 meses)

Idade em que estagnou: mesesPerda de contacto social (Comp. Autista): Aos: mesesControle cefálico: mesesPosição sentado: Sim ☐ Não ☐Idade em que adquiriu: mesesSenta-se e mantém: Sim ☐ Não ☐ Não sabe ☐Idade em que perdeu: mesesMarcha sem ajuda: Sim ☐ Não ☐ Com ajuda ☐Idade em que adquiriu: mesesMantém marcha: Sim ☐ Não ☐ Idade em que perdeu: mesesPreensão de objectos: Sim ☐ Não ☐ Não sabe ☐Idade em que adquiriu: meses

Preensão prepositada: Sim ☐ Não ☐ Não sabe ☐ **Aos:** ☐ meses

➔ **Idade em que perdeu:** ☐ meses

Aos: ☐ Anos **readquiriu:** _____ **preensão de objectos**

Aos: ☐ Anos **readquiriu:** _____ **uso voluntário das mãos para**

Aquisição de linguagem palavra: Sim ☐ Não ☐ Não sabe ☐

➔ **Idade de aquisição:** ☐ meses

Palavras propositivas: Sim ☐ Não ☐ Não sabe ☐ **Aos:** ☐ meses

➔ **Idade em que perdeu:** ☐ meses

Frases propositivas: Sim ☐ Não ☐ Não sabe ☐ **Aos:** ☐ meses

➔ **Idade em que perdeu:** ☐ meses

Aos: ☐ Anos **recuperou:** _____ **Palavras propositivas**

Aos: ☐ Anos **recuperou:** _____ **Frases propositivas**

Aos: ☐ Anos **recuperou** _____ **Canta ou repete sons**

Exame objectivo na data de observação:

Impressão geral:

Perímetro cefálico actual: ☐ : ☐

Microcefalia adquirida: Sim ☐ Não ☐ Não sabe ☐ < 3sd ☐ < 4sd ☐

Peso pPeso ; **Estatuta** P Est ; **Pé** Ppé

Comunicação intensa com os olhos: Sim ☐ Não ☐

Dificuldades de aprendizagem: profundas ☐ moderadas ☐ ligeiras ☐

Dificuldades oro motoras na alimentação: refluxo ☐ engasgamento ☐ botão ☐
gástrico

Disfunção respiratória: Não ☐ Sim ☐ **Tipo:** Não sabe ☐

➔ **Idade em que adquiriu:** ☐ anos ☐ meses

Distensão abdominal / Deglute ar: Sim ☐ Não ☐ Não sabe ☐

S. Piramidal: Sim ☐ Não ☐

Ataxia: Sim ☐ Não ☐

Sentar Chinês: Sim ☐ Não ☐

Estereotipias: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20A, 20B, 21, 22, 23, 24, 25, 26, 27, 28, 29

Idade em que adquiriu: anos meses

Frequência estereotipias 1, 2, 3, 4

Outros movimentos involuntários:

Tremor ☐ Tipo de tremor: repouso ☐ postural ☐ cinético ☐

Mioclônias ☐ Descrição:

Distonia ☐ Classificação da distonia: focal, segmentar, multifocal, generalizada

Atetose ☐ Descrição:

Coreia ☐ Descrição:

Rigidez ☐ Severidade rigidez: 1, 2, 3, 4

Distúrbios periféricos vasomotores: Sim ☐ Não ☐ Tipo:

Atrofia neurogênea: Sim ☐ Não ☐

Cipoescoliose / Cipoze alta: Sim ☐ Não ☐ Surgery ☐

Idade em que foi diagnosticado: meses

A marcha é dispráxica? Sim ☐ Não ☐ Descrição:

Epilepsia: Sim ☐ Não ☐ Não sabe ☐ Tipo:

Idade em que adquiriu: anos meses

Tratamento: Sim ☐ Não ☐ Não sabe ☐

Controlada: Sim ☐ Não ☐ Não sabe ☐

Politerapia: Sim ☐ Não ☐ Não sabe ☐

Características EEG: Sim ☐ Não ☐ Não sabe ☐ Padrão de sono: NR ☐ ANR ☐

Controle esfíncter anal: Sim ☐ Não ☐ Não sabe ☐

Idade em que adquiriu: anos meses

Alt. Comportamento: Agitação ☐ Crises de grito ☐ Crises de riso ☐ Não ☐

Alt. Sono: Sono diurno ☐ Acorda de noite ☐ Não ☐

Desde a idade de: até.....meses

Peluda: Sim ☐ Não ☐

S. RETT clinicamente diagnosticado como: rapariga ☐ rapaz ☐

Clássico; Congénito; Epilep precoce; Linguagem preservada; Regr. tardia ; F. fruste

Preencher nos S. RETT variantes ou se for possível ter estes dados

Ressonância magnética cerebral: normal ☐ anormal ☐ Não executado ☐

TAC cerebral: normal ☐ anormal ☐ Não executado ☐

Cariótipo: normal ☐ anormal ☐ Não executado ☐

Estudo metabólico: normal ☐ anormal ☐ Não executado ☐

Aminoácidos/Plasma/LCR: normal ☐ anormal ☐ Não executado ☐

Ácidos orgânicos: normal ☐ anormal ☐ Não executado ☐

Purinas: normal ☐ anormal ☐ Não executado ☐

TORCH / Inf. Congénitas: normal ☐ anormal ☐ Não executado ☐

Fundo ocular: normal ☐ anormal ☐ Não executado ☐

Registo EEG: normal ☐ anormal ☐ Não executado ☐

Pot. evocados auditivos: normal ☐ anormal ☐ Não executado ☐

Pot. evocados visuais: normal ☐ anormal ☐ Não executado ☐

EMG / velocidade condução: normal ☐ anormal ☐ Não executado ☐

Estudo cardíaco: normal ☐ anormal ☐ Não executado ☐

Observações:

Pineda severity scale

Nº	Score	Definition		Score	Definition
Age of onset of first sign	1	0 -12 mo	Respiratory function	0	no dysfunction
	2	12 – 24 mo		1	hyperventilation and/or apnea
	3	> 24 mo	Epilepsy	0	absent
Microcephaly	0	absent		1	present and controlled
	1	present		2	uncontrolled or early epilepsy
Sitting alone	0	acquired < 8 mo	Hands use	0	acquired and conserved
	1	acquired 8-16 mo <i>Seat and maintains</i>		1	lose purposefulness: 2-6 years conserved manipulation <i>.Acquired and lost</i>
	2	acquired >16 mo <i>Seat and lost</i>		2	lose purposefulness: <2 years conserved manipulation <i>Acquired and partially conserved</i>
	3	never acquired			
	+1	lost acquisition			
Ambulation	0	acquired < 18 mo	Onset of stereotypies	3	never acquired
	1	acquired < 30 mo		+1	lose all acquisitions
	2	acquired > 30 mo		0	> 10 years
	3	never acquired		1	> 36 mo – <i>At Anytime</i>
	+1	lost acquisition		2	18 – 36 mo
Language	0	preserved and propositive		3	< 18 mo
	1	lost		Date of Birth:.....	
	2	never acquired		Date evaluation:.....	

Table I – Classification of Stereotypies

(modified from Jankovick and Fernandez-Alvarez & Aicardi)

I - Motor stereotypies

1. Simple

. Head

rolling
retropulsion
grimacing
bruxism
protrusion of the lips
repetitive closure of the eyes
eye rolling

. Joined hands

washing
clapping
mouthing

. Separated hands

mouthing
hair pulling
pill rolling
one hand behind the neck
castanets
to twist two or three fingers
flapping
tapping
“sevillana”
hands twisting
visual hand behaviour

. Arms

repetitive and rhythmic flexion of the arms

. Legs

intermittent leg elevation and tapping of the floor
toe walking
jumping

. Feet

feet twisting

. Whole body

trunk rocking
shifting weight from one leg to the other

2. Complex

II- Phonic stereotypies

repetitive sounds
repetitive words or phrases

6.2. Other published articles (not included in the previous chapters)

Article 12 - Temudo T., Maciel P., Rett's syndrome. Clinical features and advances in genetics. *Rev de Neurol* 2002, 34 (suppl.1): S54-8

Article 13 - T. Temudo. 2005. Movement Disorders in Rett Syndrome. *Rev de Neurol* 2005 ; 40 (suppl1): S167-71

Article 14 - Shi J, Shibayama A, Liu Q, Santos M, Temudo T, Maciel P, Sommer SS. Detection of heterozygous deletions and duplications in the MECP2 gene in Rett syndrome by Robust Dosage PCR (RD-PCR). *Hum Mutat* 2005;25:505-511

6.2.1. Rett's syndrome: Clinical features and advances in genetics (Article 12)

Síndrome de Rett.

