

# Neurophysiology Versus Clinical Genetics in Rett Syndrome: A Multicenter Study

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Many studies have attempted to establish the genotype–phenotype correlation in Rett syndrome (RTT). Cardiorespiratory measurements provide robust objective data, to correlate with each of the different clinical phenotypes. It has important implications for the management and treatment of this syndrome. The aim of this study was to correlate the genotype with the quantitative cardiorespiratory data obtained by neurophysiological measurement combined with a clinical severity score. This international multicenter study was conducted in four European countries from 1999 to 2012. The study cohort consisted of a group of 132 well-defined RTT females aged between 2 and 43 years with extended clinical, molecular, and neurophysiological assessments. Diagnosis of RTT was based on the consensus criteria for RTT and molecular confirmation. Genotype–phenotype analyses of clinical features and cardiorespiratory data were performed after grouping mutations by the same type and localization or having the same putative biological effect on the MeCP2 protein, and subsequently on eight single recurrent mutations. A less severe phenotype was seen in females with CTS, p.R133C, and p.R294X mutations. Autonomic disturbances were present in all females, and not restricted to nor influenced by one specific group or any single recurrent mutation. The objective information from non-invasive neurophysiological evaluation of the disturbed central autonomic control is of great importance in helping to organize the lifelong care for females with RTT. Further research is needed to provide insights into the pathogenesis of

autonomic dysfunction, and to develop evidence-based management in RTT. © 2016 The Authors. *American Journal of Medical Genetics Part A* published by Wiley Periodicals, Inc.

**Key words:** Rett syndrome; neurophysiology; MECP2

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Conflict of interest: Dr. Peter Julu is the inventor of the NeuroScope, the machine used to obtain objective and quantitative neurophysiological measurements of brainstem functions in this study, but the machine is patented by Glasgow University and the right to exploit the patent was sold to MediFit Instruments Ltd in London. Dr. Peter Julu from time to time gives scientific advice to MediFit Instruments Ltd and has no financial enumeration or administrative role in the private company. The only interest Dr. Peter Julu has in this company is the proper and correct scientific use of the NeuroScope. No financial support or funding applicable.

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## INTRODUCTION

Rett syndrome (RTT) is a neurological disorder affecting almost exclusively females. It is caused by mutations in the gene encoding the methyl CpG binding protein 2 (*MECP2*) [Hagberg et al., 1983; Amir et al., 1999]. A unique pattern of neurological and behavioral symptoms appears over time [Julu et al., 2008]. Most prominent are the abnormal breathing patterns, which is a consequence of developmental brainstem immaturity in this syndrome. Abnormal breathing is the most distressing and underestimated feature in many RTT females. It may be a major determinant of the quality of daily life of the female and her family [Julu and Witt Engerström, 2005; Smeets et al., 2006; Halbach et al., 2008, 2013; Julu et al., 2008; Tarquinio et al., 2015]. Neurophysiological research has established three cardiorespiratory phenotypes in RTT (forceful, feeble, and apneustic breathers), and their clinical relevance [Julu and Witt Engerström, 2005; Smeets et al., 2006; Julu et al., 2008; Halbach et al., 2011].

Many genotype–phenotype correlation studies have been published [Huppke et al., 2002; Leonard et al., 2003, 2005; Colvin et al., 2004; Schanen et al., 2004; Charman et al., 2005; Kerr and Prescott, 2005; Smeets et al., 2005, 2009; Bebbington et al., 2008, 2012; Neul et al., 2008; Halbach et al., 2012; Cuddapah et al., 2014]. These studies are based on clinical scoring systems and/or questionnaires in providing a composite clinical phenotype. To our knowledge, the published population-based studies did not have quantitative measures of cardiorespiratory dysfunctions in Rett syndrome. Cardiorespiratory variables can be measured objectively in different clinical phenotypes, providing robust quantitative data for research. It is stated that this has important implications for life long management and future treatment in RTT [Julu et al., 2008].

The aim of this collaborative multicenter study is to correlate for the first time the RTT genotype with the quantitative cardiorespiratory data obtained by neurophysiological measurement combined with a clinical severity score.

