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Investigating genotype–phenotype relationships in Rett syndrome using an international data set

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ABSTRACT

Background: Rett syndrome is an uncommon neurodevelopmental disorder with an incidence of 1:9,000 live female births. The principal genetic cause was first reported in 1999 when the association with mutations in the methyl-CpG-binding protein 2 (or *MECP2*) gene was identified. This study uses data from a large international database, InterRett, to examine genotype–phenotype relationships and compares these with previous findings in a population-based cohort.

Method: The data set for these analyses was derived from a subset of InterRett cases with subject information collected from the family, the clinician, or both. Individual phenotypic characteristics and clinical severity using three scales were compared among those with eight known recurrent pathogenic *MECP2* mutations as well as those with C-terminal deletions ($n = 272$).

Results: Overall, p.R270X and p.R255X were the most severe and p.R133C and p.R294X were the mildest mutations. Significant differences by mutation were seen for individual phenotypic characteristics such as hand use, ambulation, and language.

Conclusions: This multicenter investigation into the phenotypic correlates of *MECP2* mutations in Rett syndrome has provided a greater depth of understanding than hitherto available about the specific phenotypic characteristics associated with commonly occurring mutations. Although the modifying influence of X inactivation on clinical severity could not be included in the analysis, the findings confirm clear genotype–phenotype relationships in Rett syndrome and show the benefits of collaboration crucial to effective research in rare disorders. **Neurology® 2008;70:868–875**

GLOSSARY

ANOVA = analysis of variance; **ARSD** = Australian Rett Syndrome Database; **IQR** = interquartile range.

Rett syndrome is a pervasive neurodevelopmental disorder found primarily in females¹ with a diagnosis incidence of 1.09 per 10,000 girls by the age of 12 years.² In a consensus statement by international clinical experts in 1988,³ there was agreement that a sequence of clinical features (including apparently normal development followed by loss of hand and communication ability and development of hand stereotypies) was required for diagnosis. These criteria were broadened slightly when revised in 2001 such that neither slowing of head growth nor early normal development were mandatory as necessary criteria and microcephaly at birth was no longer an exclusion criterion.⁴ The principal genetic cause for Rett syndrome was identified in 1999, when it was reported that causative mutations had been found in the *MECP2* gene, which encodes methyl-CpG-binding protein 2.⁵ Since then, more than 200 different *MECP2* mutations have been identified,⁶ with seven recurrent mutations accounting for more than two-thirds (70%) of cases in whom an *MECP2* gene mutation is characterized.⁷

Supplemental data at
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There is considerable clinical variation in Rett syndrome,¹ and since 1999 much research has been undertaken to investigate the possible relationships between genotype and phenotype.⁷⁻²³ However, the results, especially from the earlier studies, have not been consistent. Despite the fact that some investigations^{9,17,21} have included cases from more than one country, case numbers have still been low and statistical power has still been a problem. A further limitation, also often necessitated by small sample size, has been the grouping of mutations by type (e.g., missense, nonsense) or location, rather than the separate analysis of specific mutations. However, work with the Australian Rett Syndrome Database (ARSD)⁷ has, as has other recent research,^{18,20,21} been able to show some significant relationships by examining the common mutations individually. The ARSD study had the benefit of being population based, but was restricted in power because the Australian national population is only around 20 million, and thus the data set only contains approximately 300 diagnosed individuals born within the past 30 years. InterRett has now been funded by the International Rett Syndrome Association²⁴ to act as an international data clearinghouse and to gather data on a global scale to provide sufficient case numbers for meaningful statistical analyses. We present here an analysis of genotype–phenotype relationships among those cases reported to InterRett with the most frequently occurring *MECP2* mutations and compare the findings with previously reported data from the population-based ARSD.

METHODS Study population. A data set comprising a subset of cases was extracted from InterRett, the international Rett syndrome phenotype database, in December 2005.^{24,25} Recruitment of case families to the InterRett database commenced in December 2002 and has been primarily through the parent Listserv Rettnet,²⁶ advertisement in newsletters of parent support associations, and presentations at their meetings. Bulk submission of deidentified data has also occurred, so far from Australia, Spain, Israel, Canada, and China.