Características clínicas y avances genéticos

T. Temudo^a, P. Maciel^b

RETT'S SYNDROME. CLINICAL FEATURES AND ADVANCES IN GENETICS

Summary. Introduction. Rett's syndrome (RS) is a disorder of neurological development which is the second commonest cause of mental retardation in girls. It is normally caused by *de novo* mutations of a gene on the X chromosome. This gene encodes for the protein joining the methyl-CpG (MECP2). Mutations of this gene have been found in approximately 80% of the cases confirmed as having the classical form of RS. Mutations of the gene MECP2 were also found in about a third of the nonclassical cases of RS and in other conditions: women with mild or severe mental retardation, children with autism, even children with neonatal encephalopathy or with a clinical condition similar to RS. Development. Studies correlating the genotypes and phenotypes in classical RS suggest that the pattern of inactivation of the X chromosome is more important in determining the severity of the condition than the type or site of the mutation. However, when we consider all the phenotypes associated with mutations of MECP2, it is seen that the type of mutation correlates to a certain degree with the clinical onset and severity of the disorder. Conclusions. Recent advances in the genetics of RS therefore have specific application in the clinical field. They give further markers for diagnosis and possibly indicate the prognosis, as well as being useful in genetic counselling for the families of patients with RS. [REV NEUROL 2002; 34 (Supl 1): S54-8]

Key words. Angelman's syndrome. Autism. Encephalopathy. MECP2. Mental retardation. Rett syndrome.

INTRODUCCIÓN

Un pediatra austríaco, Andreas Rett, fue quien describió por primera vez el síndrome de Rett (SR) en 1966 [1,2], si bien no fue hasta 1983 cuando esta enfermedad fue ampliamente divulgada, gracias a la publicación de 35 casos por parte de Hagberg et al [3].

Su incidencia aproximada es de 1:15.000 niñas [4]; afecta a niñas de todas las razas y es la segunda causa más común de retraso mental grave en el sexo femenino (después del síndrome de Down) [5].

El SR afecta casi exclusivamente a niñas cuyo desarrollo psicomotor inicial es aparentemente normal durante los primeros 6 meses de vida. El perímetro cefálico es normal al nacer, y posteriormente se constata un retraso en su crecimiento que conduce habitualmente a la microcefalia. Entre los 3 meses y los 3 años de edad se observa una reducción de la adquisiciones tales como la prensión propositiva, el lenguaje y la comunicación.

Simultáneamente, las pacientes inician estereotipias manuales continuas, con las manos juntas o separadas, las cuales constituyen la señal más característica del SR. Algunas de ellas presentan, desde el inicio de la enfermedad, señales neurológicas, tales como ataxia del tronco, escoliosis neurógena, señales piramidales o distonía, pero otras las presentarán muy posteriormente, predominando en los primeros estadios de la enfermedad un cuadro de perturbación de tipo autista.

Además de los criterios obligatorios, existen criterios de apoyo al diagnóstico [6] que incluyen disfunción respiratoria, bruxismo, alteraciones electroencefalográficas, baja estatura, etc. (Tabla).

Los criterios de exclusión del SR incluyen la existencia de microcefalia al nacer y la identificación de enfermedades metabólicas degenerativas o enfermedades neurológicas adquiridas, retinopatía o atrofia óptica (Tabla).

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VARIANTES DEL SÍNDROME DE RETT

Las niñas que cumplen con la mayoría de los criterios de diagnóstico del SR son clasificadas como pacientes con SR clásico. Con el tiempo, se ha puesto en evidencia que el fenotipo del SR es mucho más variado de lo que se describió al principio. La mayoría de las variantes del SR son, comparadas con la forma clásica, más leves, especialmente en el grado de disfunción motora. Hagberg et al describieron cinco tipos de variantes [7].

- **Forma con epilepsia precoz:** estas pacientes, aunque cumplan con los criterios del SR clásico, inician los síntomas antes de los 6 meses de edad y la presentación clínica inicial está dominada por las crisis epilépticas.
- **Forma congénita:** en esta forma de SR nunca se experimentó un período de normalidad en el desarrollo psicomotor, aunque las pacientes también cumplan con los criterios del SR clásico.
- **Forma de regresión tardía:** la regresión del desarrollo psicomotor se manifiesta más tarde (generalmente después de los 4 años de edad) y de forma más insidiosa.
- **Forma 'frustré':** el curso clínico de estas pacientes es más leve e incompleto. La regresión se produce habitualmente entre el primer y el tercer año de vida, pero no es tan grave como en el SR clásico. El uso de las manos puede estar preservado y las estereotipias pueden ser mínimas o atípicas. Generalmente no presentan microcefalia ni baja estatura.
- **Forma con conservación del lenguaje:** estas pacientes, tras la fase de regresión, consiguen pronunciar algunas palabras que habían aprendido antes de manifestarse la enfermedad. Cabe señalar que las palabras pronunciadas nunca se adquieren después de la fase de regresión.

Además de estos cinco tipos descritos por Hagberg et al, también se publicaron casos de SR en niños [8-15].

Los casos descritos en la literatura corresponden a niños con un cariotipo 47 XYY [16] y en hermanos de niñas con SR familiar, que provoca frecuentemente encefalopatías letales en la infancia. Excepcionalmente, el SR en niños puede manifestarse como una encefalopatía no letal con un curso semejante al del sexo femenino [17].

Tabla. Criterios de diagnóstico para el SR clásico [6].

1. Criterios necesarios
Desarrollo prenatal y perinatal aparentemente normal
Desarrollo psicomotor aparentemente normal hasta los 5-6 meses
Perímetro cefálico normal al nacer
Desaceleración del crecimiento cefálico entre los 3 meses y los 3 años de edad
Pérdida de las adquisiciones comunicativas, del lenguaje y de la prensión manual propositiva
Aparición de retraso mental
Aparición de estereotipias manuales intensas
Anomalías de la marcha en niñas ya ambulantes
Intento de diagnóstico entre los 2 y los 5 años de edad
2. Criterios de apoyo
Disfunción respiratoria: apneas, hiperapneas, expulsión forzada del aire y la saliva
Distensión abdominal/deglución del aire
Anomalías en el EEG: actividad de base lenta o intermitente y de forma rítmica un retraso; descargas epileptiformes acompañadas o no de crisis epilépticas
Epilepsia: crisis de varios tipos
Espasticidad, distonía o atrofia muscular
Trastornos vasomotores periféricos
Escoliosis de tipo neurógeno
Pies hipotróficos, pequeños y fríos
Estatura baja y peso bajo
3. Criterios de exclusión
Organomegalia y otros síntomas de enfermedades de almacenamiento, retinopatía o atrofia óptica, microcefalia neonatal, existencia de una enfermedad metabólica o hereditaria degenerativa identificada
Enfermedad neurológica adquirida como resultado de una infección o trauma
Evidencia del retraso del crecimiento intrauterino
Evidencia de lesión cerebral adquirida en el período perinatal

EVOLUCIÓN CLÍNICA

La historia natural del SR se puede dividir en cuatro estadios [18].

Estadio I (estancamiento)

El inicio de la primera fase de la enfermedad se produce entre los 5 y los 18 meses de edad y se caracteriza por una paralización del desarrollo psicomotor y un retraso en el crecimiento cefálico. Durante semanas o meses el desarrollo psicomotor progresa lentamente, aunque generalmente se considera normal. Las niñas son hipotónicas y en la mayoría de los casos nunca llegan a gatear.

Estadio II (regresión rápida del desarrollo)

El inicio de este período tanto puede ser repentino (de un día para otro), como insidioso (duración de más de un año) y se inicia entre el primer y cuarto año de vida.

Se comprueba que existe una pérdida de las adquisiciones en la

esfera de la comunicación, con pérdida de lenguaje e implantación de un comportamiento autista. Curiosamente, al contrario de otras perturbaciones del espectro autista, el contacto visual es muy bueno, siendo ésta la única forma de comunicación conservada. Además, las niñas dejan de jugar, ya que pierden la prensión propositiva y, de manera simultánea, inician movimientos estereotipados de las manos, a veces continuos y de carácter compulsivo.

Generalmente las adquisiciones motoras ordinarias se mantienen preservadas pero, a veces, durante esta fase, las niñas que ya habían adquirido la marcha pierden esta capacidad, recuperándola más tarde en la mayoría de los casos.

Algunas pacientes empiezan a mostrar alteraciones del sueño con inversión del ritmo diurno/nocturno, presentan episodios paroxísticos de risa y llanto, bruxismo diurno, irregularidades respiratorias exclusivamente diurnas (apneas e hiperapneas) y crisis epilépticas de vez en cuando de difícil control farmacológico.

Estadio III (período pseudoestacionario)

Su inicio se produce entre los 2 y los 10 años de edad y puede durar décadas. Al contrario que las enfermedades degenerativas del sistema nervioso central, a un período de regresión del desarrollo le sigue otro con alguna recuperación e incluso progresión. Respecto a la comunicación, se produce una cierta mejoría en algunas niñas, que consiguen pronunciar algunas palabras que habían aprendido anteriormente a la aparición de la regresión. La apraxia o dispraxia manual y las estereotipias, en conjunto con el retraso en el crecimiento cefálico, constituyen las características clínicas que habitualmente permiten establecer un diagnóstico en este estadio [19]. La marcha puede adquirirse en esta fase (en aquellas niñas que no la habían adquirido o que la habían perdido en la fase de regresión), pero a partir de los 10 años es muy raro que esta circunstancia se produzca. La epilepsia es muy frecuente y a veces refractaria al tratamiento. Las anomalías respiratorias y la aerofagia con distensión abdominal están presentes en aproximadamente un 75% de los casos [7]. Entre el 85 y 90% de los casos presentan una mala evolución estaturoponderal y empeoran a medida que aumenta la edad [7].