## METHODS

Ethical approval was obtained from the Medical Ethical Committee at the Maastricht University Medical Center.

### Study Design and Participants

In order to achieve a reasonable population size using the brainstem neurophysiological technique an international multicentre study was conducted in four European countries from 1999 to 2012. The six participating centers were: Tuscany Rett Centre, Versilia Hospital (Lucca, Italy), Medical Genetic Unit, Ferrara University Hospital (Ferrara, Italy), the National Swedish Rett Centre (Frösön, Sweden), the Rett Expertise Centre Netherlands, Maastricht University Medical Centre (Maastricht, the Netherlands), Neurodegeneration and Neuroinflammation at Imperial College (London, United Kingdom), and Institute of Neurological Sciences, Southern General Hospital (Glasgow, United Kingdom).

The study cohort consisted of a group of 132 well-defined RTT females with extended clinical, molecular, and neurophysiological assessment. These females were referred to one of the participating

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centers. Neurophysiological assessment was performed in Italy and Sweden, each examining 66 RTT females. Diagnosis of RTT was based on the consensus criteria for RTT [Hagberg et al., 2002; Neul et al., 2010]. Only females with molecular confirmation were included. Males with *MECP2* related disorders were excluded from this study.

### Molecular Analysis of *MECP2*

DNA analysis of *MECP2* was performed by sequencing the coding exons and immediately adjacent intronic regions. Additional Multiplex Ligation-dependent Probe Amplification analysis of *MECP2* was done to identify large genomic rearrangements. Nomenclature was according to the *MECP2A* isoform reference sequence NM\_004992.3. Numbering started at the A of the ATG translation initiation codon. Mutations were classified by type and localization in the gene (Table I). As to mutation type, they were classified as missense (single amino acid substitutions) and truncating mutations (nonsense mutations, frame shift mutations, and large deletions/duplications). The following domains were included for mutation localization: the N-Terminal domain (NT domain), the methyl-CpG-binding domain (MBD), the transcription repression domain (TRD), and the C-terminal segment (CTS).

### ISS Scoring List

In order to evaluate the clinical severity of the common features in RTT, a modified version of the International Scoring System was used (ISS, Table II) [Kerr et al., 2001]. The clinical scoring system originally consisted of 20 items (ranging from A to T), which were scored from zero to two; the lower the score, the better the clinical condition. Based on the high prevalence of gastro-intestinal and bladder problems in females with RTT, an additional item concerning these problems was added in the adapted ISS (item U). These 21 items were grouped into five functional domains: Growth and Development (A–E), Musculoskeletal (F–H), Movement (I–L), Cortical (M–O), and Autonomic Domain (P–U). The oro-motor disturbances were included in the Autonomic domain [Julu and Witt Engerström, 2005].

### Neurophysiological Assessment

Autonomic monitoring of brainstem function was carried out using the NeuroScope™ (Medifit Instruments Ltd, London, UK). This is a cortico-bulbar neurophysiological method for

TABLE I. Mutation Type and Localization in MECP2

Domain → Type of mutation ↓	NT (n)	MBD (n)	TRD (n)	CTS (n)	Total
Truncating (Nonsense, frame shift, large deletion)	<i>p.M5fsX</i> (1)  <i>p.R9fsX</i> (9)	<i>p.D90fsX</i> (1)  <i>p.R111fsX</i> (1) <i>p.N126fsX</i> (1) <i>p.Q128X</i> (1) <i>p.Y141X</i> (2) <i>p.K144fsX</i> (2)	<i>p.R168X</i> (12)  <i>p.G237fsX</i> (1) <i>p.G238fsX</i> (1) <i>p.R255X</i> (9) <i>p.G269fsX</i> (2) <i>p.R270X</i> (11) <i>p.R294X</i> (10)	<i>p.T327fsX</i> (1)  <i>p.K347fsX</i> (1) <i>p.A358fsX</i> (1) <i>p.P362fsX</i> (1) <i>p.A378fsX</i> (1) <i>p.L386fsX</i> (11) <i>p.P388fsX</i> (1) <i>p.P389fsX</i> (2) <i>p.S401fsX</i> (1) <i>p.R453X</i> (1)	85
Missense		<i>p.R106W</i> (5) <i>p.R133C</i> (14) <i>p.S134C</i> (1) <i>p.K135G</i> (1) <i>p.P152R</i> (5) <i>p.D156E</i> (2) <i>p.T158M</i> (8)	<i>p.P225A</i> (1) <i>p.P225R</i> (1) <i>p.P302L</i> (1) <i>p.R306C</i> (8)		47
Total	10	44	57	21	132

