Rett syndrome – biological pathways leading from MECP2 to disorder phenotypes

Abstract
Rett syndrome (RTT) is a rare disease but still one of the most abundant causes for intellectual disability in females. Typical symptoms are onset at month 6–18 after normal pre- and postnatal development, loss of acquired skills and severe intellectual disability. The type and severity of symptoms are individually highly different. A single mutation in one gene, coding for methyl-CpG-binding protein 2 (MECP2), is responsible for the disease. The most important action of MECP2 is regulating epigenetic imprinting and chromatin condensation, but MECP2 influences many different biological pathways on multiple levels although the molecular pathways from gene to phenotype are currently not fully understood. In this review the known changes in metabolite levels, gene expression and biological pathways in RTT are summarized, discussed how they are leading to some characteristic RTT phenotypes and therefore the gaps of knowledge are identified. Namely, which phenotypes have currently no mechanistic explanation leading back to MECP2 related pathways? As a result of this review the visualization of the biologic pathways showing MECP2 up- and downstream regulation was developed and published on WikiPathways which will serve as template for future omics data driven research. This pathway driven approach may serve as a use case for other rare diseases, too.

Keywords: Rett syndrome, MECP2, Systems biology, Bioinformatics, Data integration, DNA methylation, Epigenetics

Background
Rett syndrome (RTT; MIM:312750) occurs in 1:10,000 girls at the age of 12 [1]. It is considered a rare disease since it affects fewer than 1 in 2000 individuals [2], but it is still one of the most abundant causes for intellectual disability in females. RTT was first described in 1966 by the Viennese pediatric Andreas Rett, who observed the typical hand movements (“hand washing”) of his patients [3, 4]. Cause of RTT is in most cases a de novo mutation of MECP2 (methyl-CpG-binding protein 2) gene; which was discovered by Amir et al. [5]. However, as stated by Neul et al., “not all mutations in MECP2 cause RTT and not all RTT patients have mutated MECP2”. Some MECP2 mutations cause not RTT but a mild intellectual disability [6] and mutations in two other genes can cause a RTT like phenotype, i.e. FOXG1 and CDKL5. These phenotypes were formerly considered as RTT but are now defined as RTT like syndrome [7].

RTT was considered a neurodevelopmental disorder but since some of the main symptoms were found to be reversible [8] researchers and clinicians tend to categorize it as a neurological disorder now [9]. RTT was also classified as an autism spectrum disorder as patients often develop autistic features like social withdrawal but only during a certain stage of development [10]. Although RTT has some autistic features/ phases these usually disappear with time and adult RTT females are quite socially active again [11]. MECP2 mutations are rarely found in autism patients and if, they are termed “Autism with MECP2 mutation” [7].

Recent research was able to find a correlation between certain MECP2 mutations (or MECP2 variants) and some phenotypes, e.g. cardiorespiratory phenotype [12], but most of the biological pathways between gene and phenotype are not yet fully understood. Especially the
molecular pathways leading from MECP2 gene to scoliosis, epilepsy or decreased growth are currently not known. In this review, we summarize the knowledge about the molecular interactions of MECP2 gene and protein, their known downstream effects and discuss how pathway and omics data based research can elucidate the pathways towards RTT phenotypes. This review integrates database knowledge from Ensembl [13], OMIM [14], UniProt [15], The Human Protein Atlas [16], and Gene Ontology [17] and a biologic pathway was developed and published on WikiPathways [18] to visualize the mechanistic action of MECP2 and serve as a template for future omics data analysis also in other rare disease models.

**Rett syndrome phenotype development within life**

Typical development of RTT starts with an “asymptomatic” first stage followed by decreased, arrested and retarded development of motor and communication skills after 6–18 months of normal postnatal development, development of stereotypic movements and loss of purposeful movement. Although the onset of typical disorder symptoms after the age of 6 months is characteristic for RTT, observations of parents that “something is wrong with this child”, are often made before. This matches with newer research which indicates that severe changes in neuronal development are apparent already at this age but due to their mild and uncertain symptoms not able to be diagnosed [19]. After a stagnation stage (2 – 10 years) which can last for years and can include some recovery and secondary gain of abilities the fourth stage typically reduces again mobility (by abnormal muscle tonus and scoliosis) while communication and cognition is preserved. RTT females are typically severely intellectually disabled, have microcephaly and seizures. Additionally, they often develop symptoms like cardiac and breathing abnormalities, gastro-intestinal problems like constipation, low muscle tension, autistic like behavior, scoliosis (and other osteopathies), sleeping problems and hormone disequilibrium. In summary, MECP2 affects epigenetic regulation of gene expression, which changes neurobiological activity, network formation and function, which causes the major phenotype. In summary, MECP2 affects epigenetic regulation of gene expression, which changes neurobiological activity, network formation and function, which causes the major phenotype. Recent longitudinal studies on the lifelong development of RTT stated that survival at the age of 25 years was 77.6% and 59.8% at 37 years [20]. The most abundant causes of death were lower respiratory tract infection, aspiration/asphyxiation and respiratory failure. Two-thirds of RTT females had seizures on some point in their lives of which 36.1% were drug resistant [20]. About half of the females completely lost the ability to walk while independent walking was preserved in 17.8%. Scoliosis (85.5%) and abnormal breathing patterns (up to 88.7%) were very abundant [20]. Typical noticeable laboratory results are EEG (and EKG) abnormalities, atypical brain glycolipids, altered neurotransmitter, creatine and growth factor levels, and alkalosis. Most of the symptoms can be related to disturbed neuronal function but some of them are caused or influenced by alterations which are not yet elucidated [4, 21, 22]. It is also unknown whether it is the often in RTT observed changes of carbon dioxide metabolism that cause respiratory problems or that dysfunctional brain stem neurons are responsible for breathing abnormality [4].

**MECP2 gene, transcript and protein**

The MECP2 gene is highly conserved in Euteleostomi (bony vertebrates). The NCBI HomoloGene/UniGene database gives detailed information about gene homologues in 10 mammalian, 2 amphibian and 1 bony fish species [23]. The human MECP2 is located on chromosome X, position 154,021,573-154,137,103 (reverse strand) (according to Ensembl, version 84, genome build GRCh38.p5 (GCA_000001405.20)) and there are currently 21 transcripts known, two of these are protein coding (Fig. 1). Due to dosage compensation MECP2 is inactivated in one X-chromosome in females and the degree of inactivation is assumed to contribute to the difference in phenotypes for RTT [24]. RTT is more often observed in females due to its location on the X-chromosome. Hemi-zygous males with a severe mutation are generally not viable, but there are several non-lethal mutations which can lead to severe congenital encephalopathy, RTT-like syndrome, and mild to severe intellectual disability in males [25]. Mosaic expression with only wild-type MECP2 active in females are possible but supposed to be extremely rare [24].