Aunque de manera lenta, en esta fase existe una regresión neuromotora que culmina con la pérdida de la marcha autónoma y la dependencia de la silla de ruedas, momento en el que las pacientes pasan automáticamente al estadio IV (IVa). Las pacientes que nunca adquirieron la marcha en el estadio III, también pasan, a partir de los 10 años al estadio IV (IVb), dado que es muy poco probable que se adquiera esta capacidad con posterioridad a esa edad, tal como se mencionó anteriormente.

Estadio IV (deterioro motor tardío)

El inicio de esta fase coincide con la pérdida de la marcha o con la no adquisición de la misma a los 10 años de edad. En la mayoría de los casos, el tránsito a este estadio se produce en la segunda década, pero algunas pacientes mantienen la marcha autónoma durante varias décadas.

En esta fase predominan generalmente los síntomas extrapiramidales; las pacientes presentan una rigidez mixta (piramidal y extrapiramidal), distonía, atrofia muscular marcada, bradicinesia con desaparición casi total de estereotipias, temblor e hipomimia. La escoliosis está presente en cerca de la mitad de los casos y las irregularidades respiratorias y las crisis epilépticas desaparecen o se reducen considerablemente. Los pies y las manos son frecuentemente muy pequeños, fríos y presentan deformidades debido a la distonía. La práctica desaparición de las estereotipias manuales en esta fase hace difícil el diagnóstico del SR a quien lo observa

por primera vez en pacientes en edad adulta. La mayoría de las pacientes de esta edad presentan un retraso mental profundo, apraxia manual, tienen frecuentemente baja estatura, dependencia de la silla de ruedas y un síndrome parkinsoniano. El diagnóstico sólo puede realizarse gracias a su historia anterior.

Esta fase puede durar décadas, y la edad de fallecimiento es variable. En un tercio de los casos, la muerte acontece durante los primeros veinte años de vida, sobre todo durante la noche, no está relacionada con crisis epilépticas y, por ahora, no existe una explicación.

GENÉTICA

La base genética y el tipo de herencia del SR fueron objeto de controversia desde que esta enfermedad se describió por primera vez. Esto es comprensible si pensamos en que la mayoría de los casos de SR se producen de manera esporádica, con una recurrencia familiar muy baja (cerca del 1/1.000) [4]. Este hecho dificultó el reconocimiento de su naturaleza hereditaria y complicó enormemente el planteamiento del mapa genético y el clonaje posicional del gen causante, que se basaban en la segregación del gen en familias afectadas. Sin embargo, con las familias existentes y gracias a tratamientos de exclusión de ligamiento genético, fue posible delimitar la región de interés a la Xq28 [20-23], donde se situaban varios genes candidatos a ser estudiados en pacientes con SR.

Recientemente, la identificación de mutaciones en el gen que codifica la proteína de unión a las metil CpG (MECP2) [24] confirmó la hipótesis inicial de que el SR era una nueva enfermedad ligada al cromosoma X dominante, y que sigue la regla general de las mutaciones *de novo*; es decir, además de dificultar su reconocimiento como enfermedad hereditaria, tiene implicaciones para el consejo genético en el SR.

La proteína MECP2 se une al ADN metilado, independientemente de su secuencia, y se piensa que está implicada en procesos de silenciamiento de la expresión génica. Las mutaciones que afectan a la función de esta proteína podrán conducir a la expresión inapropiada de numerosos genes en diferentes momentos, o podrá tener consecuencias nefastas en el desarrollo y la maduración del sistema nervioso. La identificación de los genes cuya expresión no está regulada en ausencia del MECP2 funcional, será, sin duda alguna, crucial para la comprensión del mecanismo de la patogénesis del SR.

El gen MECP2 tiene una longitud de 76 Kb [25] y está compuesto por cuatro exones, que constituyen una secuencia codificante de aproximadamente 1,5 Kb. En este gen se encontraron mutaciones en aproximadamente un 80% de los casos comprobados con la forma clásica del síndrome, en diferentes poblaciones [24,26-32]. A pesar de que la gran mayoría de las mutaciones eran *de novo*, existen ocho mutaciones que son aún más comunes y que explican prácticamente el 65% de los casos [30]. En total, se describieron 60 mutaciones diferentes, distribuidas por toda la región codificadora del gen, aunque se localicen en mayor número en el exón 4. Es incluso posible que existan otras mutaciones, por ejemplo, en regiones no codificadoras del gen (tales como el promotor y los intrones), pero éstas son más difíciles de localizar, y son objeto de investigación en la actualidad. También se encontraron mutaciones en el gen MECP2 en casos de SR no clásico, aunque en un porcentaje de casos mucho menor [26].

La hipótesis tradicionalmente propuesta para explicar el predominio de pacientes de sexo femenino es la letalidad embriológica o perinatal de la mutación en individuos del sexo masculino (homoci-

góticos para los genes del cromosoma X). No obstante, de acuerdo con lo anteriormente mencionado, existen niños con mutaciones en el gen MECP2 que nacen y sobreviven durante un año o más tiempo. Además, hay que añadir que no se observa una tasa superior de abortos espontáneos en madres de pacientes con SR [33], lo cual sería de esperarse si hubiera letalidad embriológica. Una explicación alternativa sería la mayor tasa de mutación de este gen en la gametogénesis masculina al compararse con la femenina [33,34]. Si las mutaciones se produjeran de manera preferente en las células germinales de los padres, y éstos transmiten obligatoriamente el cromosoma X a sus hijas, se podría explicar de esta manera el sesgo observado.

La identificación del gen causante de la mayoría de los casos del SR tiene implicaciones prácticas importantes en términos de diagnóstico, pues existe ahora la posibilidad de confirmar un diagnóstico clínico de SR; también parece posible realizar un diagnóstico prenatal de esta enfermedad en el ámbito de una consulta de consejo genético, aunque, como se mencionó anteriormente, el riesgo de recurrencia familiar del SR sea bajo y sólo en los casos de mosaicismo somático o inactivación no aleatoria del cromosoma X de la madre (que permite que ésta sea portadora de la mutación pero que no esté afectada, situación muy rara) se justifique claramente esta intervención. En las otras situaciones es menos claro el beneficio, y la decisión de la pareja de optar o no por el diagnóstico prenatal debe tomarse sopesando los riesgos asociados en cada una de las decisiones.

CORRELACIÓN FENOTIPO-GENOTIPO

Ahora que se sabe que el gen MECP2, ligado al cromosoma X, está sujeto a la inactivación del cromosoma X (ICX), se espera que los patrones del ICX tengan una influencia sobre el fenotipo [25]. Así, hace mucho tiempo que se conoce que, además del SR clásico, existen formas leves en las que faltan algunos síntomas [4], así como formas muy graves que no tienen un período de normalidad en el desarrollo inicial y causan hipotonía y espasmos infantiles [35].

A medida que se llevan a cabo un mayor número de análisis mutacionales, la clasificación de estas diferentes entidades como variantes de un síndrome están siendo verificadas genéticamente [14,28,36,37]. De hecho, se ha hecho evidente que las mutaciones en el gen MECP2 producen un abanico aún mayor de fenotipos que el sospechado previamente.

Con el objetivo de determinar de qué manera los diferentes tipos de mutaciones del gen MECP2 contribuyen en la variabilidad fenotípica del SR, varios grupos han efectuado estudios de correlación genotipo-fenotipo. Amir et al estudiaron pacientes con inactivación equilibrada del cromosoma X y clasificaron los genotipos en mutaciones de delección (en las que la proteína MECP2 resultante es más pequeña de lo normal, siendo en la mayoría de ocasiones degradada por las células) o 'missense' (en la que existe producción normal de la proteína, pero en la que un aminoácido es sustituido por otro, lo cual puede alterar la función de la proteína); no se encontró correlación entre estos tipos de mutaciones y la gravedad clínica del síndrome, pero se comprobó que las pacientes que presentaban las mutaciones de delección padecían más anomalías respiratorias y una disminución del ácido homovanílico en el líquido cefalorraquídeo, mientras que las que tenían mutaciones 'missense' presentaban una mayor incidencia de escoliosis [38].

Ni en los estudios de Bienvenu et al [26], ni en los de Huppke et al [27], se encontró correlación entre el tipo de mutación y las características clínicas. Sin embargo, Cheadle et al verificaron que las mutaciones de delección situadas en la parte anterior de la

proteína se correlacionan con un pronóstico peor que el de las mutaciones de delección más posteriores [29].

Por lo tanto parece que, en el SR clásico, la correlación entre el tipo de mutación y el fenotipo todavía no se ha aclarado del todo, si bien la ICX es un determinante mayor de las manifestaciones clínicas [38], al estar relacionada con los niveles de expresión de la proteína MECP2 mutante.

En el futuro será importante basar las correlaciones genotipo-fenotipo en una escala de gravedad estandarizada que podrá aplicarse a pacientes con SR.

INVESTIGACIÓN DE MUTACIONES EN EL GEN MECP2 EN OTRAS ENFERMEDADES

El abanico de enfermedades asociadas a la mutación del gen MECP2 afecta actualmente a los dos sexos. En el sexo femenino se encontraron mutaciones en las formas clásica y variantes del SR, así como en mujeres con dificultades entre moderadas y graves de aprendizaje.

En el sexo masculino, se descubrieron mutaciones en el gen MECP2 en niños con un fenotipo de SR clásico con el síndrome de Klinefelter [16], en dos niños de la misma familia con retraso mental ligado al cromosoma X y espasticidad progresiva [36], en un niño con un fenotipo semejante al del SR [17] y en otros cuatro niños con encefalopatía progresiva de aparición neonatal [13,14].