NT, N-Terminal segment; MBD, Methyl-CpG-Binding Domain; TRD, Transcription Repression Domain; CTS, C-Terminal Segment; n, number of patients (nomenclature according to the MECP2A isoform reference sequence NM\_004992.3).

monitoring brainstem autonomic functions and cortical activity simultaneously in real-time and synchronizing the various autonomic signs. The NeuroScope, a neurophysiology piece of equipment is used in routine clinical examination in Rett syndrome [Julu and Witt Engerström, 2005] and other neurodevelopmental disorders like the autistic spectrum disorders (ASD) [Ming et al., 2016] to monitor brainstem autonomic functions. The cardiac sensitivity to baroreflex (CSB) was measured as previously described [Julu et al., 2003], and is defined as the increase in pulse interval per unit increase in systolic blood pressure. This quantifies the negative feedback control of blood pressure (BP) beat by beat. The CSB is calculated according to the formula previously published [Julu et al., 2003]. The method detects rapid changes in CSB in real time within a continuous measurement, facilitating the evaluations of response latencies. A non-invasive continuous index of cardiac vagal tone (CVT), described as “pulse synchronized phase shifts in consecutive cardiac cycles” is a form of cardiac cycle jitter was quantified in real time using the NeuroScope as previously described [Little et al., 1999]. The CVT is quantified in clinically validated units of a linear vagal scale (LVS) with zero reference point equivalent to full atropinization in humans [Julu, 1992a,b]. The electrocardiogram (ECG) for deriving all the cardiovascular indices was recorded via three chest leads conforming to Einthoven’s Lead II and the non-invasive BP waveform was quantified continuously using volume-clamp photoplethysmography through a finger cuff of the Nexfin<sup>TM</sup>-Monitoring System (BMEYE, Amsterdam, The Netherlands) or Portapres<sup>TM</sup> blood pressure monitor (Finapres Medical Systems, Amsterdam, The Netherlands). A TCM4 or

TCM3 monitor (Radiometer, Copenhagen, Denmark) is used to quantify the partial pressures of oxygen (pO<sub>2</sub>) and carbon dioxide (pCO<sub>2</sub>) transcutaneous through a special sensor placed on the abdominal skin in the sub-costal region in the mid-clavicular line close to the liver and the signal is transmitted live continuously to the NeuroScope and displayed in real-time synchronously with all other physiological parameters. Other raw data were fed into the NeuroScope for further processing and real-time derivation of the autonomic indices using VaguSoft<sup>TM</sup> software (Medifit Instruments Ltd) as follows. The peaks of blood pressure (BP) waveforms during cardiac cycles provided values for the systolic pressure (SBP, in mmHg). The lowest BP before the ejection period of the cardiac cycle is diastolic BP (DBP, in mmHg) and the arithmetic mean of arterial pressure during the whole cardiac cycle is mean arterial pressure (MAP, in mmHg). The instantaneous heart rate (HR, in bpm) was calculated continuously in real time from the intervals between consecutive electrocardiographic R-waves (“R-R intervals, in msec.”) Cortical activity was monitored using electroencephalography (EEG) and synchronized with autonomic function. A continuous video record time-locked with the physiological data was kept for behavioral analysis. The breathing movements measured using a stretch sensitive plethysmograph placed around the chest at the level of the xiphisternum were analyzed using the LARS respiratory analysis software (MediFit Instruments Ltd). The individual breathing movement was scored for types of rhythm and amplitudes according to the criteria previously described for Rett syndrome [Julu and Witt Engerström, 2005]. The total time of each specific type of breathing movement was compared with the