The transcription and translation of MECP2 is highly regulated [26] (Table 1 and Fig. 2). There are several cis- and trans-regulatory elements for MECP2 gene expression regulation known. Cis regulatory elements, including promotor elements, are loci on the DNA which act as binding sites for transcription factors and activate or repress gene expression. Trans-elements affect the regulation in an indirect way and can be located close or far away. They can for instance include genes that encode transcription factors for this specific gene. Translation of MECP2 can be regulated by a set of microRNAs [27–35]. MicroRNAs are small non-coding RNAs, that repress translation mRNA into protein by binding to the 3’ untranslated region of the mRNA. The regulation of MECP2 expression, stimulation and repression, is visualized in Fig. 2 (transcriptional and translational regulation of MECP2) which is derived from WikiPathways [18] pathway ID 3584.
The two coding transcripts are isoforms of MECP2, long e1 and short e2, while e1 seems to be the more important one [36, 37] (Fig. 1). Itoh et al. observed that specific deactivation of e2 did not influence normal neurodevelopment while loss of e1 led to RTT [38]. The MECP2 protein has at least six biochemically distinct domains [39]. Two of them are most important for the protein function: the (84 amino acids) methyl-CpG binding domain (MDB) which is the one which selectively binds 5MeCyt and the transcriptional repression domain (TRD) (102 amino acids) which binds cofactors attracting histone deacetylase and finally leading to transcription repression as explained in the chapter 4 (Fig. 1) [39, 40]. Interestingly, MBD is the only structured domain (α-helix) while 60% of MECP2 is unstructured [39]. There are several post translational modifications of MECP2 known which contribute to its multi-functional properties, phosphorylation, acetylation, SUMOylation, and ubiquitination [41].

MECP2 protein is most abundant in brain but also enriched in lung and spleen tissue [42]. However, according to The Human Protein Atlas database MECP2 protein (and its transcript) is found in quite high amounts in almost every tissue, too. This may actually be the most underestimated part in RTT research which typically focus on neuronal development and function [16]. Many phenotypes and symptoms may be as well deriving from dysfunctional cellular regulation in other organs than central nervous system. In neurons an expression level of about 1.6x10^7 protein copies per nucleus was estimated by Skene et al. It is about the same number as nucleosomes or 5-methyl-cytosine (5MeCyt) spots on the DNA, leading to the suggestion that every spot might be covered by one MECP2 [43].

### MECP2 function

MECP2 is a multifunctional protein which influences gene expression and metabolism on many levels [9] (Fig. 3). The main function of MECP2 is to recognize and bind specifically methylated cytosine residues in the DNA (namely 5MeCyt) that are enriched with A/T bases adjacent [44]. MECP2 binds also but with lesser affinity to hydroxymethylated DNA (namely 5-hydroxy methylated cytosine, 5OHMeCyt). Mutations in MECP2, especially in the MBD, which lead to loss of specific 5MeCyt binding functions are known to cause RTT [45] (Fig. 1).

### Table 1 Regulation of MECP2 expression by transcription factors and microRNA

| Type                                                                 | Transcription factors/microRNA                                      |
|----------------------------------------------------------------------|--------------------------------------------------------------------|
| Transcription factors targeting MECP2 cis-elements [27–29]          | Activation by BRN3, MYT1, SP1, SP3, C/EBP, CTCF, E2F1, TAF1, TAP1 |
| Repression by REST, BRN2, BCL6,                                      |                                                                    |
| Trans-regulatory elements of MECP2 [30]                             | Activation by HNRNPF                                              |
| miRNA (posttranscriptional repression) [31–35]                      | Repression by HNRNPH1                                             |
|                                                                       | hsa-miR-483-5p, hsa-miR-132-5p, hsa-miR-152-3p, hsa-miR-199a-3p,   |
|                                                                       | hsa-miR-30a-3p, and hsa-miR-130b-3p                                  |
This is in line with the Gene Ontology classification for the main molecular functions of MECP2: DNA binding, namely double-stranded methylated DNA and protein binding, namely histone deacetylase. The actual full Gene Ontology annotation of MECP2 can be found online e.g. Ensembl database MECP2 entry [13].

The molecular functions of MECP2 are known to influence various biological mechanisms, which are summarized and visualized in the pathway Fig. 2, namely 1) MECP2 influences global translation by enhancing the AKT/mTOR signaling pathway [46], 2) Alternative splicing of downstream gene products is affected because MECP2 forms a complex with YB1, an important splicing factor [29, 47–51], 3) Expression of various microRNAs and long non-coding RNAs is regulated by MECP2 (20, 45, 47–49), and 4) MECP2 triggers the chromatin compaction at methylated DNA sites which regulates the transcription of adjacent genes (34, 37–41). The last one is an important (and best investigated) pathway and will be explained in detail below.

**MECP2 as epigenetic regulator**

Methylation of DNA is part of epigenetic gene expression regulation, where DNA is modified without changing the genetic code. Most transcription factors are unable to bind to methylated DNA so methylation usually silences a gene. Furthermore, methylated DNA is via MECP2 mediated cofactor binding - a binding site for histone deacetylase (HDAC) which increases DNA compaction by removing certain acetyl residues from lysine at the histone tail allowing them to get closer to each other (Fig. 2, MECP2-HDAC complex). So, methylated DNA is tightly wrapped around histone proteins and the access of transcription factors is physically
About 1% of DNA is methylated in humans and the methylation sites are often in regions with a high occurrence of CG, so-called CpG islands. CpG islands are present in the promoter regions of most human genes (60%). Methylation patterns play a role in cellular differentiation and tissue specific gene expression already during early development [52–54]. The methylation pattern is continuously modified and maintained during mitosis and cell differentiation throughout life to grant cellular function [55, 56]. Figure 4 visualizes this circular pathway of DNA methylation, hydroxylation and de-methylation and shows the mode of action of involved proteins including MECP2.

MECP2 recognizes and binds specifically to 5MeCyt present in DNA. After binding it attracts co-repressor
complexes containing SIN3A, NCOR and SMRT. This co-repressor complex finally recruits histone deacetylase (HDAC) [29, 40, 47, 57] (Fig. 2, see MECP2 – HDAC complex). The complex acts by removing certain acetyl groups from histone proteins leading to chromatin condensation around methylated DNA [58, 59]. Cell culture experiments with an inhibitor of HDAC resulted in the same phenotype as MECP2-KO cells [60]. The NCOR-SMRT interaction domain (NID) of MECP2 is located within the TRD domain and the association with NCOR-SMRT is responsible for transcription repression activity of MECP2.

MECP2 was generally considered as a transcription inhibitor but recent research found also a conditional transcription activation function. Skene et al. identified MECP2, because of its mere amount, being equal to number of histone octamers or methylated DNA sites, as a global damper of gene transcription in mature mouse brain cells [43]. During MECP2 absence H3 (histone family 3) acetylation levels were globally elevated and H1 (histone family 1) levels doubled suggesting MECP2 alters global chromatin state towards condensation and represses transcription. Li et al. on the other hand found global transcription activation by MECP2 in human embryonic stem cells which underwent differentiation to neuronal cells but repression by MECP2 in mature neuronal cells indicating that MECP2 can be both, an activator and repressor [61]. The activation mechanism is explained as follows: MECP2 recruits CREB1 as a cofactor to target gene promoters [62] (Fig. 2, Activation of transcription): MECP2 binding to 5OHMeCyt was even interpreted as a marker of active genes in neurons [62]. MECP2 was also found to form a TET1 containing complex which leads to 5MeCyt hydroxylation and further to demethylation of DNA, enabling transcription [63]. This mechanism was found to activate expression of downstream genes, namely BDNF, SST, OPRK1, GAMT, GPRIN1, and CREB1 [28] and is contradictory to other findings which describe MECP2 to block DNA demethylation by TET complex (Fig. 4) [64].