También se describieron mutaciones del gen MECP2 en tres casos con fenotipo semejante al síndrome de Angelman, sin anomalía molecular en el cromosoma 15 [39].

En la actualidad aún no existe suficiente información que permita predecir cuáles son los trastornos del neurodesarrollo que pueden ser explicados por las mutaciones del gen MECP2. Parece claro que las niñas que presentan todos o algunos de los criterios del SR deben ser evaluadas, así como debemos considerar cuidadosamente el análisis de mutaciones en niñas con retraso mental no sindrómico, autismo con algunas características de encefalopatía progresiva y en otras enfermedades del desarrollo neurológico ligadas al cromosoma X [40].

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SÍNDROME DE RETT.**CARACTERÍSTICAS CLÍNICAS Y AVANCES GENÉTICOS**

Resumen. Introducción. El síndrome de Rett (SR), una enfermedad neurológica del desarrollo que constituye la segunda causa de retraso mental profundo en el sexo femenino, es provocada en la mayoría de los casos por mutaciones de novo en un gen situado en el cromosoma X, gen que codifica la proteína de unión a las metil-CpG (MECP2); se han descubierto mutaciones en este gen en aproximadamente un 80% de los casos comprobados que presentan la forma clásica del SR. También se encontraron mutaciones en el gen MECP2 en aproximadamente un tercio de los casos de SR no clásicos e incluso en otras enfermedades: mujeres con retraso mental leve o profundo, niños con autismo e incluso niños con encefalopatía neonatal o con un cuadro clínico similar al del SR. Desarrollo. Los estudios de correlación genotipo-fenotipo en el SR clásico sugieren que el patrón de inactivación del cromosoma X tiene un efecto más importante en la determinación de la gravedad de la enfermedad que el tipo o la localización de la mutación. Sin embargo, cuando se considera la totalidad de los fenotipos asociados a las mutaciones MECP2, se comprueba que el tipo de mutación tiene alguna correlación con la presentación clínica o la gravedad de la enfermedad. Conclusión. Los recientes avances en la genética del SR presentan, por lo tanto, aplicaciones concretas en el panorama clínico, y proporcionan marcadores auxiliares de diagnóstico y posibles indicadores de pronóstico, así como para el consejo genético a las familias de pacientes con SR. [REV NEUROL 2002; 34 (Supl 1): S54-8]

Palabras clave. Autismo. Encefalopatía. MECP2. Retraso mental. Síndrome de Angelman. Síndrome de Rett.

SÍNDROME DE RETT.**CARACTERÍSTICAS CLÍNICAS E AVANÇOS GENÉTICOS**

Resumo. Introdução. A síndrome de Rett (SR), uma doença neurológica do desenvolvimento que constitui a segunda causa de atraso mental severo no sexo feminino, é causada na maioria dos casos por mutações de novo no gene situado no cromossoma X que codifica para a proteína de ligação às metil-CpG (MECP2), tendo-se encontrado mutações neste gene em cerca de 80% dos probandos com a forma clássica de SR. Também foram encontradas mutações no gene MECP2 em cerca de um terço dos casos de SR não clássicos e ainda numa variedade de outras patologias: mulheres com atraso mental ligeiro ou severo, crianças com autismo e ainda rapazes com encefalopatia neonatal ou com uma apresentação clínica semelhante à da SR. Desenvolvimento. Os estudos de correlação genótipo-fenótipo na SR clássica sugerem que o padrão de inativação do cromossoma X tem um efeito mais importante na determinação da gravidade da doença do que o tipo ou localização da mutação. Contudo, quando a totalidade de fenótipos associados com as mutações MECP2 é considerada, verifica-se que o tipo de mutação tem alguma correlação com a apresentação clínica e gravidade da doença. Conclusão. Os avanços recentes na genética da SR têm assim implicações concretas para a clínica, fornecendo marcadores auxiliares de diagnóstico e possíveis indicadores de prognóstico, e também para o aconselhamento genético das famílias de doentes com SR. [REV NEUROL 2002; 34 (Supl 1): S54-8]

Palavras chave. Atraso mental. Autismo. Encefalopatia. MECP2. Síndrome de Angelman. Síndrome de Rett.

6.2.2. Movement Disorders in Rett Syndrome (Article 13)

Discinesia en el síndrome de Rett

T. Temudo

MOVEMENT DISORDERS IN RETT SYNDROME

Summary. Introduction. *Rett syndrome is a progressive neurodevelopmental disorder with onset in early childhood occurring almost exclusively in females and caused by mutations in methyl-CpG-binding protein 2 (MECP2) and is one of the most frequent causes of mental retardation in females. Regression is a defining feature of Rett syndrome and during the regression period the patients develop autistic behaviour. This is the reason why Rett syndrome has been classified as a pervasive developmental autistic spectrum disorder. However movement disorders are so exuberant, characteristic and unique in Rett syndrome that is very difficult to an experienced clinician mistake both situations. Development. We can define Rett syndrome as a condition which manifests in the majority of the cases as a hyperkinetic movement disorder and progresses (at varying rates) to a bradykinetic disorder. The aim of this publication is to characterize and describe movement disorders in Rett syndrome based on a revision of the literature and the personal experience of the author. [REV NEUROL 2005; 40 (Supl 1): S167-71]*

Key words. Movement disorders. Rett syndrome.

INTRODUCCIÓN

Andreas Rett, un pediatra vienés, descubrió el síndrome de Rett (SR) cuando observó que dos niñas que estaban esperando en su consulta presentaban la misma discinesia: estereotipias manuales. En 1966, publicó los datos de 22 niñas con atrofia cerebral progresiva, las estereotipias manuales, demencia, alalia, apraxia de la marcha y una tendencia a las crisis epilépticas [1,2]. Este trastorno se denominó síndrome de Rett en 1983 por Bent Hagberg, Jean Aicardi, Karin Dias y Ramos, que estudiaron a 35 pacientes de sexo femenino de Suecia, Francia y Portugal, e hicieron hincapié en el comportamiento autista, microcefalia adquirida, ataxia del tronco con sacudidas y alteraciones vasomotoras [3]. Los criterios de diagnóstico para este síndrome se describieron con más precisión en 1988 [4]. Después de que Andreas Rett descubriera el síndrome, se tardó más de 30 años en determinar su base genética –las mutaciones de la proteína MeCP2– en buena medida porque la enfermedad es fundamentalmente esporádica y porque los casos familiares son poco comunes [5].

Entre los criterios de diagnóstico del SR necesarios y los complementarios (Tablas I y II) se encuentran algunas discinesias como la distonía, las estereotipias manuales y la apraxia de la marcha [6]. Sin embargo, puede haber otras discinesias que no estén tan bien descritas como los movimientos estereotipados del tronco, la cabeza o los miembros inferiores, temblor, corea, mioclonía, rigidez y anomalías de la marcha.

PRESENTACIÓN CLÍNICA

Estereotipia

Fisiopatología de las estereotipias

El sistema dopaminérgico en los ganglios basales ha sido relacionado con la producción de estereotipias. La inyección de dopamina intraestriatal y la administración sistémica de agonis-

tas de la dopamina que son activos presinápticamente (como las anfetaminas) y de agonistas de la dopamina que son activos postsinápticamente (como la apomorfina) en ratas provocan conductas repetitivas dependientes de la dosis como olisquear, roer, lamer, morder, erguirse, menear la cabeza y otras conductas estereotipadas aprendidas [7]. Estas estereotipias se pueden bloquear con fármacos neurolépticos [8] como el SKF 38393, un agonista D₁, que no provoca estereotipias pero que aumenta las estereotipias inducidas por apomorfina (un agonista mixto D₁ y D₂) y por PHNO, un agonista D₂ selectivo [9]. Esto indica que los receptores D₂ actúan como mediadores de las estereotipias y que los receptores D₁ potencian este efecto. Los neuropeptidos también pueden modular la conducta estereotipada. La microinyección de pancreocimina y neurotensina en el núcleo *accumbens* medial, aunque no en el cuerpo estriado, potencia la estereotipia inducida por apomorfina [10].

Presentación clínica de las estereotipias en el síndrome de Rett

Las estereotipias manuales son el rasgo distintivo del síndrome de Rett y aparecen cuando se pierde el uso útil de las manos. Los automatismos casi continuos, repetitivos y compulsivos desaparecen durante el sueño y pueden agravarse con la ansiedad.

Las estereotipias manuales pueden realizarse sobre el eje vertical, con movimientos simétricos de las dos manos (frotarse las manos, aplaudir, dar golpecitos, retorcerse las manos, ponérselas en la boca) o con las manos actuando por separado –más frecuente– haciendo cada una un movimiento diferente (tirarse del pelo con una mano y con la otra darse golpecitos en el tronco; tirarse del pelo con una mano y la otra en la boca; agitar una mano; *pill-rolling* con una mano, es decir, los dedos y la muñeca se mueven como si estuviesen manipulando pequeños objetos).

A menudo, cuando se ponen las manos en la boca, se producen mutilaciones y las estereotipias se deben inhibir mediante métodos mecánicos o utilizando dosis bajas de risperidona [11].