TABLE II. Modified Version of the ISS Scoring List

## International scoring system

Growth and development	
A	Head circumference during the first year
2	Already below the 3rd percentile at birth
1	Normal at birth but decelerating
0	Normal at birth with no deceleration
B	Early developmental progress (birth to 12 months)
2	No or virtually no progress
1	Suboptimal progress
0	Normal progress
C	Present head circumference—(percentile/standard deviations SD)
2	Below 3rd percentile
1	3rd–10th percentile
0	Above 10th percentile
D	Weight (kg)
2	Below 3rd percentile
1	3rd–10th percentile
0	Above 10th percentile
E	Height (cm)
2	Below 3rd percentile
1	3rd–10th percentile
0	Above 10th percentile
Musculoskeletal	
F	Muscle tone (also describe)
2	Severe hypotonia, dystonia, or hypertonia
1	Tone mildly abnormal
0	Normal
G	Spine posture
2	Severe scoliosis
1	Mild scoliosis
0	No deviation
H	Joint contractures
2	Severe contractures
1	Minor contractures
0	None
Movement	
I	Gross motor function
2	Cannot walk with support
1	Walking impaired
0	Walks normally
J	Hand stereotypy (patting, squeezing, wringing, mouthing)
2	Dominating or constant
1	Mild or intermittent
0	None
K	Other involuntary movements (e.g., tremor, dystonia, chorea, athetosis)
2	Dominating or constant
1	Mild or intermittent
0	None
L	Voluntary hand use (e.g., self-feeding)
2	None
1	Reduced or poor
0	Hand use normal
Cortical	
M	Intellectual disability (=learning disability, retardation)
2	Apparent profound (infant level)
1	Any except profound
0	No impairment

TABLE II. (Continued)

## International scoring system

N	Speech
2	Currently uses no real words with meaning
1	Currently uses some real words with meaning
0	Normal speech
O	Epilepsy
2	Uncontrolled or poorly controlled
1	Previous epileptic seizures or well-controlled with medication
0	Never
Autonomic features	
P	Oro-motor difficulty
2	Severe (e.g., feeding aversion; gagging, choking, tube/button fed)
1	Slight (e.g., delayed chewing, swallowing, on supplements)
0	None
Q	Disturbed awake breathing rhythm (e.g., hyperventilation, breath holding, panting)
2	Severe, with vacant spells and color changes
1	Mild, without vacant spells and color changes
0	Normal breathing pattern
R	Peripheral circulation of extremities
2	Cold or discolored with atrophic changes
1	Cold or discolored without atrophic changes
0	Normal color and temperature of extremities
S	Mood disturbance
2	Prominent or disruptive agitation/crying spells
1	Abnormally prone to agitation
0	Normal
T	Sleep disturbance
2	Prominent/disruptive day sleeping or night waking
1	Present, not prominent
0	Normal sleep pattern
U	Gastro-intestinal and bladder
2	Reflux oesophagitis, severe constipation, or neurogenic bladder dysfunction
1	Gastrointestinal dysmotility without secondary complications
0	No signs of gastrointestinal dysmotility or difficulties to empty bowel or bladder

Adapted from Kerr et al. [2001].

duration of the whole monitoring session. The prevailing breathing phenotype determines the cardiorespiratory phenotype where Forceful Breathers would have longer duration of forceful breathing, Feeble Breathers would have longer duration of weak ineffective breathings and Apneustic Breathers would have longer duration in apneusis during the whole monitoring session [Julu and Witt Engerström, 2005].

## Data Analysis

Data were collected from all centers into a unified, anonymous database. Clinician experts in RTT made the ISS scoring list. Baseline brainstem functions were measured during normal breathing without agitation, with normal blood gases (pCO<sub>2</sub>

**TABLE III. Recurrent Mutations Including Number and Percentage of RTT Females**

Recurrent mutation	Number of RTT females	Percentage of RTT females (%)
p.R133C	14	11
p.T158M	8	6
p.R168X	12	9
p.R255X	9	7
p.R270X	11	8
p.R294X	10	8
p.R306C	8	6
C-terminal deletions	21	16
Total	93	70

and  $pO_2$ ) and in the absence of epileptiform activity on EEG. We considered a baseline state of the subjects when there was no visible contraction of neck muscles and breathing was quiet (regular breathing curve) with  $<10\%$  fluctuations about the means in both blood pressure and heart rate from their peaks to troughs for at least 10 min. Details of the assessment of brainstem functions to determine cardiorespiratory phenotype are published elsewhere [Julu and Witt Engerström, 2005; Ming et al., 2016].