Data collection. Data are submitted to InterRett from families and clinicians in the form of online or paper-based questionnaires. The questionnaire for families is modeled on

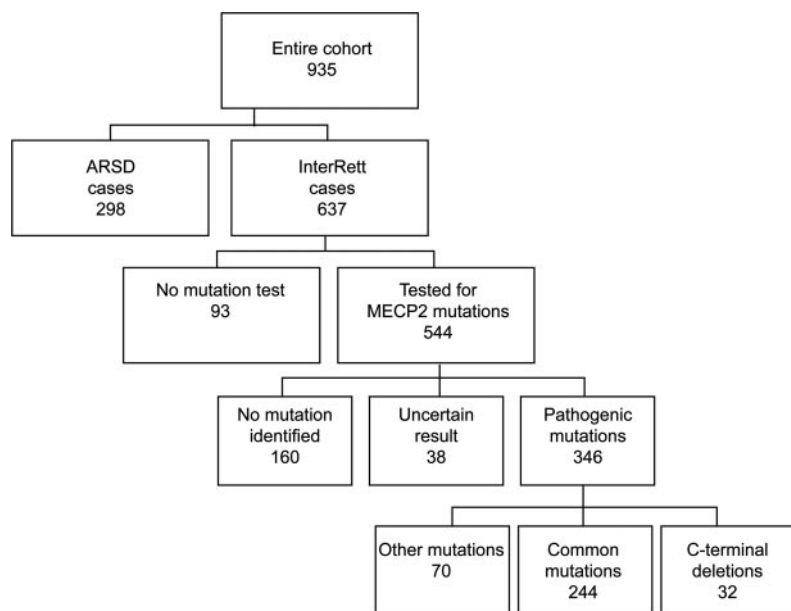
the ARSD family questionnaire.^{27,28} The clinician questionnaire was specifically designed with input from the InterRett reference panel²⁵ to gather information that allows severity to be defined so that international comparisons can be made.^{7,12,20,29} Clinician questionnaires have been submitted both on individual cases (on whom family data had already been provided) and in the form of deidentified cases from patient records. Collaborative international networks with clinical and research groups have been crucial in preparing the groundwork for the bulk data submission process. Deidentified data have also been supplied by one of the authors (M.P.) from a protocol of her own design¹² on 259 Spanish cases (Pineda data) known to her at the time of the study.

Data management and coding. Three severity scoring systems, referred to as the Kerr,²⁹ Percy,²⁰ and Pineda¹² scales, have been applied in the past²⁷ to provide quantitative estimates of clinical severity (with higher scores indicating greater severity). The attributes of the severity scales have previously been described in detail.²⁷ Each scale is a summation of individual items related to Rett syndrome phenotypic characteristics. The items are based on the severity or degree of abnormality of each characteristic on a discrete scale (e.g., 0, 1, 2), with the highest level corresponding to the most severe or most abnormal presentation. For example, the Pineda scale is based on the sum of scores for items measuring age at regression/loss of social interaction, head circumference, sitting, ambulation, speech/language, respiratory function, epilepsy, hand use, air swallowing, and age at onset of hand stereotypy. The Kerr scale (19 items, maximum possible score 38) contains more items focusing on current functioning, and the Pineda scale (10 items, maximum possible score 31) contains more on developmental characteristics, whereas in the Percy scale (15 items, maximum possible score 45), there is more of a balance between current functioning and developmental characteristics.²⁷ Where information from both the clinician and the family questionnaire was available, the source that was thought to be more applicable, more specific, and most valid was used for this analysis. Pineda data could not be used to develop Kerr or Percy scores that were comparable with the scores based on the family or clinician's questionnaires, and hence only Pineda scale scores were calculated for the 259 Spanish cases. A number of items from the three severity scales were similar and could be substituted for each other. For example, the Percy scale item for respiratory function was replaced by the Kerr scale item for disturbed awake breathing dysfunction. Modifications to the original severity scales are as detailed (tables e-1 through e-3 on the *Neurology*[®] Web site at www.neurology.org).²⁷

Information about gene mutations was sought in all three questionnaires. This analysis is restricted to assessment of cases with the nine most frequently reported *MECP2* mutations—p.R106W, p.R133C, p.T158M, p.R168X, p.R255X, p.R270X, p.R294X, p.R306C, and C-terminal deletions, which together comprise more than three-quarters (78%) of pathogenic mutations reported in subjects with Rett syndrome.⁶

Statistical methods. When items contributing to the severity scales were not available, the missing scores were imputed before an overall severity was calculated using a method similar to hot deck imputation³⁰ with the imputed values coming from a random sample of values from the same probability distribution as the original, nonmissing values.