Detrás de las estereotipias manuales hay también otros tipos de estereotipias que afectan a otras partes del cuerpo (observación personal): dar vueltas con la cabeza y también con los ojos, sacudir el tronco, elevar las piernas de forma intermitente y dar golpecitos en el suelo, caminar de puntillas, movimientos de balanceo de todo el cuerpo con cambio de peso de una pierna a

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la otra. Las estereotipias también pueden ser muy complejas al principio de la enfermedad: algunas niñas presentan una especie de 'baile estereotipado' [observación personal].

Cada niña tiende a tener las mismas estereotipias a lo largo de su vida pero tienen tendencia a disminuir y a simplificarse y ralentizarse con la edad, cuando las pacientes con el síndrome de Rett se vuelven hipocinéticas y rígidas.

Distonía

La asimetría postural se observa con frecuencia en el síndrome de Rett, y en las fases tardías de la enfermedad siempre está presente en los miembros inferiores.

FitzGerald et al [12] describen la presencia de distonía en 19 de 32 pacientes (59%), con más frecuencia distonía crural o generalizada aunque también distonía focal, que afecta a las extremidades superiores o inferiores. Normalmente, las alteraciones son asimétricas, y el lado derecho del cuerpo es el más afectado [13,14]. La escoliosis, una característica común en el síndrome de Rett, puede ser un signo de distonía del tronco, y en las formas graves que nunca logran la ambulación está presente ya desde las primeras fases de la enfermedad.

Síndrome rígidoacínético

Una cara 'de máscara' inexpressiva es un signo clínico temprano en pacientes con síndrome de Rett, acompañado de una habilidad desproporcionada para la comunicación ocular y de un parpadeo normal. En una serie de 32 pacientes con el síndrome de Rett examinadas por FitzGerald et al [12], la hipomimia era más grave y más común en las niñas más mayores.

La mayoría de pacientes adquieren una ambulación independiente. A medida que la enfermedad evoluciona (la velocidad de la evolución es variable) la marcha se vuelve más rígida, con un balanceo deficiente de los brazos, y algunas pacientes presentan una marcha rígida y de base amplia característica con contracciones de los músculos abdominales e hiperextensión de las piernas [observación personal]. También pueden experimentar congelación cuando quieren iniciar un movimiento.

La bradicinesia y la rigidez también son frecuentes en las niñas más mayores [12] y algunas formas graves que nunca adquieren la deambulacion independiente presentan rigidez a una edad muy temprana [observación personal].

Bruxismo

El bruxismo casi siempre está presente en el síndrome de Rett. Se produce durante la vigilia y cesa durante el sueño [3,15]. Se produce normalmente en individuos que presentan retraso mental [16] y puede asociarse a trastornos dentales y psicológicos [17,18].

FitzGerald et al [12] consideraron que el bruxismo puede ser una forma de distonía focal pero otros autores consideran que el bruxismo es un tipo de conducta autoagresiva. Algunas niñas mayores con el síndrome de Rett que padecen bruxismo presentan una morfología maxilar particular con prognatismo, debido quizá a este movimiento continuo característico [observación personal].

Temblor

Algunas niñas con el síndrome de Rett son predominantemente atáxicas y presentan un temblor postural del tronco y de los miembros superiores.

El temblor postural también puede estar presente en algunas niñas que tienen el síndrome de Rett con rigidez y acinesia.

Tabla 1. Criterios de diagnóstico revisados para el síndrome de Rett.

Criterios necesarios
Antecedentes prenatales y perinatales aparentemente normales
Desarrollo psicomotor normal en buena parte durante los primeros seis meses o que puede ser retardado desde el nacimiento
Circunferencia normal de la cabeza en el nacimiento
Desaceleración postnatal del crecimiento de la cabeza en la mayoría
Pérdida de las habilidades manuales adquiridas entre los seis meses y los dos años y medio de edad
Movimientos estereotípicos de las manos, como retorcerse/apretarse las manos, dar palmadas/golpecitos, lavarse/frotarse las manos y ponerlas en la boca
Retraimiento social emergente, disfunción de la comunicación, pérdida de las palabras aprendidas y trastorno cognitivo
Trastorno (dispráxico) o falta de locomoción
Criterios de apoyo
Trastornos de la respiración durante la vigilia (hiperventilación, apnea forzada, expulsión forzada del aire o la saliva, aspiración de aire)
Bruxismo
Alteración del patrón del sueño desde la primera infancia
Tono muscular anormal sucesivamente asociado a la emaciación muscular y la distonía
Alteraciones vasomotoras periféricas
Evolución de la escoliosis/cifosis durante la infancia
Retraso del crecimiento
Pies pequeños hipotróficos y fríos; manos pequeñas y delgadas
Criterios de exclusión
Organomegalia u otros signo de tesarismosis
Retinopatía, atrofia óptica o cataratas
Signos de daños cerebrales perinatales o posnatales
Existencia de trastornos metabólicos identificables u otros trastornos neurológicos progresivos
Trastornos neurológicos adquiridos por infecciones o traumatismos craneoencefálicos graves

Corea

Los movimientos coreoatetoides pueden afectar a las manos [12], pero no es un signo común en pacientes con el síndrome de Rett.

Mioclónia

Se informó de casos de mioclónia en el 34% de 32 pacientes con el síndrome de Rett examinadas por FitzGerald et al [12], que afectaban predominantemente a la cabeza o al tronco.

Guerrini et al [19] estudiaron a 10 pacientes con el síndrome de Rett y observaron una mioclónia que afectaba a los miembros distales en 9 de las pacientes. La gravedad de la mioclónia no estaba relacionada con otros síntomas o con la edad. Realizaron estudios neurofisiológicos y llegaron a la conclusión de que las pacientes con SR manifiestan un patrón característico de la mioclónia refleja cortical.

Tabla II. Relación revisada de los fenotipos variantes.

Criterios de inclusión
Cumplen al menos 3 de los 6 criterios principales
Cumplen al menos 5 de los 11 criterios de apoyo
Criterios principales
Ausencia o disminución de las habilidades manuales
Disminución o pérdida del balbuceo
Pautas monótonas de las estereotipias manuales
Disminución o pérdida de las habilidades comunicativas
Desaceleración del crecimiento de la cabeza desde los primeros años de vida
Perfil del síndrome de Rett: una fase de regresión seguida de una recuperación de la interacción que contrasta con una regresión neuromotora lenta
Criterios de apoyo
Irregularidades respiratorias
Hinchazón/aspiración de aire
Bruxismo, respiración con sonido áspero
Locomoción anómala
Escoliosis/cifosis
Atrofia de los miembros inferiores
Pies fríos y morados, normalmente trastorno del crecimiento
Alteraciones del sueño incluyendo gritos por la noche
Accesos de risa/grito
Disminución de la respuesta al dolor
Contacto visual/señalización con la mirada intensos

ESTUDIOS BIOQUÍMICOS

Un análisis de las aminas biogénicas y sus metabolitos arrojaron unos resultados discordantes que pusieron de manifiesto o bien un descenso de los niveles de ácido homovanílico (HVA), MHPG y ácido 5-hidroxiindolacético (5-HIAA) [20,21] o bien ningún cambio significativo de estos metabolitos [22]. Los niveles reducidos de 5-HIAA en el LCR están acompañados por niveles bajos de 5-metiltetrahydrofolato (5-MTHF) pero por niveles normales de folato sérico, mientras que los aportes complementarios orales de ácido fólico restablecieron los valores de 5-MTHF y el recambio de serotonina [21]. Las concentraciones en LCR de ácido gamma-aminobutírico (GABA) y de otros aminoácidos eran normales [23], mientras que la concentración de glutamato era bastante elevada [24]. Los descensos de los niveles en LCR de β -fenilalanina, una amina endógena sintetizada por la descarboxilación de fenilalanina, puede reflejar una alteración del sistema dopaminérgico nigroestriado [25]. Los niveles en plasma de β -endorfina y de prolactina son un poco inferiores a los normales [26], con un aumento de los niveles de β -endorfina en LCR en algunas de las pacientes con SR, aunque no en todas [27]. Los niveles en LCR de sustancia P son deficientes [28]. Los niveles de la bipterina, un cofactor en la sín-

tesis de las aminas biogénicas en sangre y orina, son normales, con un ligero descenso en LCR, aunque sin carencias en la enzima crítica, dihidropteridina reductasa [20]. Los lípidos cerebrales de la membrana presentan un descenso en el LCR [29]. Algunos estudios *post mortem* pusieron de manifiesto una reducción de los niveles de dopamina, serotonina y de sus respectivos metabolitos HVA y 5-HIAA en la sustancia negra de las niñas mayores con SR, aunque no se registró ninguna carencia generalizada de dopamina [22], mientras que otros revelaron un descenso de los niveles de dopamina, norepinefrina, serotonina y de sus metabolitos en la mayoría de las regiones cerebrales [30]. Un aumento de la proporción entre la dopamina en el estriado y el HVA y un nivel más elevado del ácido 3,4 dihidroxifenilacético indica un aumento del recambio de dopamina en el cerebro, con un descenso de la serotonina y con un aumento local de su precursor triptófano en el estriado-palidal y la sustancia negra [22]. Una reducción de la enzima AChT cortical indica una disfunción del sistema colinérgico prosencefálico [22].