The genotype–phenotype analyses were performed after grouping together all mutations of similar types, localizations or putative biological effect on the MeCP2 protein. Consequently, mutations were then subdivided into the following five groups: (i) truncating mutations in the NT and MBD domain, causing loss of function or disruption of the two functional domains MBD and TRD; (ii) missense mutations in the MBD domain, giving rise to a modified or non-functional MBD; (iii) truncating mutations in the Interdomain and TRD, causing a loss of functional TRD; (iv) missense mutations in TRD giving rise to a modified or non-functional TRD; and (v) small truncations in the CTS leading to protein with an altered C-terminus. A further separate genotype–phenotype analysis was performed on eight recurrent mutations, defined as mutations present in at least 5% of the RTT females in this cohort (Table III).

We used descriptive statistics to analyze mutation types, ISS scores, and cardiorespiratory data including Valsalva's manoeuvre type of breathing (Valsalva breathing). Linear regression analysis was used to analyze the relationships among ISS scores (total and functional domain scores), CVT, HR, and mutation groups or recurrent mutations. Checks for the normality assumption were done employing Q–Q plots. If the normality assumption was in doubt, the analyses were done by ordinal logistic regression. Relationships among cardiorespiratory phenotypes, Valsalva breathing (present or absent), and mutation groups or recurrent mutations, were examined using nominal logistic regression. The mutation groups and the recurrent mutations were used as predictor variables through dummy coding in the regression analyses. Since age may be a confounding factor for the ISS scores and HR, this variable was included as

an extra predictor for these outcomes. First a statistical test was done to check whether there was a relation between mutation groups or recurrent mutations and the outcome in question. If present, it was re-examined in a pairwise fashion to determine which groups differed from each other with respect to the outcome variable. The level of statistical significance for all tests was set to a probability value of  $\leq 0.05$ , and all analyses were carried out using SPSS18.

## RESULTS

The age of the RTT females ranged between 2 and 43 years (mean age: 12.46 years, SD = 9.36). According to the clinical criteria, 74% (n = 98) of the females were typical RTT and 26% (n = 34) atypical RTT.

### Mutation Analysis

*MECP2* mutations were classified by mutation type, localization, and putative protein function, as shown in Table I. Truncating mutations were present in 64% (n = 85) and missense mutations in 36% (n = 47). Forty-three percent had a mutation affecting the TRD (n = 57), whereas 41% (n = 54) had a mutation affecting the MBD. The mutations localized in the MBD were predominantly missense mutations (82%), while those affecting in the TRD were mostly truncating (81%). Half of the truncating mutations, affecting both the MBD and TRD, were due to a large deletion of both exon three and most of the coding part of exon four. Sixteen percent (n = 21) displayed a truncating mutation in the CTS leading to an extensive replacement of the C-terminus, which is likely to have an unfavorable effect on the natural protein function due to its putative effect on protein structure.

Table III shows the recurrent mutations included in this study, together comprising 70% of the pathogenic mutations in this cohort.

### ISS Scoring List

The mean severity score on the ISS scoring list was 20.7 points (range 2–36, SD = 7.59). Separating the scores into the functional domains, the mean scores were: Growth and Development, 3.79 points (range 0–8, SD = 2.42); Musculoskeletal, 2.54 points (range 0–6, SD = 1.84); Movement, 4.76 points (range 1–8, SD = 1.59); Cortical, 4.10 points (range 1–6, SD = 1.37); Autonomic, 5.53 points (range 0–12, SD = 2.59), respectively.