Figure 1 Cohort distribution by source and genetic status



ARSD = Australian Rett Syndrome Database.

Comparisons of overall severity score between mutations were performed using analysis of variance (ANOVA).³¹ Categorical variables were analyzed for significant associations using Pearson χ^2 and Fisher exact tests.^{32,33} Age was defined as age at collection of the family questionnaire or age at last update of clinical records. Age correction, where necessary, was done by subtracting the mean score for each age group from the score for each case in that age group (equivalent to using age as a covariate in linear regression). Stata version 9 was used for analysis.³⁴ Correction for multiple comparison was not made.³⁵ Significance tests for differences between mutations were only calculated for the overall test of any differences between mutations, not for any specific comparisons.

RESULTS Cohort description. As at December 2005, InterRett had collected information on 935 cases of Rett syndrome. These cases came from 31 different countries, with the largest proportion com-

ing from Australia (308 cases, 32.9%), Spain (259 cases, 27.8%), the United States (160 cases, 17.1%), Israel (71 cases, 7.6%), and the United Kingdom (47 cases, 5%). To permit valid comparison with previously published information from the Australian cohort,⁷ the Australian cases born since January 1976 known to be in the ARSD ($n = 298$) were not included within the current analysis. Cases in whom *MECP2* gene mutation testing had not been arranged ($n = 93$), in whom a mutation had not been detected ($n = 160$), or for whom the clinical significance of any detected gene variant was uncertain ($n = 38$) were also not included (figure 1). This study is therefore restricted to the remaining 346 cases with an unequivocally pathogenic mutation. Age at data collection ranged from 2 months to 45 years, with a mean age at data collection of 10 years 4 months. The mean ages at data collection differed for different mutations, with cases with p.R106W, p.R294X, p.R306C, and C-terminal deletions tending to be older and cases with p.R255X or p.T158M tending to be younger (table 1).

Severity scales and age. There was an increasing (implying greater severity) trend with age in the Kerr score (linear regression coefficient 0.130, $p < 0.001$), with the mean Kerr score increasing by age group up to 26 years and then decreasing thereafter (table 2). This necessitated age correction of the overall scores for each mutation in the Kerr scale. No such age variation existed in the Percy or Pineda scale scores for the mutation-positive subset (Percy score: linear regression coefficient -0.015 , $p = 0.73$; Pineda score: linear regression coefficient -0.024 , $p = 0.40$; table 2).

Overall severity and mutation type. The mutations p.R106W, p.R133C, p.T158M, p.R168X, p.R255X, p.R270X, p.R294X, p.R306C, or a de-

Table 1 Age at data collection for cases with common mutations

Common mutations	No. of cases, $n = 272^*$	Mean age at data collection, y	Median age at data collection, y	Range of ages at data collection
p.R106W	18	14.64	14.32	16 mo to 31 y
p.R133C	27	9.66	7.93	2 y to 33 y
p.R168X	36	9.27	7.57	18 mo to 31 y
p.R255X	47	8.17	6.28	18 mo to 22 y
p.R270X	26	9.73	5.44	18 mo to 37 y
p.R294X	21	12.54	9.28	3 y to 44 y
p.R306C	22	12.00	7.68	4 y to 45 y
p.T158M	43	9.18	7.07	19 mo to 29 y
C-terminal deletions	32	11.76	9.67	3 y to 41 y

* Includes 244 cases with common mutations and 32 with C-terminal deletions. Does not include 4 cases with date of birth unspecified.

Table 2 Severity (Kerr, Percy, and Pineda) descriptive statistics for *MECP2*-positive cases

Age group, y	n	Mean (IQR) Kerr score	Mean (IQR) Percy score	n	Mean (IQR) Pineda score
0-3	24	16.3 (14-18.5)	21.2 (17.5-24)	57	16.6 (14-19)
3-6	48	18.4 (16-20)	20.9 (17-23)	80	14.4 (11-17)
6-11	50	19.1 (17-22)	21.1 (18-25)	80	15.6 (12.5-18.5)
11-18	37	20.6 (18-23)	19.7 (15-24)	68	15.1 (12-18)
18-26	18	22.3 (21-25)	21.7 (17-25)	39	16.4 (13-20)
>26	16	20.6 (18-23.5)	20.6 (17-24)	17	14.1 (12-15)
Total	193*	19.3 (16-22)	20.8 (17-24)	341*	15.4 (12-18)

* 197 mutation-positive cases without Pineda scores; 4 cases with date of birth unspecified.