MECP2 is additionally responsible for neuronal activity triggered transcription. Neuronal membrane depolarization and Ca$^{2+}$ influx leads to phosphorylation of MECP2 which makes it detach from DNA, allowing decondensation and transcription (Fig. 2) [65–69]. Specific blocking of MECP2 phosphorylation sites led to RTT like symptoms [70]. For a conclusion, MECP2 is responsible for the epigenetic regulation of gene expression, which changes neurobiological activity, network formation and function which is likely to cause a severe disorder phenotype if the protein is affected by mutation.

**Mutations of MECP2 leading to RTT**

At the moment, several hundred different mutations have been reported leading to RTT by loss or impaired function of MECP2 protein due to truncation, abnormal folding, or binding instability [25] (see also MECP2 varieties on LOVD database [71]). This contributes to the variety of RTT phenotype and symptom severity.

The effects of total absence of MECP2 protein were investigated in several model systems. Deletion of MECP2 from glial cells had only mild phenotypic consequences [72]. In a mouse model specific deletion of MECP2 in the forebrain caused behavioral abnormalities, limb clasping, impaired motor coordination, anxiety, and abnormal social behavior but not locomotor activity or changes in fear conditioning [73]. In another mouse model, silencing MECP2 in GABAergic neurons led to severe RTT like phenotype [74].

Sixty-seven percent of all MECP2 mutations found in humans are in eight hot spots: R106 (corresponding RS number from dbSNP: rs28934907), R133 (rs28934904), T158 (rs28934906), R168 (rs61748427), R255 (rs61749721), R270 (rs61750240), R294 (rs61751362) and R306 (rs28935468). Most of the mutations which cause RTT occur in the MBD region of MECP2 [70] (Fig. 1). A 100-fold reduction of binding affinity of MECP2 to methylated DNA is documented for the mutations R106W (rs28934907), R133C (rs28934904), and F155S (rs28934905) and binding affinity reduction of about 2-fold was found in mutation T158M (rs28934906) [45].

**The most important metabolites, genes and pathways affected by MECP2 in RTT**

The examination and investigation of RTT females (and model systems) revealed that an impaired MECP2 influences biological pathways on many levels. Several genes have been found to be increased or decreased in expression, levels of various metabolites are changed and several biological pathways were found to be typically affected although the molecular mechanisms are not yet clear. In this chapter the main metabolites, genes and pathways which are influenced or changed by RTT are summarized and as far as known integrated in the MECP2 mechanistic pathway (Fig. 2). For getting these results, samples from human RTT females were often used but many results come from studies with Mecp2$^{-/-}$ mice (e.g. the Bird model [75]). These mice do not express Mecp2 at all and they display the same symptoms as humans, such as normal development until about 6 weeks, regression, reduced movement, clumsy gait, irregular breathing, and the mice have a reduced life span of about 3 months. Postmortem analysis revealed reduced brain and neuronal cell size which is similar to observations in humans. Other mouse strains or in vitro models with mutated MECP2, reduced or overexpressed MECP2 levels are also commonly used to study RTT.
Recently, researchers started to use iPSCs (induced pluripotent stem cells) from human or murine origin [76].

Metabolites

Early autopsies revealed reduced levels of catecholamines, namely dopamine, serotonin and norepinephrine while markers for bioaminergic metabolism in general were higher [77, 78] (summary of metabolites in Table 2 and see also the list of metabolites on WikiPathways [18] (pathway ID 3584). This was confirmed by a study of Panayotis et al. [79] who revealed time-dependent levels of dopamine, norepinephrine, serotonin, and their catabolites in the brain tissues of MeCP2-/y mice. Viemari [80] et al. showed that MeCP2-/y mice have a deficiency in norepinephrine and serotonin content in the medulla and a drastic reduction of medullary TH (catecholamine producing) neurons indicating that dysfunctional neuronal development may be the reason for decreased catecholamine levels. The phosphorylation of MECP2 is triggered by neuronal activity which is caused by release of neurotransmitters (Fig. 2). Hutchinson et al. [81] demonstrated that phosphorylation of MECP2 is dependent on dopamine, serotonin and norepinephrine-activated pathways (Fig. 2, MECP2 phosphorylation).

Another metabolite shown to be present in high levels in RTT females is glutamate. Increased glutamate production (or decreased glutamate consumption) may lead to over excitation of glutamaticer neurons and can trigger increased uptake and conversion of glutamate to glutamine. Over excitation feedback may cause the downregulation of the glutamate receptor which was observed before [82]. Moreover, severe downregulation in gene expression for the glutamate D1 receptor GRID1 (GluD1) was found (Fig. 2). This receptor links in gene expression for the glutamate D1 receptor observed before [82]. Moreover, severe downregulation of the glutamate receptor which was found (Fig. 2). This receptor links and see also the list of metabolites on WikiPathways [18]. BDNF, which shows downregulation of the glutamate receptor GRID1 [83] are downregulated (Table 3 and see also the list on pathway 3584 [18]). BDNF, which shows in all investigated models the most consistent effect, is a MECP2 regulated protein and it is necessary for neuronal development and function, too (Table 3). The BDNF molecular pathway and its influence on neuronal development and function is currently the best investigated: For GAMT it is known that this gene is involved in creatine metabolic and biosynthetic process which was found to be dysregulated in some (not all) Rett females and may cause or consequence to breathing problems [4]. DLX5 and DLX6 are homeobox genes and involved in regulation of gene expression in general. DLX5 is especially responsible for ectodermal differentiation processes. The activity of both is highly regulated by methylation and for the maintenance of this methylation pattern MECP2 is required: binding sites for MECP2 have been found in both promoters. There is an ongoing discussion about DLX5 and DLX6 expression being influenced by MECP2 deficiency [87], the final argument seems that it is at least true for DLX5 [88]. FKBP5 and SGK1 were identified as potential MECP2 targets because they are involved in regulation of

Table 2 Metabolites in RTT

| Changes in RTT | Metabolites                                      |
|---------------|--------------------------------------------------|
| Increased levels [77–79] | Catabolites of catecholamine metabolism, glutamate |
| Decreased levels [77–80] | Dopamine, norepinephrine (noradrenaline), serotonin, melatonin, myo-inositol, phospholipids, GABA |

Table 3 Genes, which are up- or downregulated in human or model system without functional MECP2

| Changes in RTT | Genes                                      |
|---------------|--------------------------------------------|
| Upregulated [25, 89, 90] | BDNF, FKBP5, IG2, DLX5, DLX6, SGK1, MPP1, GAMT, FXYD1 |
| Downregulated [25, 83] | UBE3A, GRID1 |

A metabolomics investigation found changed phospholipid profiles in MeCP2-/-mice [82]. Phospholipid metabolism is directly associated with cell growth since it provides membrane material for inner and outer hull structure. Still, it remains unclear whether this is the cause or just one of the consequences of reduced neuronal cell size and network connectivity of RTT. But as MECP2 basic function is global transcription dampener in combination with activity dependent activation of transcription this suggests that reduced membrane material production is a consequence of lack of activity specific transcription activation.