### Cardiorespiratory Status of the RTT Cohort

Forty-nine percent were diagnosed as feeble breathers (n = 65), 41% as forceful (n = 54), and 10% as apneustic (n = 13). Valsalva breathing was present in 62% (n = 82), and occurred in all three cardiorespiratory phenotypes. We assessed both sympathetic and parasympathetic functions of the autonomic nervous system by measuring HR and CVT of these females. HR varied between 66 and 172 beats/min (mean rate = 99.2, SD = 17.4). Mean CVT was 4.50 (range 0.9–13.9, SD = 2.53).

## Genotype–Phenotype Analysis

**Clinical severity.** *Total ISS score.* The total ISS score did differ significantly between the different mutation groups ( $F(df_1 = 4, df_2 = 126) = 3.02, P = 0.02$ ). Females with a CTS mutation scored significantly lower than females with a mutation in the NT domain or nonsense mutation in the MBD ( $t = 2.53, P = 0.01$ ), and with a nonsense or missense mutation in the TRD ( $t = 3.22, P < 0.01$  and  $t = 2.31, P = 0.02$ , respectively).

The total ISS score also differed significantly between the different recurrent mutations ( $F(df_1 = 7, df_2 = 83) = 4.47, P < 0.001$ ). Females with a p.R133C, p.R294X, or CTS mutation scored significantly lower than females with a p.T158M ( $t = 2.32–2.57, P = 0.01–0.02$ ), p.R168X ( $t = 2.70–2.92, P < 0.01$ ), p.R255X ( $t = 3.54–3.70, P < 0.001–0.01$ ), p.R270X ( $t = 2.55–2.79, P = 0.01$ ), or p.R306C mutation ( $t = 2.05–2.35, P = 0.02–0.04$ ).

**Growth and development domain.** The ISS score in the Growth and Development domain did differ significantly between the different mutation groups ( $F(df_1 = 4, df_2 = 126) = 3.22, P = 0.02$ ). Females with a CTS mutation scored significantly lower than females with a mutation in the NT domain or nonsense mutation in the MBD ( $t = 3.00, P < 0.01$ ), missense mutation in the MBD ( $t = 2.53, P = 0.01$ ), and nonsense or missense mutation in the TRD ( $t = 2.92, P < 0.01$  and  $t = 2.76, P < 0.01$ , respectively).

The ISS score also differed significantly between the different recurrent mutations ( $F(df_1 = 7, df_2 = 83) = 4.57, P < 0.001$ ). Females with a p.R294X or CTS mutation scored significantly lower than females with a p.T158M ( $t = 3.08, P < 0.01$  and  $t = 2.89, P < 0.01$ , respectively); p.R168X ( $t = 2.31, P = 0.02$  and  $t = 2.03, P = 0.05$ , respectively), p.R255X ( $t = 3.88, P < 0.001$  and  $t = 3.84, P < 0.001$ , respectively), p.R270X ( $t = 3.10, P < 0.01$  and  $t = 2.93, P < 0.01$ , respectively) or p.R306C mutation ( $t = 3.00, P < 0.01$  and  $t = 2.78, P < 0.01$ , respectively). Also females with a p.R133C mutation scored significantly lower than females with a p.T158M mutation ( $t = 2.00, P = 0.05$ ) or p.R255X mutation ( $t = 2.84, P = 0.01$ ).

**Musculoskeletal domain.** The ISS score in the Musculoskeletal domain did not differ significantly among the different mutation groups ( $F(df = 4) = 9.42, P = 0.051$ ), but did differ significantly among the different recurrent mutations ( $F(df = 7) = 15.00, P = 0.04$ ). Females with a p.R133C or p.R294X mutation scored significantly lower than females with a p.T158M ( $F(df = 1) = 12.23, P < 0.001$  and  $F(df = 1) = 9.39, P < 0.01$ , respectively), p.R168X ( $F(df = 1) = 13.44, P < 0.001$  and  $F(df = 1) = 10.00, P < 0.01$ , respectively), p.R255X ( $F(df = 1) = 14.83, P < 0.001$  and  $F(df = 1) = 11.47, P = 0.001$ , respectively), p.R270X ( $F(df = 1) = 9.19, P < 0.01$  and  $F(df = 1) = 6.64, P = 0.01$ , respectively) or p.R306C mutation ( $F(df = 1) = 5.86, P = 0.02$  and  $F(df = 1) = 4.11, P = 0.04$ , respectively). Also females with a CTS mutation scored significantly lower than females with a p.T158M ( $F(df = 1) = 5.84, P = 0.02$ ), p.R168X ( $F(df = 1) = 6.38, P = 0.01$ ) or p.R255X mutation ( $F(df = 1) = 7.89, P = 0.01$ ).