† 346 mutation-positive cases with Pineda scores; 5 cases with date of birth unspecified.

IQR = interquartile range.

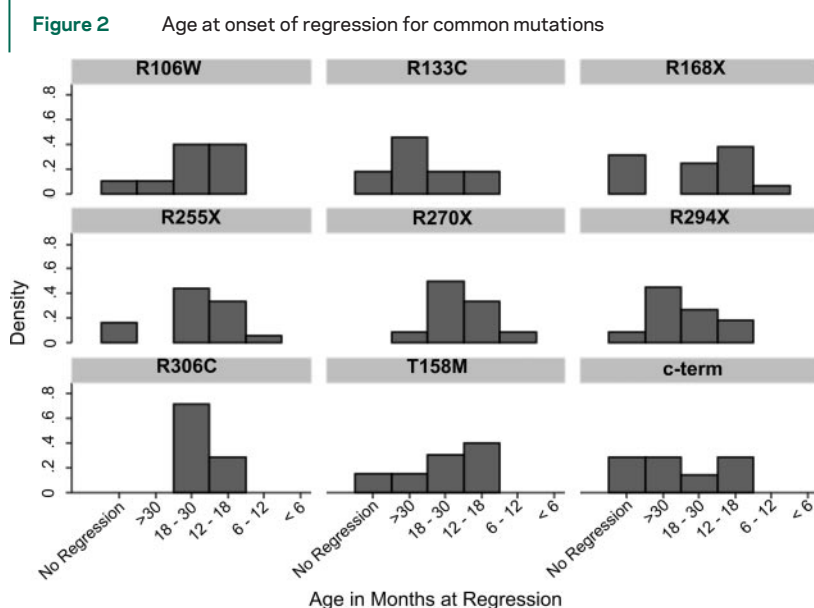
letion of the C-terminal region occurred among 276 of 346 cases (79%) with an unequivocally pathogenic mutation of *MECP2*. Differences between mean severity scores for different mutations were confirmed with ANOVA (Kerr score: $p < 0.001$; Percy score: $p < 0.001$; Pineda score: $p < 0.001$). The mutation associated with the lowest mean severity score for all three of the severity scales was p.R133C (figures e-1 through e-3). Other mutations associated with a milder phenotype included p.R294X, p.R306C, and the C-terminal deletions. The p.R270X mutation was associated with a higher severity score on all scales and was the most severe mutation overall using the Percy scale. Significant differences between pairs of common mutations can be seen in the tables (tables e-1 through e-3) of mean severity scores, confidence intervals, and results of t tests and can be inferred where confidence intervals in figures e-1 through e-3 do not overlap. The

relationships between the mean scores for each mutation in each cohort are similar, with high scores in InterRett associated with high scores in the ARSD for both the Percy severity scale and the Pineda severity scale (Percy: Pearson $r = 0.88$, $p = 0.01$ $df = 7$, figure e-2; Pineda: $r = 0.91$, $p = 0.004$ $df = 7$, figure e-3).

Individual aspects of phenotype and mutation type.

In addition to the differences in overall severity, cases with each common mutation showed varying levels of severity for individual characteristics of the Rett syndrome phenotype. These are best demonstrated using items from the Pineda scale (figures 2 and e-4 through e-6), which could be applied to the maximum number of cases ($n = 276$). A delayed onset of regression (figure 2) occurred most frequently in subjects with p.R133C and p.R294X mutations, in particular when compared with p.R168X, p.R255X, and p.R270X mutations (overall χ^2 test: $p < 0.001$). Delayed onset of hand stereotypies was more likely among cases with p.R133C, p.R294X, p.R306C, and C-terminal deletions, with around 40% of subjects in these groups developing hand stereotypies after 3 years. In comparison, less than one-third of subjects with p.R106W, p.R168X, p.R255X, and p.R270X had delayed onset of stereotypies (overall χ^2 test: $p = 0.001$). As shown in figure e-4, approximately two-thirds of those with p.R133C and p.R294X and just less than half of those with p.R306C and C-terminal deletions had conserved hand function compared with less than one-third of subjects overall (overall χ^2 test: $p < 0.001$).