Genes/gene products

MECP2 is a global transcription and translation influencing factor but there are also single specific genes which are found to be up- or downregulated in the absence of functional MECP2. The expression of the MECP2 target genes is affected in human, mouse and in vitro models. In the absence of functional MECP2 the expression of BDNF, GAMT, DLX5, DLX6, FKBP5, SGK1, FXYD1 and MPP1 is upregulated whereas the expression of UBE3A [25] and GRID1 [83] are downregulated (Table 3 and see also the list on pathway 3584 [18]). BDNF, which shows in all investigated models the most consistent effect, is a MECP2 regulated protein and it is necessary for neuronal development and function, too (Table 3). The BDNF molecular pathway and its influence on neuronal development and function is currently the best investigated: For GAMT it is known that this gene is involved in creatine metabolic and biosynthetic process which was found to be dysregulated in some (not all) Rett females and may cause or consequence to breathing problems [4]. DLX5 and DLX6 are homeobox genes and involved in regulation of gene expression in general. DLX5 is especially responsible for ectodermal differentiation processes. The activity of both is highly regulated by methylation and for the maintenance of this methylation pattern MECP2 is required: binding sites for MECP2 have been found in both promoters. There is an ongoing discussion about DLX5 and DLX6 expression being influenced by MECP2 deficiency [87], the final argument seems that it is at least true for DLX5 [88]. FKBP5 and SGK1 were identified as potential MECP2 targets because they are involved in regulation of
glucocorticoid responding gene regulation (stress response). Mecp2-0 mice showed anxiety behavior, had elevated levels of those transcripts but normal levels of glucocorticoids [89]. FXYD1 is a transmembrane modulator of Na+/K+ -ATPase activity involved in neurite outgrowth. It was found to be overexpressed in a Mecp2-0 mouse model inhibiting neuronal growth [90] and reduced arborization is generally found in RTT. MPP1 (signal transduction, neuronal homeostasis) and IGF2 (cell proliferation) were identified in a transcriptomics expression study to be strongly overexpressed in a study with human lymphoblastoid cells of Rett patients and controls [91]. MECP2 interacts with E6AP, the protein of the UBE3A gene and they are regulating the expression of several target genes [92]. GRID1 encodes for glutamate D1 receptor (GluD1) and is downregulated in the absence of functional MECP2 [83]. Glutamate receptors play a major role in neuronal signal transduction.

Pathways

Bedogni et al. mentioned the difficulty to identify unique target pathways of MECP2 because MECP2 is both a repressor and an activator of transcription and the balancing and timing of transcription levels seems to contribute more to disorder development than activation of single pathways [70]. Several transcriptomics studies using samples from RTT females, mouse and in vitro model systems showed about 60 significantly enriched pathways, for example inflammation, MAPK signaling, ERBB signaling, neurotropin intracellular cascade, sterol biosynthesis [93], cholesterol metabolism [93], cytoskeleton formation, and apoptosis [70].

While animal and in vitro models often use MECP2-KO models (e.g. Mecp2-/-/ mouse) human RTT patient derived samples often have a residual MECP2 activity due to the various mutations which impair the function rather than inhibiting the expression completely. Tanaka et al. [76] investigated gene expression profiles of iPSC lines derived from six different RTT females with different MECP2 mutations. They created and compared expression profiles of two iPSCs cell lines derived from each of the patients, one with the X chromosome active that has the wild type MECP2 and the other one with the chromosome with the mutated gene. The differently expressed genes and altered pathways are very different for each patient. This is an interesting result, as there are so many different MECP2 mutations which lead to RTT. A specific mutation affects protein function differently and may trigger different pathways leading to different phenotypes. Linking genetic data to molecular analysis (transcriptome, metabolome) and phenotype will be a future challenge to elucidate the pathways of RTT.

MECP2-related pathways involved in RTT phenotypes

In this chapter we discuss how mutated MECP2 leads to failure in neuronal synapsis formation and function which are one of the major causes of RTT phenotype. MECP2 acts in a biological (molecular) network of constant interaction by regulating and being regulated. This complex molecular network interaction leads to effects in cellular morphology (e.g. arborization), synapsis function and neuronal network growth, development, and maintenance.

According to Lyst and Bird [9] MECP2 mutations cause RTT by disrupting two major functions: 1. co-repressor recruitment, and 2. chromatin compaction, which are both basic molecular functions. Skene and Bedogni specified this assumption of MECP2 function as a global dampener of transcription in neurons plus the activity dependent transcription activation which leads to proper synapsis function and development [43, 70].

The differences in global gene expression of RTT and wild-type control groups are not substantial – neither in fold change nor number of genes differently expressed - indicating that more subtle dysregulation events in several pathways are responsible for RTT [62, 94–97]. MECP2 is a global repressor of transcription [43], a global activator of gene translation [61] and it reacts on neuronal pathway signals which lead to phosphorylation of MECP2 and detachment from DNA. Therefore, MECP2 dampens neuronal transcription globally and allows activity related responses which seem to be necessary for learning activity and specific synapsis formation [98]. Bedogni et al. found a direct connection between MECP2 and molecular pathways leading to cytoskeleton re-formation [70] indicating structural changes leading towards neuronal network formation. BDNF and FXYD1 seem here to be the link between MECP2 and the cellular phenotype [90]. Li et al. found in human embryonic stem cells, which are developing from stem cells to neuronal precursor cells to neurons, that in a premature state, MECP2 acts as an activator of transcription while transcription repressor activity was only found in mature neurons [61]. The levels of MECP2 start to increase postnatally and the protein is quite abundant in mature nervous systems [43, 99]. The expression of MECP2 is not uniform in different neuronal cell populations [100], parts of the brain and changes with age [101]. Mouse Mecp2-KO neuronal precursor cells are not different from wildtype ones in respect to expression patterns, proliferation, and differentiation (morphology), they change only during maturation [99]. Together with the observation that symptoms of RTT do not appear before about month 6, this led to the assumption that MECP2 has less to do with neurogenesis but more with neuronal function and maintenance, and synapsis formation and
function. This may explain why the RTT phenotype becomes visible only at the quite late age of 6–18 months. In RTT females brains a decreased number of synapsis was found [102–104]. Synaptogenesis again is mostly observed in the period of RTT symptom development (month 6 – 18) which may explain the development of learning disability. In MECP2 null mouse model reduced neuronal differentiation [105] and synaptic deficits [98] were observed. Mice studies and postmortem brains of RTT females reveal alterations in neuron structures which may be due to decreased dendritic complexity because of an immature synaptic spine morphology leading to malfunction of synaptic development and plasticity [106–108]. Changed neuronal tubulin expression was found directly in the brain tissue of RTT and Angelman syndrome patients [109]. Dysfunctional MECP2 led also to changes in synaptic transmission, short and long-term synaptic plasticity, deficits in short and long term potentiation (LTP and LTD) in mice [110].