**Movement domain.** The ISS score in the Movement domain did not differ significantly among the different mutation groups ( $F(df_1 = 4, df_2 = 126) = 1.27, P = 0.29$ ) or recurrent mutations ( $F(df_1 = 7, df_2 = 83) = 1.55, P = 0.16$ ).

**Cortical domain.** The ISS score in the Cortical domain did not differ significantly among the different mutation groups ( $F(df_1 = 1, df_2 = 5) = 12.78, P = 0.21$ ) or recurrent mutations ( $F(df_1 = 1, df_2 = 8) = 14.83, P = 0.34$ ).

**Autonomic domain.** The ISS score in the Autonomic domain did not differ significantly among the different mutation groups ( $F(df_1 = 4, df_2 = 126) = 2.11, P = 0.08$ ) or recurrent mutations ( $F(df_1 = 7, df_2 = 83) = 1.93, P = 0.08$ ).

**Autonomic assessment.** *Cardiorespiratory phenotype.* There was no significant correlation between cardiorespiratory phenotype and groups of mutations ( $\chi^2(df = 8) = 4.77, P = 0.78$ ). Excluding the apneustic breathers for the analysis did not change these results ( $\chi^2(df = 4) = 3.37, P = 0.50$ ).

Also no significant correlation was seen among the cardiorespiratory phenotypes and recurrent mutations ( $\chi^2(df = 14) = 13.42, P = 0.49$ ). Excluding the apneustic breathers for this analysis also did not change these results ( $\chi^2(df = 7) = 8.91, P = 0.26$ ).

*Valsalva manoeuvre type of breathing.* There was no significant correlation among the presence and absence of Valsalva breathing and the groups of mutations ( $\chi^2(df = 4) = 2.50, P = 0.64$ ) or recurrent mutations ( $\chi^2(df = 7) = 10.81, P = 0.15$ ).

*Cardiac vagal tone (CVT).* There was no significant correlation between CVT and the groups of mutations ( $F(df_1 = 4, df_2 = 126) = 2.40, P = 0.06$ ) or recurrent mutations ( $F(df_1 = 7, df_2 = 83) = 1.17, P = 0.33$ ).

*Heart rate (HR).* There was no significant correlation between HR and the groups of mutations ( $F(df_1 = 4, df_2 = 126) = 0.29, P = 0.88$ ) or recurrent mutations ( $F(df_1 = 7, df_2 = 83) = 0.31, P = 0.95$ ).

## DISCUSSION

This multicenter study was the result of an international collaborative network set up to create a database with sufficient numbers of robust clinical, molecular, and neurophysiological data for further analyses. The grouping of the mutations in *MECP2* was based on the putative biological effects of these mutations on the MeCP2 protein as explained in our Methods. Despite the minor methodological differences, the clinical severity and general genotype–phenotype results were similar to those in previous studies. We used a modified version of the internationally accepted clinical scoring system (ISS) to quantify the effects of RTT on growth, development, and other bodily dysfunctions in our genotype–phenotype analyses. The ISS provided us with a form of quantifiable clinical severity of the various bodily dysfunctions in this RTT cohort, which indeed is comparable with previous research [Bebbington et al., 2008, 2010; Halbach et al., 2012]. We have elucidated a less severe clinical phenotype in females with CTS, p.R133C, or p.R294X mutations. However, clinical severity varies even within one specific type or group of mutations. It means genotypes have very limited use for clinical management in RTT.