All cases with p.R133C and p.R294X learned to walk (figure e-5). In contrast, just less than one-third of subjects with p.R168X and just more than one-third with p.R255X and p.R270X muta-



tions did not learn to walk (overall χ^2 test: $p = 0.002$). Cases with p.R133C mutations were more likely to conserve some of their language skills (figure e-6), with 18% described as currently using single words and 21% currently using phrases, compared with 13% and 2% overall (overall χ^2 test: $p < 0.001$). Cases with p.R270X and p.R255X were least likely to acquire the ability to speak.

Severe feeding difficulties occurred most commonly in subjects with p.R168X and p.R270X (both 75%) and least commonly in cases with p.R133C (1.9%) and p.R306C (5.6%) (overall χ^2 test: $p = 0.009$). Only a single case with a C-terminal deletion was reported to swallow air (3.9%), compared with 26% to 56% for the other common mutations (overall χ^2 test: $p = 0.011$).

For a range of other characteristics, including epilepsy prevalence (58.6%), scoliosis (54.6%), breathing problems (64.2%), peripheral circulation problems (71.3%), mood disturbances (66.9%), and sleep problems (67.3%), no significant relationships with mutation type were found even after performing stratified χ^2 tests over age groups.

DISCUSSION This study made use of the international database InterRett to identify 276 cases with highly recurrent *MECP2* mutations. Clinical severity of each of these cases was defined using the three scoring systems,^{12,20,29} used previously to measure clinical severity.^{7,17,27} All three scoring systems revealed that the mutations p.R133C and p.R294X were associated with the least severe phenotype. However, there was some variation by scoring system in relation to the most severe phenotype, with the mutations p.R255X and p.R270X being associated with the most severe outcomes on the Pineda scale, and the p.T158M mutation being the most severe on the Kerr scale. Milder mutations such as p.R133C, p.R294X, p.R306C, and C-terminal mutations tended to have the latest onset of regression as well as later onset of stereotypies. The same pattern was seen with hand skills, which were most likely to be retained by those with these mutations. Cases with an underlying p.R133C mutation were most likely to retain “language,” and those with p.R270X and p.R255X least likely to develop “speech.” Our data reveal that the Pineda scale reflected the core characteristics more effectively than the other two scales, with a greater number of categories in most items, allowing greater discrimination of variation in Rett syndrome phenotype. It also displayed resilience to the potential for bias

brought about by age-related developments in the progression of the Rett syndrome phenotype, particularly by including aspects of developmental changes (losing hand skills, losing previous mobility) and age at acquisition of skills.

A key reason for conducting this analysis of InterRett data was to replicate in a larger independent sample the research undertaken using the population-based ARSD. Comparison of scores in both studies derived from the Percy and Pineda scales revealed similar patterns. In both studies, the Percy scale indicated that the p.R133C and the p.R294X mutations were the least severe overall. While overall in the InterRett data the p.R133C was mildest, the Australian data showed that p.R294X was marginally milder using this scale. However, the p.R270X and p.R255X mutations were associated with the most severe phenotype in both studies. In contrast to the InterRett investigation, the Australian analysis did not find any significant difference between the p.R255X cases and the other mutations, though significant differences did exist for the p.R270X. Using the Pineda scale to identify those mutations with greatest and least clinical severity, the InterRett results more or less mirrored the findings from the previous Australian analysis. The only exception was cases with the p.R306C mutation, which in the InterRett data were significantly less severe in the Pineda scale than cases with p.R255X, p.R270X, or p.R168X. This was a result not found in the Australian investigation but is consistent with findings from elsewhere.²⁰

We were unable to carry out a direct comparison between the findings from the InterRett data from the Kerr scale and those from the Australian data because of the age adjustment we needed to make for the InterRett data. This showed a slightly different picture from the other two scales, with the p.T158M significantly more severe than both the p.R133C and the p.R294X mutations. This suggests perhaps that the developmental features (as emphasized by the Percy and Pineda scales) with this mutation might play less of a role in its overall phenotype than with mutations such as p.R270X and p.R255X.