The abnormal levels of neurotransmitters and differently expressed neurotransmitter receptors lead to an imbalance between excitatory and inhibitory neuronal activity (namely imbalance of GABAergic, glutamatergic and dopaminergic neuronal pathways). Such abnormal ratio of excitation/inhibition in brain activity was also found in autistic patients before [111–113] and it is a known effect in Parkinson’s disease which shares the motoric disabilities with RTT [114]. The gene in the neurotransmitter pathway which is known to be down-regulated in the absence of functional MECP2 is GRID1 which could here be the link between mutation and phenotype [83]. RTT models using murine and human induced pluripotent stem cells showed some RTT features and these symptoms were documented for MECP2 overexpression models, too, leading again to the assumption that MECP2 function is dose dependent [26].

In summary, MECP2 affects epigenetic regulation of gene expression, which changes neurobiological activity, network formation and function which causes the major phenotype.

**Current gaps in understanding RTT pathways**

Although these processes could indeed explain many neuronal function related symptoms of RTT there is still lack of evidence for other phenotypes, especially those which occur in many but not in all RTT females. 1) Breathing patterns: breathing is regulated by brain stem function which gets its signals from receptors for blood pH, carbon dioxide and (to a lower amount) oxygen levels. Abnormal RTT breathing patterns could be

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**Table 4** Olfactory vs. visual system: tissue specific MECP2 influence

| Olfactory epithelium | Visual system |
|----------------------|--------------|
| Observation: A study on olfactory bulb biopsies of RTT females revealed less olfactory receptors indicating less sensitive olfactory sense [119]. The olfactory epithelium in postnatal rodents experiences strong upregulation of MECP2 [120, 121]. | Observation: Vertebrate eyes are originally specialized brain tissue. Though being expected to be subjected to MECP2 dysfunctionality symptoms the visual system, retina, visual nerve and visual cortex seem to be less affected by RTT. Patients are able to focus, blink, eye-track, and do not perform worse in visual tests than healthy population [122]. Their families report often that they are using eye contact as communication method and therefore eye tracking systems are a promising method to improve communication. |
| Molecular/histological data: MECP2 deficiency induces also an imbalance in glutamatergic/GABAergic innervation in the olfactory bulb. The excitation in MECP2 KO mice is reduced and there is generally an imbalance between excitatory and inhibitory pathways observed leading to premature death of olfactory neurons in RTT mice models [121]. MECP2 seems to regulate the activity dependent transcriptional responses in olfactory sensory neurons the same way as in central nervous system and model systems [123]. This cycle of neuronal activity dependent transcription activation (fast feedback loop based on Ca**2+/-calmodulin) seems to be responsible for neuronal circuitry refinement; playing a role in olfactory sensory nerve maturation and olfactory learning. MECP2 affects the expression of olfactory sensory cell adhesion molecules KIRREL2 and PCHD20 directly, KIRREL3, and CNTN4 indirectly. It represses KIRREL2 but is required for activity dependent upregulation of KIRREL2 after odor stimulation [123]. KIRREL3 is an autism related gene [124] and the family of KIRREL genes is known to be widely expressed in neuronal tissue for synaptogenesis and synaptic specificity [125]. | Molecular/histological data: Jain et al. investigated ocular MECP2 expression in post mortem brains of RTT females and compared it to healthy controls. Although the RTT females show the typical severe neurological deficits their visual functions are well preserved. There were no gross or microscopic aberrations detected and no significant MECP2 level differences [101]. Another study investigating MECP2 expression levels in many neuronal and non-neuronal tissues found MECP2 to be expressed weak or moderate in the nucleoplasm of retinal cells while there were peaks of strong MECP2 presence in chromocenters [126]. Although it was shown in a previous study with MECP2 KO mice that their visual system (acuity) is affected with disorder onset [127] and visual systems also need refinement by circuits and MECP2 dependent synapse remodeling [128] this was not confirmed by the study of Song et al. [126]. Their retina samples from MECP2 deficient mice did not show any differences to control concerning immunochemical markers, cellular and histological anatomy, synapsis formation and neurotransmitters [126]. |
| Conclusion: Data indicates that the olfactory sense is less functional in Rett females due to the strong dependency of the molecular signal processing pathways on MECP2. | Conclusion: Together with the measured visual performance in human RTT females [122] these observations indicate that MECP2 surprisingly does not play a major role in ocular function. |
| Gap: Why is the olfactory sensory system affected in RTT females? Is there a measurable difference in response to olfactory stimulants of Rett females and controls? | Gap: What is the mechanistic explanation of the rather unaffected visual system? Why do neuronal cells of the visual system not need MECP2 for proper function? |
caused by neuronal dysfunction of the brain stem or neuronal pathways but metabolic dysregulation involving abnormal creatine levels due to GAMT over/under expression is also an influencing factor [4]. GAMT is one of the MECP2 downstream activated genes. 2) Cardiac abnormalities: Heart beat is generally regulated by central nervous system but has its own nerve knots for signal production, too. Specialized neurons in the heart ensure proper electric signal transition. These might be affected by RTT directly, by brain stem function, or both. RTT patients are indeed known for higher incidence of sudden death and heart problems like tachy-, brady- and arrhythmia were observed before (. Furthermore, vascular dysfunctions have been found which are directly related to MECP2 dysfunction although the molecular pathway between MECP2 and effector genes is not yet elucidated [115]. 3) Digestion and nutrient uptake problems: Stomach and intestines are covered with a complex network of nerve cells. Dysfunctional nerve cells may lead to constipation and malabsorption of nutrients, e.g. Vitamin D but there may also be other nutrient processing pathways involved. 4) Tissue specific effects: Tissue specific neuronal cells are differently influenced by MECP2 mutation, e.g. olfactory sensory vs. visual system (Table 4). Recently it was found that MECP2 mutations contribute to hypersensitivity of mechanoreceptors [116] which aligns with the observation of clinicians and caregivers. It is currently unknown in which neuronal cell subpopulations MECP2 is more or less necessary for normal function although there are indications that there are differences [100].

To understand these processes integration of different levels of biological knowledge and research results is necessary, and interactive biological pathways help to organize, analyze, and visualize existing knowledge. To integrate the information the exact mutation of MECP2 gene needs to be combined with molecular data (e.g. gene expression data, metabolomics) and a detailed description of the RTT females’ phenotype (including clinical laboratory measurements). This process will increase the understanding of the underlying pathways of the variety of RTT phenotypes. Gathering this knowledge and bringing it properly together needs collaboration of biomedical and bioinformatics researchers, physicians and patients [117]. Furthermore, knowing the essential pathways and their components which contribute to a certain phenotype or symptom may lead to the discovery of drug targets. These are not likely to be able to cure RTT itself but may help to reduce the severity of specific symptoms.