NEUROPATOLOGÍA

Hay una reducción generalizada del tamaño neuronal con un aumento de la densidad de la concentración de células en la corteza cerebral, el tálamo, los ganglios basales, la amígdala y el hipocampo [31]. Otros estudios ponen de manifiesto una disminución del número de neuronas en la corteza frontal y temporal sin un descenso del grosor cortical, lo que afecta fundamentalmente a las células piramidales grandes, más prominentes en las capas II y III que en las capas profundas, y a la preservación de la corteza visual [32]. Estos cambios están asociados a una falta de especialización dendrítica del área en el modelo de orientación de las dendritas y los axones, un descenso de la ramificación dendrítica [33], neuronas pequeñas con un aumento de la concentración neuronal [34], pérdidas de las dendritas de las células piramidales en las áreas frontal, motora y subicular [35], reducción de las ramas dendríticas apical y basilar en las capas 3 y 5 de la corteza frontal, motora y temporal inferior [36] y 'dendritas desnudas' sin espinas en las neuronas piramidales, lo que indica una reducción en el número de contactos sinápticos con las neuronas aferentes [32]. Los estudios de Golgi pusieron de manifiesto una modificación selectiva en el tamaño de las dendritas de las neuronas piramidales en las cortezas frontal, motora y temporal, mientras que las dendritas en las neuronas piramidales en el hipocampo y la corteza visual no manifestaron ningún cambio. Algunas anomalías similares caracterizan las estructuras del temporal medio en el autismo infantil [37].

Se observó hipocomasia ligera en el cuerpo estriado e hiperromía de las neuronas grandes en el cuerpo estriado y el palio interno, aquí con dendritas anormales y degeneración de las fibras mielínicas gruesas [32,38], así como gliosis difusa leve.

Otro signo es la hipomelanización de la sustancia negra *pars compacta* sin una pérdida neuronal determinada, en la que una mayoría de las neuronas nigricas grandes contienen poca o ninguna neuromelanina [39,40]. Solamente en una paciente de 21 años de edad con el síndrome de Rett, las células nigricas sufrieron una reducción de alrededor de un 30% comparado con los controles ajustados según edad, y la presentación de gránulos de pigmento libres en el neurópilo como se ve en la enfermedad de Parkinson. Esto y los datos fundamentales que ponen de manifiesto el etiquetado del ADN intranucleosomal fragmentado, utilizando el método TUNEL en las neuronas de la sustancia negra,

indican que sufren una degeneración activa [40]. No se observan anomalías aparentes en otros núcleos pigmentados del tronco encefálico, y ningún estudio morfométrico de los núcleos dorsales serotoninérgicos del rafe puso de manifiesto una reducción de las neuronas, aunque se observó un número reducido de células en el núcleo basal colinérgico de Meynert [41].

El cerebelo puede manifestar una atrofia importante de todos los lóbulos del vermis y una pérdida progresiva de las células de Purkinje, con conservación de las células en cesta y estrelladas independientemente de la edad [42], con simplificación del núcleo olivar inferior sin pérdida de células o con gliosis. Estos signos, que también se observan en el autismo infantil,

[37] indican una interrupción del desarrollo que empieza antes del nacimiento [31].

ENSAYOS DE FÁRMACOS

El beneficio de los fármacos dopaminomiméticos, por ejemplo, los agonistas de la dopamina y la levodopa, es controvertido pero puede mejorar las habilidades motoras [43], mientras que la naltrexona, un antagonista opiáceo, puede ayudar a estabilizar las irregularidades respiratorias [44].

Las dosis bajas de risperidona pueden ser útiles para disminuir las estereotipias que provocan algún tipo de automutilación [11].

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DISCINESIA EN EL SÍNDROME DE RETT

Resumen. Introducción. El síndrome de Rett es un trastorno progresivo del neurodesarrollo que aparece en la primera infancia y que afecta casi exclusivamente a las niñas y mujeres. Está provocado por mutaciones de la proteína 2 de fijación de metil-CpG (MeCP2) y es una de las causas más frecuentes del retraso mental en las niñas y mujeres. La regresión es un rasgo característico del síndrome de Rett, y durante el período de regresión las pacientes desarrollan una conducta autista. Esta es la razón por la que el síndrome de Rett se ha clasificado como un trastorno generalizado del desarrollo del espectro autista. Sin embargo, las discinesias son tan exuberantes, características y únicas en el síndrome de Rett que es difícil que un médico experimentado confunda las dos situaciones. Desarrollo. Podemos definir el síndrome de Rett como una enfermedad que se manifiesta en la mayoría de los casos como un trastorno hipercinético y que evoluciona (a ritmos variables) hacia un trastorno bradicinético. El objetivo de esta publicación es caracterizar y describir las discinesias en el síndrome de Rett basándose en una revisión bibliográfica y en la experiencia personal de la autora. [REV NEUROL 2005; 40 (Supl 1): S167-71]

Palabras clave. Discinesia. Síndrome de Rett.

PERTURBAÇÃO DO MOVIMENTO NO SÍNDROMA DE RETT

Resumo. Introdução. O síndrome de Rett é uma perturbação progressiva do desenvolvimento neurológico que surge na primeira infância e que afecta quase exclusivamente o sexo feminino. É causado por mutações da proteína 2 da fixação de metil-CpG (MeCP2) e é uma das causas mais frequentes do atraso mental nas mulheres. A regressão é um traço característico do síndrome de Rett, e durante o período de regressão as doentes desenvolvem um comportamento autista. Esta é a razão pela qual o síndrome de Rett tem sido classificado como uma perturbação generalizada do desenvolvimento do espectro autista. Contudo, os distúrbios do movimento são tão exuberantes, característicos e únicos no síndrome de Rett que é difícil que um médico experiente confunda as duas situações. Desenvolvimento. Podemos definir o síndrome de Rett como uma doença que se manifesta na maioria dos casos como um distúrbio hipercinético e que evolui (a ritmos variáveis) até ao distúrbio bradicinético. O objectivo desta publicação é caracterizar e descrever os distúrbios do movimento no síndrome de Rett com base numa revisão bibliográfica e na experiência pessoal da autora. [REV NEUROL 2005; 40 (Supl 1): S167-71]

Palavras chave. Perturbação do movimento. Síndroma de Rett.

6.2.3. Detection of heterozygous deletions and duplications in the MECP2 gene in Rett syndrome by Robust Dosage PCR (RD-PCR) (Article 14)

MUTATION IN BRIEF

Detection of Heterozygous Deletions and Duplications in the MECP2 Gene in Rett Syndrome by Robust Dosage PCR (RD-PCR)

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Fifty to eighty percent of Rett syndrome (RTT) cases have point mutations in the gene encoding methyl-CpG-binding protein-2 (MECP2). A fraction of MECP2 negative classical RTT patients has large heterozygous deletions. Robust Dosage PCR (RD-PCR) assays were developed as a rapid, convenient and accurate method to detect large heterozygous deletions and duplications. A blinded analysis was performed for 65 RTT cases from Portugal by RD-PCR in the coding exons 2-4 of the MECP2 gene. Neither the patients with point mutations nor the non-classical RTT patients without point mutation had a deletion or duplication. One of remaining eight female patients with classical RTT without point mutation had a heterozygous deletion. This is the first report of a deletion spanning the entire MECP2 gene. The deletion was confirmed by southern blotting analysis and the deletion junction was localized 37kb upstream from exon 1 and 18kb downstream from exon 4. No duplications were detected. Our results suggest that RD-PCR is an accurate and convenient molecular diagnostic method. © 2005 Wiley-Liss, Inc.

KEY WORDS: Rett Syndrome; MECP2; RD-PCR; heterozygous deletion

INTRODUCTION

Fifty to eighty percent of Rett syndrome (RTT; MIM# 312750) cases have point mutations in the MECP2 gene (Methyl-CpG-binding protein 2; MIM# 300005)(Amir et al., 1999; Miltenberger-Miltenyi., 2003). Southern blotting analysis, quantitative PCR and MLPA (Multiplex Ligation-Dependent probe amplification) have been used to detect large deletions and duplications in the MECP2 gene of RTT patients without point mutation (Schollen et al., 2003; Erlandson et al., 2003; Ariani et al., 2004; Laccone et al., 2004). In those reports, 12 out of 59 (20.3%) classical RTT patients without point mutation have large deletions in MECP2 gene.

Robust dosage PCR (RD-PCR) has been developed as a rapid, convenient and accurate method to detect heterozygous deletions and duplications (Liu et al., 2003). The accuracy and consistency of RD-PCR has previously been validated in multiple blinded analyzes with 100% accuracy (Liu et al., 2003; Nguyen et al., 2004). RD-PCR has the advantages of rapid optimization and validation of new assays, and inclusion of positive controls without the requirement of the heterozygous deletion. The enhanced RD-PCR protocol has the additional advantages of tolerance toward genomic DNA of variable quality and uniform and unbiased performance across

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regions of variable sequence context and GC content (Nguyen et al., 2004; Shi. et al., 2004).

In our study, we used, for the first time, RD-PCR to detect heterozygous deletions in the *MECP2* gene. A blinded analysis was performed for 65 RTT cases from Portugal. One of eight patients with classical RTT without point mutation had a heterozygous deletion spanning the entire coding sequence. The deletion was confirmed by southern blotting. This is the first biological analysis report on RD-PCR.

MATERIALS AND METHODS

Samples

The DNA was prepared from peripheral blood by Puregene DNA Isolation Kit (Gentra, Minneapolis, MN). The concentrations were measured by UV spectrophotometer at 260 nm and adjusted to a working concentration of 30 ng/μl in TE buffer. Forty-eight control samples were analyzed in a blinded validation analysis, in which gender was used as a surrogate for heterozygous deletions.