The InterRett data provide a composite summation of the findings from elsewhere, such as those of a German study¹⁶ that was the first to observe the increased severity associated with mutations in the nuclear localization signal region of the transcription repression domain where the mutations p.R270X and p.R255X are situated; those of a US study²⁰ that found a milder phenotype associated with p.R306C; those of a Belgian study²³ that described the milder clinical severity

of the C-terminal deletions as well as the work of ourselves¹⁷ and others¹⁴ relating to the mild phenotype associated with p.R133C; and those of a UK study³⁶ that, using a simple scoring system based on current functioning, found p.T158M to be one of the more severe mutations. On this occasion, we did not include the Australian data as a subset of the InterRett data to allow us to replicate independently our earlier findings.¹⁷ However, by doing this in the future, the power of InterRett will be further increased.

The greatest strength of this study is that it has made use of an international collaborative network²⁴ to collect a large number of cases for genotype–phenotype analysis. The need for and value of this has previously been expressed, but it has taken the infrastructure of this international collaboration to allow this to happen. The infrastructure is significant because it has enabled the involvement of families who have directly submitted information about their own children—information that has usually been complemented by data from the child’s clinician. It also has brought together data submitted from widely geographically dispersed clinicians and research groups from different countries. This process of collaboration and cooperation is crucial to effective research in rare disorders, and the experience of InterRett to date demonstrates the value of such a model. For Rett syndrome, there is also the added complexity brought about by the locus and allelic genetic heterogeneity associated with the disorder.

The InterRett infrastructure, which encourages simultaneous submission of clinical details by parents, treating clinicians, and research groups, also offers the advantage of data quality improvement brought about by comparing data (on the same individual) from different sources. As well as permitting recognition of concordant and discordant data, a much more detailed clinical description is ascertained by capturing the complementary observations of health practitioners and parents or caregivers. In instances where data were discordant, care was taken to weight the source considered most likely to reflect accurately the information being sought. Other strengths include the adoption of appropriate statistical techniques to account for missing data, clearly an anticipated problem when combining data from different sources which may have been collected in different ways. The effect of age on the different scales was also assessed and, when confounded, appropriately age adjusted. We believe that by using the aforementioned statistical approaches, we have translated into strengths

what some might regard as weaknesses in the structure of a data set defined from heterogeneous sources.

We have to acknowledge that InterRett, unlike the ARSD, is not population based. Although countries such as Spain, Israel, Canada, and France have made commitments to contributing their national data to this endeavor, there have been countries where as yet we have not been able to establish this collaborative process even for the submission of deidentified data. Therefore, although components such as the Spanish data are most likely representative of the underlying population, this is much less likely with family-initiated data from the United States and the United Kingdom. We have previously shown that families participating in an Internet study were more socioeconomically advantaged in comparison with the families in our national Australian register,³⁷ although this bias is likely to reduce over time as Internet access globally becomes more equitable. InterRett would nevertheless benefit from further research to compare the distribution of sociodemographic, clinical, and genetic characteristics of its subjects with those in the ARSD population.

X inactivation is a gene dosage compensation mechanism in mammals that equalizes the expression levels from genes positioned on the X chromosome among females (XX) and males (XY). Thus, the difference in severity between individuals with the same mutation may be influenced by the proportion of cells that have inactivated the normal X chromosome.⁸ We are now aware that X inactivation does contribute to the variability in phenotype in Rett syndrome at least with some of the common mutations of intermediate severity (p.T158M and p.R168X),³⁸ although there is need for further research on its role with milder and more severe mutations. In the previous study that combined data from the ARSD with data from the United Kingdom, we were able to demonstrate the protective effect of skewing of X inactivation on clinical severity.³⁸ We acknowledge that the absence of data on X inactivation is a shortcoming of the present study.

A defining characteristic of Rett syndrome genotype–phenotype comparisons in the past has been the inconsistency of results, with some studies finding no differences between mutations or mutation groups and others (such as the ARSD) finding several relationships. Despite the methodologic differences, the correspondence of the results between this InterRett investigation and the previous ARSD study indicates consistency be-

tween a large international and a population-based study. Thus, we can finally confirm the evidence for clear indisputable genotype–phenotype relationships in Rett syndrome. What still remains inaccessible, however, is more detailed analysis of the effects of X inactivation. Nevertheless, we have clearly shown that basic genetic testing is warranted in Rett syndrome not only for the molecular confirmation of a clinical diagnosis, but also to provide information on the likely clinical profile for the affected child. The overall aim of research in Rett syndrome and in any disabling condition is the provision of greater information that can be translated into better clinical practice. With this large study into the genetic and phenotypic aspects of Rett syndrome, at the very least this aim has been met.

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Investigating genotype phenotype relationships in Rett syndrome using an international data set

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