Conclusions
The present review summarizes the current knowledge about MECP2 structure and function, how it influences levels of metabolites, gene expression and biological pathways, and tries to bridge the different types of data available to explain the development of typical RTT phenotype by visualizing in form of a pathway (Fig. 2). Although the dysregulation events in neuronal cells can be already be explained quite well, the mechanistic explanation of several additional symptoms is still missing. Integrating the knowledge about the individual mutation, molecular data and phenotype information will help to find biological pathways and therefore explanations for these symptoms. Finding the right target genes, proteins, or metabolites can build a bridge between genotype and phenotype, and possibly to drug targets and pathways like this will be a great help in visualization and analyses.

Abbreviations
- Genes: Transcripts and proteins are abbreviated according to the human genome name consortium.; RTT: Rett syndrome; 5MeCyt: 5-methyl cytosine; 5OHMeCyt: 5-hydroxy methyl cytosine; iPSCs: Induced pluripotent stem cells.
Developmental delay in Rett syndrome: data from the natural history study

Abstract

Background: Early development appears normal in Rett syndrome (OMIM #312750) and may be more apparent than real. A major purpose of the Rett Syndrome (RTT) Natural History Study (NHS) was to examine achievement of developmental skills or abilities in classic and atypical RTT and assess phenotype-genotype relations in classic RTT.

Methods: Developmental skills in four realms, gross and fine motor, and receptive and expressive communication from initial enrollment and longitudinal assessments for up to 7 years, were assessed from 542 females meeting criteria for classic RTT and 96 females with atypical RTT divided into two groups: 50 with better and 46 with poorer functional scores. Data were analyzed for age at acquisition and loss of developmental features and for phenotype-genotype effects. Acquired, lost, and retained skills were compared between classic RTT and atypical RTT with better or poorer functional scores using Fisher's Exact test. To examine if the mean total score from the Motor Behavioral Assessment during follow-up differed for acquiring a skill, we used a generalized estimating equation assuming compound symmetry correlation structure within a subject. A general linear model was used to examine whether the mean age of acquisition or loss of a developmental skill differed by mutation type. P values <0.05 were considered significant and were two-sided without adjustment for multiple testing. Statistical analyses utilized SAS 9.3 (SAS Institute, Cary, NC, USA).

Results: Early developmental skills or abilities were often acquired albeit later than normal. More complex motor and communication acquisitions were delayed or absent. Clinical severity was less in those achieving the respective skill. Individuals with R133C, R294X, and R306C point mutations and 3′ truncations tended to have better developmental outcomes.

Conclusions: Early developmental skills were acquired by many, but clear differences from normal emerged, particularly in skills expected after age 6 months. When comparing clinical severity, greater acquisition of specific skills was associated with specific mutations, confirming the impression that these mutations confer milder developmental abnormalities. These data may serve for planning and interpretation of early intervention studies in RTT.

Trial registration: This NHS study, clinicaltrials.gov (NCT00296764), represents the largest group of RTT participants assessed repeatedly by direct examination.

Background

Rett syndrome (RTT), OMIM #312750, a neurodevelopmental disorder predominantly affecting females, has been characterized by ‘apparently’ normal initial development followed by frank regression of fine motor and communication skills typically between 6 and 18 months of age [1-3]. Despite absence of prospective evidence of delayed early development, retrospective review has suggested that abnormalities are evident within the first 6 months [4-7]. Infants have often been described as being hypotonic and occasionally being excessively irritable or having postural stiffness often belying the underlying hypotonia. Assessments of development have been hampered by relatively small numbers of participants, possibly allowing phenotypic variability to skew assessments, involved data derived from questionnaires without direct
assessment of the participants by clinicians experienced in the diagnosis of RTT, or focused on specific skills or time periods rather than acquisition of developmental skills longitudinally [4-10]. Videotaped assessments have provided important retrospective observations with regard to specific early developmental skills. These prior studies indicate that the early period of development in RTT could be regarded as abnormal [6,7,10] and evidence of abnormal deceleration in head growth occurring as early as age 1.5 months based on recent data from the NICHD-sponsored Rare Disease Natural History Study (NHS) provides neuroanatomical support [11].

Information obtained over the past 7 years through the NHS has yielded extensive longitudinal data on a large cohort of individuals with classic and atypical RTT, providing definitive evidence for developmental patterns regarding the achievement of specific milestones that deviate from normal. Here, we capture and compare the acquisition of specific skills or abilities and whether these fall within the limits for achieving accepted milestones for the respective skills or abilities. Further, we extend the relationship between these developmental trajectories and specific MECP2 mutations and compare these trajectories in participants with classic and atypical RTT.

Methods
Data from initial enrollment in the NHS were assessed from 542 females who met criteria for classic RTT and 96 females who met criteria for atypical RTT and were enrolled into the study at an initial age under 10 years old. Although our overall cohort of individuals with RTT exceeded 900, we chose to restrict the analysis to subjects seen initially before 10 years of age in order to increase accuracy of parental recall for the acquisition of developmental skills or abilities (in the following sections, we will use the term ‘skills’ for both). Age at enrollment for this group ranged from 7 months to 10 years of age; median age was 4 for classic RTT, 4.5 for higher function atypical RTT, 3.5 for lower function atypical RTT. As individuals with atypical RTT have a bimodal distribution, those less severely affected and those more severely affected, this group was divided into 50 having better functional scores (clinical severity score ≤20) and 46 having poorer functional scores (clinical severity score >20). Criteria for enrollment were based on clinical assessment by experienced clinicians, requiring each participant (1) to fulfill consensus criteria for RTT [12,13] and (2) to have genetic testing for the responsible gene, MECP2 (Methyl-CpG-binding protein 2), although identification of a MECP2 mutation was not essential for inclusion. Nevertheless, 530 (97.8%) of 542 classic RTT participants and 83 (86.5%) of 96 atypical RTT participants had a MECP2 mutation.

Developmental achievement of specific skills was obtained in four categories through a detailed, direct interview with the parents or responsible caregivers based on specific recall aided by baby books and pictures, association with key events or time points such as birthdays, holidays, or other celebrations, and the review of prior medical evaluations by primary care physicians and any subspecialists: gross motor, fine motor, receptive communication, and expressive communication. In regard to developmental categories, parents were asked to provide information in three phases: the specific age at which the skill was acquired; whether and at what age it was lost; and whether and at what age it was regained. These participants were evaluated every 6 months if less than 6 years of age and annually thereafter. For the majority of this cohort, 337 (62%) of participants with classic RTT and 66 (69%) with atypical RTT were below age 6 at the time of enrollment. Developmental data were reviewed for each participant at semi-annual if less than age 6 or annual visits.

The present report is restricted to acquisition, loss, and overall retention of skill. Retained skills were determined as follows: Acquired skill – Loss of skill + Regained skill/Group total = % Retained skill. For classic RTT, specific ages could be assigned to each skill in >95% and for atypical RTT, in greater than 97%. The remaining data could not be recalled. These were updated, particularly for children <3 years of age, at periodic exams conducted semi-annually for the first 5 years of age and annually thereafter. Clinical severity scores (CSS) [14] and motor behavioral assessments (MBA) [15] were acquired at each visit and results compared with each developmental achievement. The CSS assessed the ordinal scores in 13 domains (age at regression, age at stereotypy onset, degree of deceleration of head growth, growth (BMI) status, sitting, walking, hand function, scoliosis, vocalization/verbalization, eye contact, periodic breathing, hand/foot skin temperature, and seizures). Total score range was 0 to 58 with higher scores representing greater clinical severity. The MBA examined 37 ordinal scores in three domains: behavioral-social (16 items), orofacial/respiratory (7 items), and motor/physical (14 items). Total score range was 0 to 148, with higher scores representing greater severity.