The main reason for conducting this study was to evaluate the influence of *MECP2* mutation on brainstem instability, which includes breathing dysrhythmia, so characteristic of RTT. Breathing dysrhythmia is a major reason for seeking medical attention and for secondary referral of persons with

RTT. Cardiorespiratory data must be obtained using objective and quantitative neurophysiological measurements of brainstem functions, because clinical management of brainstem autonomic dysfunction in RTT is a profound challenge. Each cardiorespiratory phenotype requires a unique and specific clinical approach [Julu et al., 2008]. In this cohort of RTT females, up to 49% were feeble breathers, 41% were forceful breathers, and 10% were apneustic breathers. There is some but little difference in the distribution of the three cardiorespiratory phenotypes in this cohort compared with that previously reported [Julu and Witt Engerström, 2005]. This cohort of RTT confirms that Valsalva breathing is a common complication of breathing dysrhythmia in RTT, affecting all three cardiorespiratory phenotypes as previously reported [Julu and Witt Engerström, 2005]. The wildcat excitatory effects of Valsalva's manoeuvre on the autonomic nervous system in general and other brainstem functions can cause clinical deterioration in RTT [Julu and Witt Engerström, 2005; Smeets et al., 2006]. Detailed correlation analyses of the cardiorespiratory data showed that the cardiorespiratory phenotypes in RTT are not influenced by genetic mutations. This provides further proof that the cardiorespiratory phenotypes needs as much attention in clinical management as the genetic mutations.

The baseline brainstem functions were severely affected in all RTT females similar to the results in previous studies [Julu et al., 1997, 2001; Julu and Witt Engerström, 2005]. The mean CVT in this cohort was lower than the normal mean value in young adults [Julu, 1992b]. Since CVT is the only central inhibitory output to the heart, it is very important in brainstem cardiorespiratory integration. Its role in rapid cardiovascular responses is very important and it is a major contributor to integrative inhibition within the cardiovascular system [Guyenet et al., 1996]. The HR values in this cohort were within the normal limits for the age group. It implies that the resting sympathetic tone is within normal limits [Julu et al., 1997], and not exaggerated above normal as previously thought [Naidu et al., 1987]. This normal but unrestrained sympathetic tone due to little or no parasympathetic negative feedback is the cause of a type of sympatho-vagal imbalance unique to RTT. This may contribute to the increased sudden deaths of up to 26% of females with RTT, compared with only 2.3% in the general population of the same age range [Kerr et al., 1997; Hagberg et al., 2001]. Although this figure may be overestimated, in more recent studies sudden unexplained deaths are not specifically addressed [Laurvick et al., 2006] or the cause of death incompletely reported [Kirby et al., 2010]. The discussion above and the high ISS score in the Autonomic Domain both reflect the great impact of brainstem dysfunction on the clinical severity in RTT. We provide here sufficient reasons for carers of persons with RTT to seek medical attention starting early in childhood. This is because "bedside" clinical evaluation including ISS scoring cannot determine the contributions of autonomic dysfunction to the clinical severity in individual cases [Halbach et al., 2012]. In persons with RTT, objective quantitative clinical and neurophysiological assessment must be done early after diagnosis or promptly following the onset of brainstem autonomic symptoms. For the moment there is lack of appropriate neurophysiological facilities for brainstem assessment in most hospitals and therefore this study is a pledge to make them more available to a larger number of girls and women with RTT.

## CONCLUSION

This is the first study to use objective and robust data of cardiorespiratory variables in the investigation of genotype–phenotype correlation in RTT. All females with RTT had dysautonomia, and this was not restricted to nor influenced by one specific group or single recurrent mutation. The clinical variability within a specific genetic mutation or within a similar group of mutations makes genotype–phenotype correlations a difficult task. The robust and objective information obtained from non-invasive neurophysiological evaluation of the brainstem autonomic functions will contribute to the understanding of the ongoing pathology in RTT and its life-long management. Although considerable progress is being made in understanding the mechanisms of autonomic dysfunction in RTT [Weese-Mayer et al., 2006; Rohdin et al., 2007; Katz et al., 2009; Lioy et al., 2011; Abdala et al., 2014], further research is needed for a better understanding of the pathogenesis of autonomic dysfunctions in this syndrome. This will facilitate future development of evidence-based management strategies in RTT.

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