Sixty-five RTT patient samples from Portugal and two blinded male controls were screened in the study. Patients were diagnosed according to the RTT diagnostic criteria defined by Hagberg et al (Hagberg et al., 1985 and 2002), which includes psychomotor regression after a period of normal development, severe mental retardation, deceleration of head growth and loss of purposeful hand skills with appearance of stereotypical hand movements. 30 of the RTT samples were classical RTT patients, and 8 of these were negative *MECP2* mutation according to our previous work.

RD-PCR assay

Genomic DNA samples were incubated at 90°C in TE buffer for 10 minutes to minimize RD-PCR bias (Shi *et al.*, 2004). Four RD-PCR assays for the three coding exons of *MECP2* gene were designed (Table 1) according to Liu et al (Liu et al., 2003), except for the 5' universal tail of 5' ggccaagtgt- 3'. These assays were divided into two groups depending on whether the *ATM* or *FUT* gene was used as the autosomal control segment. Group I had two assays in exon2 and exon3 of the *MECP2* gene, exon 12 of the ataxia telangiectasia mutated (*ATM*) gene was the internal control. Group II had the other two assays in the coding part of exon 4 of the *MECP2* gene; the fucosyltransferase 2 (*FUT*) gene was the internal control.

Ten more RD-PCR assays were developed in the 3' and 5' flanking regions of the *MECP2* gene to localize the deletion junction. The primers were designed according to the genomic sequence from NT_025965 (GenBank accession number) (data not shown).

The PCR mixtures contained a total volume of 25μl: 1xExpand™ High Fidelity buffer#3 (Roche), 4.5 mM MgCl₂, 200 μM of each dNTP for Group I or 3.0 mM MgCl₂ and 150 μM dGTP/50 μM deaza-dGTP, 200 μM of each other dNTPs and 10% DMSO for Group II, 0.1-0.2 μM of each pair of primers, 1U Platinum *Taq* DNA polymerase (Invitrogen) and 1U platinum *Taq* DNA polymerase High Fidelity (Invitrogen), 0.5 μg of BSA, and 60 ng of genomic DNA. The cycling entailed denaturation at 94°C for 15 sec, annealing at 55°C for Group I or 65°C for Group II for 30 sec, and elongation at 72°C for 1 min for 23 cycles.

Quantitation

Twelve μl of PCR product was electrophoresed through a standard 2% agarose gel. Gels were stained in 0.2μg/ml Ethidium Bromide for 1 hour and scanned by Typhoon 9410 Imager (Amersham) with the following parameters: focal plane =+3 mm, laser wavelength= 532 nm, Green, emission filter =610 BP 30, photomultiplier voltage =600 V, pixel size =100μm and sensitivity =normal.

ImageQuant™ software was used to quantitate the PCR yield. Net signal of a product band was obtained by subtracting local background signal from total signal in arbitrary unit. The ratio of yields (ROY) is calculated by dividing the target net signal by the internal control net signal. For normalization, the ROY of the patient sample was divided by the average ROY of the normal females.

Table 1. List of Primer Pairs and PCR Segments

	3' sequence-specific region		T _m (°C)	GC%	Core PCR segment ^c				
	Name ^a	Sequence ^b (5' - 3')			Region	Size	T _m (°C)	GC%	
Group I	Assay 1								
	Target								
	MECP2-2(709463)D	5' TTTAGTCTTTGGGGTACTTTTA3'	45.4	32	Exon 2 of MECP2	492	74.1	38	
	MECP2-2(709954)U	5' GGCTTGTGATAGTGTTGATTCT3'	47.8	41					
	Control ^d								
	ATM(38415)D	5' ATCCTGCAAGTTTACCTAAC3'	44.9	41	Exon 12 of ATM	418	75.2	41	
	ATM(38829)U	5' GATCAGGGATATGTGAGTGT3'	46.4	43					
Group II	Assay 2								
	Target								
	MECP2-3(649492)D	5' ACCTGGTCTCAGTGTTTCATTGT3'	50.0	46	Exon 3 of MECP2	486	81.2	55	
	MECP2-3(649977)U	5' CTTTCAGGGAAGAAAAGTCAGAA3'	49.8	41					
	Assay 3								
	Target								
	MECP2-4-1(647695)D	5' CTTTGTGTCAGAGCCCTACCCATA3'	52.7	50	Exon 4-1 of MECP2	447	83.5	61	
	MECP2-4-1(648141)U	5' CCACCATCACCACCACTCAGAG3'	56.5	59					
Group II	Control								
	FUT(502)D	5' TTCACCGGCTACCCCTGCTC3'	58.6	65	FUT2	504	83.5	61	
	FUT(1006)U	5' GGAGTCGGGGAGGGTGTAAAT3'	54.1	60					
	Assay 4								
	Target								
	MECP2-4-2(648547)D	5' CCCCCTGGCGAAGTTTGAAAAG3'	60.5	55	Exon 4-2 of MECP2	400	81.2	56	
	MECP2-4-2(648946)U	5' CCACCATCCGCTCTGCCCTATC3'	61.4	64					

a. For example, MECP2-2(709463)D: MECP2=methyl CpG binding protein 2, Xq28, its sequence is from NT_025965.13 (GenBank accession number); 5' end of the 3' sequence-specific region of the primer begins at 709463; and D, downstream. The precise sizes and locations of the PCR fragment can be obtained from the information names. ATM=ataxia telangiectasia mutated, 11q22-q23, its sequence is from U82828; FUT=fucosyltransferase 2,19q13, its sequence is from D82933.

b. The sequence of the 3' sequence-specific region is shown. A 10-nucleotide universal tail (5' ggccaagtgt 3') is attached to the 5' end of each primer. Note that the control primers have been redesigned relative to previous report (Liu et al., 2003) to incorporate the 10-nucleotide universal tail.

c. The core PCR segment does not include the tails.

d. Exon 12 of ATM gene and FUT gene were internal controls of the Group I and Group II. They are listed in one assay in each group and left out in others.

Southern blotting analysis

Southern blot was performed using probes RTT2 (709610-709766, sequence is from NT_025965.13), RTT3 (649518-650043) and p(A)10 (639141-639564) that hybridized with exon 2, exon 3 and the end of the 3'UTR. Probes were generated by PCR from genomic DNA, purified from 1% agarose gel by QIAEX II (QIAGEN, Valencia, CA) and labeled with ³²P dCTP by Prime-It II Random primer (Stratagene, Cedar Creek, TX). The genomic DNA (8μg) of female control, male control and patient P3 was digested with *Hind III* and *Pst I* for probe RTT2, *Sac I* for probe RTT3 and *Hind III* and *Sac I* for probe p(A)10. Digested DNA fragments were separated in

a 1.5% agarose gel and blotted into a nylon membrane (Hybond H-N+; Amersham Pharmacia Biotech, Buckinghamshire, England). Hybridization was performed overnight at 65°C and washings were carried out in a series of SSC/SDS solutions (0.1%SDS, 2%-0.1% SSC). Membranes were exposed to storage phosphor screen, scanned by Typhoon 9410 Imager (Amersham, Molecular Dynamics, Sunnyvale, CA). ImageQuant™ software was used to quantitate the signals.

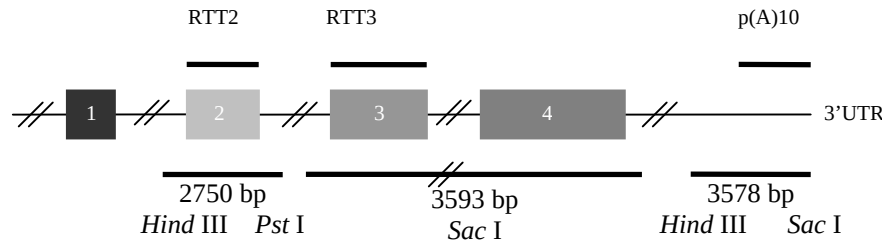


Figure 1. Schematic representation of the *MECP2* gene regions analysed by Southern blotting and localization of the probes used in the assay (figure is not to scale).

RESULTS AND DISCUSSION

Validation by blinded analysis with 100% accuracy

RD-PCR, a duplex PCR, amplifies an endogenous internal control and a target locus. The internal control has a known gene copy number per cell while the target has an unknown number per cell. The ROY was directly proportional to the ratio of the two input templates, so the copy number of the *MECP2* gene could be obtained according to the ROY and the known copy number of the internal control.

For validation of the four assays in the *MECP2* gene, a blinded analysis was performed with 48 blinded genomic DNA samples where either the sex status or the number of each status were unknown (Fig. 2A). The male sample was functionally equivalent to a RTT patient with large heterozygous deletion. All the males and females were determined with 100% accuracy. The standard deviations of ROY were around 0.04 in both male and female samples in each of the assay.