Statistical analysis
The study group consisted of 542 classic RTT and 96 atypical RTT. The atypical RTT group was further subdivided as noted above. Whether or not the proportion of acquiring, losing, or retaining a developmental skill was different among groups is summarized in Tables 1 and 2. The proportion of lost skills was Number lost/Number who acquired; the proportion of overall retention of skills was the Number retained/The group total. Skills for acquired, lost, and retained were compared between
Table 1 Acquired developmental milestones: gross and fine motor

| Skill                | Classic (N = 542, 5 without answers) | Atypical better (N = 50) | Atypical poorer (N = 46, 1 without answers) |
|----------------------|--------------------------------------|--------------------------|---------------------------------------------|
|                      | Acquired    | Lost | Retained | Acquired      | Lost | Retained | Acquired      | Lost | Retained |
| Rolling              | 95 (511)    | 23 (118) | 77 (414) | 96 (48)      | 6.3 (3) | *          | 96 (48)       | 87 (39)       | 28 (11)       | 62 (28)*       |
| Sit with support     | 97 (520)    | 12 (62) | 88 (473) | 100 (50)     | 2.0 (1)  | *          | 100 (50)      | 93 (42)       | 19 (8)        | 84 (38)        |
| Sit alone            | 80 (427)    | 16 (70) | 69 (369) | 94 (47)      | 0 (0)    | *          | 94 (47)       | 31 (14)       | 21 (3)        | 24 (11)        |
| Crawl                | 69 (370)    | 38 (139) | 45 (244) | 88 (43)      | 7.0 (3)  | *          | 82 (41)       | 22 (10)       | 40 (4)        | 13 (6)         |
| Pull to stand        | 62 (331)    | 34 (112) | 43 (231) | 92 (46)      | 4.3 (2)  | *          | 88 (44)       | 16 (7)        | 43 (3)        | 8.9 (4)       |
| Walk with help       | 79 (422)    | 13 (54) | 71 (383) | 94 (47)      | 4.3 (2)  | *          | 92 (46)       | 31 (14)       | 14 (2)        | 27 (12)        |
| Walk alone           | 53 (284)    | 14 (41) | 47 (253) | 78 (39)      | 13 (5)   | *          | 70 (35)       | 6.7 (3)       | 67 (2)        | 2.2 (1)       |
| Climb steps          | 20 (106)    | 26 (27) | 15 (81)  | 62 (31)      | 23 (7)   | *          | 52 (26)       | 0 (0)         | 0 (0)         | 0 (0)         |
| Ride tricycle        | 4 (22)      | 32 (7)  | 3.2 (17) | 16 (8)       | 0 (0)    | *          | 16 (8)        | 2.2 (1)       | 0 (0)         | 2.2 (1)       |

* (N = number): p values for Classic vs. Atypical better and Classic vs. Atypical poorer are indicated for acquired, lost, and retained skills ●<0.05; *<0.01; **<0.001.

Human studies approval
Each site obtained and maintained IRB approval for the performance of this study. Parental approval for study conduct and publication of results was obtained before entry into the study. The study has been registered with ClinicalTrials.gov: NCT00299312 since 3 March 2006.

Results
Data for acquisition of developmental skills were derived from 542 participants with classic RTT and 96 with atypical RTT, all less than 10 years of age at the initial assessment. The atypical RTT group displayed a bimodal distribution of clinical severity resulting in two groups representing better (50) or poorer (46) CSS. This atypical RTT grouping resulted in two distinct patterns of involvement and specific MECP2 mutations (Table 5). The CSS value for classic RTT had a mean of 21.8 (range 7 to 43; SD 6.81); for atypical better, the CSS value was 12.0 (range 3 to 20; SD 6.81); and for atypical poorer, the CSS value was 27.6 (range 21 to 39; SD 4.15). Mutations in the better functioning group were concentrated among three point mutations (R133C, R294X, and R306C) and 3’ truncations, representing 58% (29/50). Conversely, 15.2% (7/46) of individuals in the poorer functioning group had one of these mutations. The poorer functioning group had more than twice as many lacking a MECP2 mutation. In both instances, this distribution differed from the classic RTT group (p = 0.0004) where the eight common point mutations comprise 59% and 3’ truncations, large deletions, and other insertions/deletions represent 25% of the total.
Table 2 Acquired developmental milestones: expressive and receptive language

| Skill | Classic (N = 542, 5 without answers) | Atypical better (N = 50) | Atypical poorer (N = 46, 1 without answers) |
|-------|--------------------------------------|--------------------------|---------------------------------------------|
|       | Acquired | Lost | Retained | Acquired | Lost | Retained | Acquired | Lost | Retained |
| Fix and follow | 94 (503) | 28 (143) | 86 (464) | 94 (47) | 8.5 (4)* | 94 (47) | 89 (40) | 7.6 (3)* | 85 (39) |
| Quiet to voice | 85 (457) | 26 (118) | 77 (416) | 84 (42) | 7.1 (3)* | 84 (42) | 71 (32)* | 0 (0) | 71 (32) |
| Inhibit to no | 66 (357) | 25 (88) | 56 (303) | 76 (38) | 7.9 (3) | 72 (36)* | 47 (21)* | 10 (2) | 42 (19) |
| Follow command with gesture | 55 (295) | 36 (107) | 42 (225) | 74 (37)* | 16 (6) | 68 (34) | 29 (13) | 7.7 (1)* | 27 (12) |
| Follow command with no gesture | 45 (241) | 32 (76) | 36 (195) | 70 (35) | 20 (7) | 62 (31) | 24 (11)* | 9.1 (1) | 22 (10) |

Expressive communication

| Skill | Classic (N = 542, 5 without answers) | Atypical better (N = 50) | Atypical poorer (N = 46, 1 without answers) |
|-------|--------------------------------------|--------------------------|---------------------------------------------|
|       | Acquired | Lost | Retained | Acquired | Lost | Retained | Acquired | Lost | Retained |
| Social smile | 99 (535) | 16 (85) | 96 (518) | 100 (50) | 10 (5) | 100 (50) | 98 (44) | 14 (6) | 96 (43) |
| Coo | 93 (499) | 36 (178) | 67 (360) | 98 (49) | 14 (7)* | 86 (43) | 78 (35)* | 31 (11) | 56 (25) |
| Babble | 95 (511) | 51 (259) | 64 (346) | 98 (49) | 18 (9) | 90 (45) | 80 (36) | 33 (12) | 62 (28) |
| Single words | 77 (412) | 86 (354) | 21 (113) | 94 (47)* | 55 (26) | 68 (34) | 36 (16)* | 13 (2) | 36 (16)* |
| Phrases | 18 (98) | 76 (74) | 50 (28) | 54 (27) | 11 (1)* | 42 (21) | 6.7 (3) | 0 (0) | 6.7 (3) |
| Gestures | 53 (286) | 86 (246) | 10 (56) | 70 (35)* | 46 (16) | 44 (22) | 31 (14)* | 50 (7)* | 18 (8) |
| Points for wants | 23 (121) | 65 (79) | 9.5 (51) | 58 (29) | 26 (8) | 44 (22) | 4.4 (2)* | 100 (2) | 0 (0) |

% (N = number): p values for Classic vs. Atypical better and Classic vs. Atypical poorer are indicated for acquired, lost, and retained skills ●<0.05; *<0.01; **<0.001.