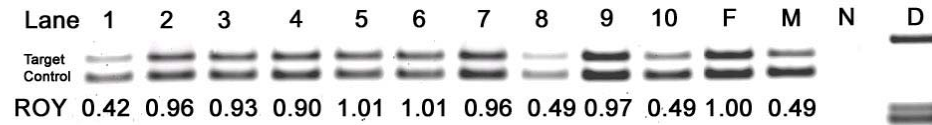
Large heterozygous deletion found in one patient

Exons 2, 3 and 4 of the *MECP2* gene were analyzed for deletions by four RD-PCR assays. ROYs of each assay were obtained and the copy numbers of the three coding exons 2-4 of the *MECP2* gene were determined in the 65 patient samples and two blinded male controls (Fig. 2B). All the 65 patient samples were previously scanned for point mutation in coding exons and immediate flanking intronic regions of *MECP2* gene by DOVAM-S (Detection of virtually all mutations-SSCP). The RD-PCR analysis was performed blinded to previous point mutation scanning. Only one of eight female patients with classical RTT without point mutation (P3) and two blinded male controls, P1 and P2 showed much lower ROY values than all the other female patients in all the four assays. ROYs of the female patient P3 were 0.44, 0.49, 0.51, and 0.52, indicating that the patient carried a large heterozygous deletion spanning the completely coding region of the *MECP2* gene. None of 22 patients with non-classical RTT with point mutations had a heterozygous deletion. No patient with duplication was observed.

Except for the three samples, P1, P2 and P3, the means and standard deviations of the ROYs were 1.00 ± 0.09 , 1.02 ± 0.08 , 1.00 ± 0.09 and 1.00 ± 0.10 respectively; the ranges of ROYs were 0.83-1.22 for Assay 1, 0.83-1.21 for Assay 2, 0.83-1.21 for Assay 3 and 0.79-1.22 for Assay 4. The two blinded male controls were both detected as heterozygous deletions in every assay. All female patients without deletions or duplications had ROYs clearly distinguishable from the male controls and the patient with deletion. These strongly supported the accuracy of the RD-PCR assays.

Southern blotting analysis was used to confirm the deletion identified by RD-PCR method. Signal intensity of patient P3 was similar to that of the male control with probes RTT2, RTT3 and p(A)10 (Fig. 3), indicating only one copy of the *MECP2* gene in patient P3.

A



B

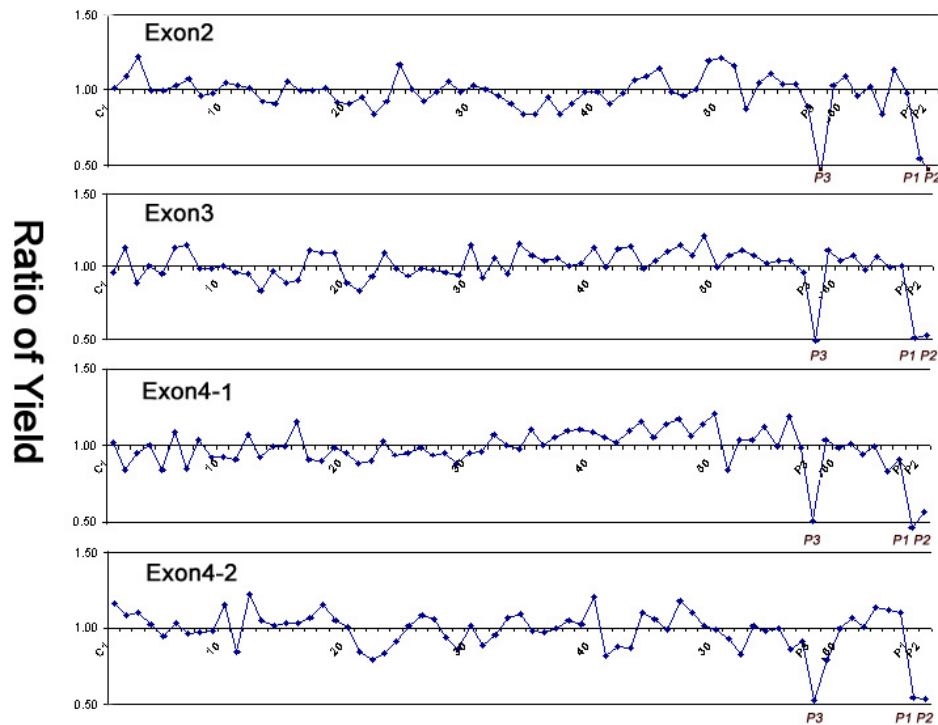


Figure 2. Analysis of copy number of the coding region of the *MECP2* gene. **A:** Blinded RD-PCR analysis for exon2. Lanes 1 to 10 are blinded normal control samples, where the gender is unknown. F is a normal female control, M is a normal male control, N is a negative control without DNA added. D is PhiX174 DNA/Hae III Markers, in which three bands of 603bp, 310bp, 281bp+217bp were shown. The ROY of each sample is indicated. **B:** ROY for four RD-PCR assays. Sixty-seven samples were tested for each assay. Y-axis is ROY, crossed with X-axis at 1.0. P1, P2, and P3 have much lower ROYs, indicating only one copy of the *MECP2* gene. P3 is the RTT patient with the deletion; P1 and P2 are male controls.

Characterization of the large deletion in the female patient P3

To localize the deletion junction, ten more RD-PCR assays were developed flanking the *MECP2* gene. The deletion junction was located within a region of 37.2kb upstream from 5' end of exon 1 and 18.1kb downstream from 3' end of exon 4 (Fig. 4). Long-distance PCR approaches were designed, but unfortunately failed, probably because the DNA was partially degraded.

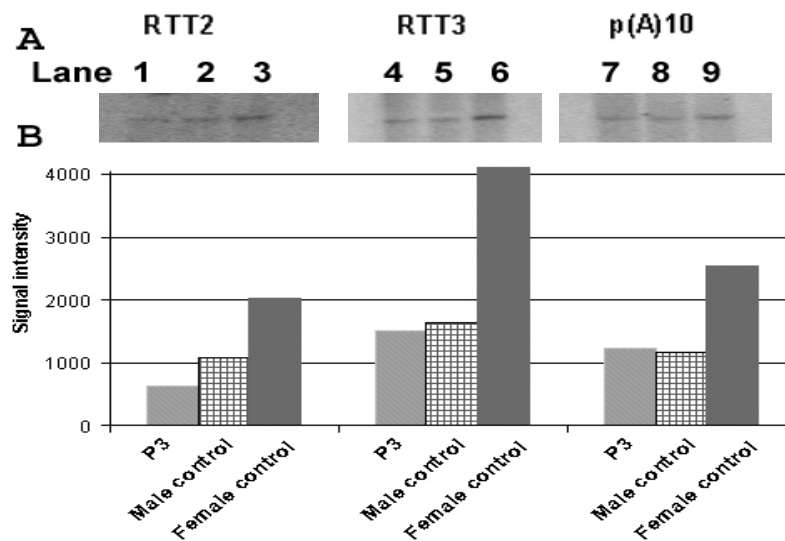


Figure 3. Southern blotting analysis. **A:** Images of southern blotting with probes RTT2, RTT3, and p(A)10. Lanes 1, 4, and 7 are patient P3; Lanes 2, 5, and 8 are male control; and lanes 3, 6, and 9 are female control. **B:** Quantitation of each individual. Signal intensity of patient P3 was similar to that of the male control, indicating only one copy of the *MECP2* gene in patient P3.

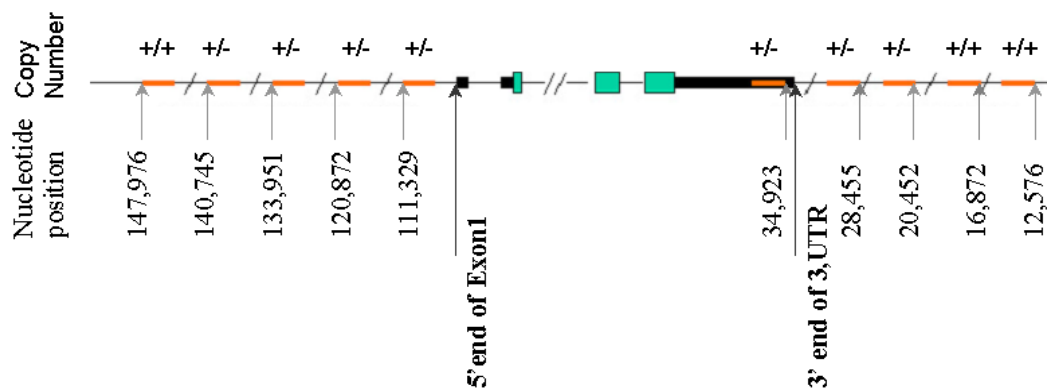


Figure 4. Localization of the deletion junction in the female patient P3. Ten RD-PCR assays were developed in the flanking region of the *MECP2* gene on X-chromosome. Primers were designed according to the genomic sequence from NT_025965 and the nucleotide positions are shown. +/+ indicates two gene copies at the test locus, while +/- indicates only one copy. The deletion junction was located within a region of 37kb upstream from 5' end of exon 1 and 18kb downstream from 3' end of exon 4 (3'UTR).

RD-PCR was used for detection of heterozygous deletions and duplications in the *MECP2* gene in RTT patients. One large deletion was identified in one of eight classical RTT patients without point mutations. The prevalence of *MECP2* gene heterozygous deletions detected by RD-PCR in our patients is 12.5% (1 out of 8), not significantly lower than the aggregate of the previous reports (12 out of 59) (Schollen et al., 2003; Erlandson et al., 2003; Ariani et al., 2004; Laccone et al., 2004). As illustrated by the ten additional dosage assays developed to characterize the deletion, rapid assay development and optimization are two important advantages of RD-PCR.

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