Nearly all participants in all diagnostic groups acquired early gross motor skills such as rolling and sitting with support (Table 1); however, other gross motor skills were less likely to be acquired. In classic RTT, the percentage of participants acquiring a particular skill decreased as the skill became more advanced. A minority of participants was able to achieve the most advanced skills. In classic RTT, the better functioning atypical groups had distinct patterns of gross motor skill acquisition compared with classic RTT. The better functioning atypical group did not show the decline in percentage of individuals gaining more advanced skills, with nearly 90% acquiring skills up to walking with help. In contrast, the poorer functioning atypical group showed an even sharper decline in percentage of participants gaining gross motor skills, with a minority gaining the ability to sit alone or other more advanced skills.

Among fine motor skills in the classic group, reaching for an object and finger feeding were most likely to be advanced skills.

Table 3 Motor-behavioral assessment and acquired developmental milestones: gross and fine motor

| Developmental skill | Parameter estimate ± Standard error, from the generalized estimating equation after adjusting for age at enrollment (Skill not gained vs. gained) |
|---------------------|-----------------------------------------------------------------------------------------------------------------------------------|
| Rolling             | 7.9 ± 2.6 (p = 0.003)                                                                                                           |
| Sit with support    | 14.8 ± 2.6 (p < 0.0001)                                                                                                          |
| Sit alone           | 8.7 ± 1.0 (p < 0.0001)                                                                                                           |
| Crawl               | 8.4 ± 0.9 (p < 0.0001)                                                                                                           |
| Pull to stand       | 9.3 ± 0.9 (p < 0.0001)                                                                                                           |
| Walk with help      | 11.0 ± 1.0 (p < 0.0001)                                                                                                          |
| Walk alone          | 10.9 ± 0.8 (p < 0.0001)                                                                                                          |
| Climb steps         | 11.2 ± 1.2 (p < 0.0001)                                                                                                          |
| Ride tricycle      | 10.6 ± 3.0 (p = 0.0004)                                                                                                          |
| Hold bottle         | 6.4 ± 1.1 (p < 0.0001)                                                                                                           |
| Reach               | 6.5 ± 2.7 (p = 0.015)                                                                                                            |
| Transfer            | 4.2 ± 1.1 (p < 0.0001)                                                                                                           |
| Pincer grasp        | 6.0 ± 1.0 (p < 0.0001)                                                                                                           |
| Finger feed         | 8.3 ± 1.4 (p < 0.0001)                                                                                                           |
74% to 78%. As with gross motor capabilities, participants with classic RTT were significantly better than the poorer functioning atypical group whereas the higher functioning atypical group did better than those with classic RTT.

Loss of acquired motor skills occurred in all groups. In classic RTT, fine motor skills were more likely to be lost than gross motor skills (Table 1). The better functioning atypical group showed a similar pattern of greater fine motor than gross motor loss; however, the percent losing

| Developmental skill                  | Parameter estimate ± Standard error, from the generalized estimating equation after adjusting for age at enrollment (Skill not gained vs. gained) |
|--------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------|
|                                      | Classic                                                                 | Atypical better                                                                 | Atypical poorer                                                                 |
| Fix and follow                       | −2.3 ± 2.2 (p = 0.287)                                                                                                          | 8.4 ± 3.0 (p = 0.005)                                                                 | 2.7 ± 6.4 (p = 0.679)                                                                 |
| Quiet to voice                       | 1.6 ± 1.5 (p = 0.269)                                                                                                           | 4.3 ± 3.2 (p = 0.184)                                                                 | 7.5 ± 3.3 (p = 0.025)                                                                 |
| Inhibit to no                        | 5.2 ± 1.0 (p < 0.0001)                                                                                                          | 1.6 ± 2.9 (p = 0.586)                                                                 | 5.1 ± 2.5 (p = 0.038)                                                                 |
| Follow command with gesture          | 4.1 ± 0.9 (p < 0.0001)                                                                                                          | 6.8 ± 2.6 (p = 0.010)                                                                 | 0.1 ± 3.2 (p = 0.972)                                                                 |
| Follow command with no gesture      | 3.5 ± 1.0 (p = 0.0002)                                                                                                          | 4.2 ± 2.6 (p = 0.102)                                                                 | −2.4 ± 3.0 (p = 0.430)                                                                 |
| Social smile                         | 10.0 ± 2.9 (p = 0.0007)                                                                                                          | N.A.                                                                                       | 1.7 ± 1.6 (p = 0.299)                                                                 |
| Coo                                  | 2.6 ± 1.8 (p = 0.152)                                                                                                           | 13.2 ± 2.4 (p < 0.0001)                                                                | −2.3 ± 2.8 (p = 0.423)                                                                 |
| Babble                               | 3.0 ± 2.2 (p = 0.165)                                                                                                           | −2.3 ± 2.8 (p = 0.410)                                                                 | 6.4 ± 3.9 (p = 0.097)                                                                 |
| Single words                         | 6.3 ± 1.1 (p < 0.0001)                                                                                                           | 7.0 ± 1.8 (p < 0.0001)                                                                | 3.0 ± 2.3 (p = 0.194)                                                                 |
| Phrases                              | 7.3 ± 1.2 (p < 0.0001)                                                                                                           | 2.9 ± 2.5 (p = 0.248)                                                                 | 11.8 ± 1.7 (p < 0.0001)                                                               |
| Gestures                             | 2.8 ± 0.9 (p = 0.003)                                                                                                           | 6.5 ± 2.4 (p = 0.007)                                                                 | 3.1 ± 2.3 (p = 0.191)                                                                 |
| Points for wants                     | 7.2 ± 1.1 (p < 0.0001)                                                                                                           | 8.1 ± 2.3 (p = 0.0004)                                                                | 4.4 ± 6.4 (p = 0.490)                                                                 |

Figure 1 Inverse Kaplan-Meier graphs were created for acquired developmental milestones. Expected upper limits of normal are represented by the vertical blue line [17]. These plots represent the early four skills, (A) sitting alone, (B) reaching, (C) fixing and following, and (D) social smile. The remaining acquired skills and the Kaplan-Meier graphs for lost skills are displayed as additional files. In each case, more than 95% (517/542) of classic RTT and more than 97% (93/96) of atypical RTT participants reported a specific age of acquisition for each skill.
either skill was far less than in classic RTT. This ultimately led to a greater percentage of those with better functioning atypical RTT demonstrating retained motor skills. The poorer functioning atypical group had similar loss of both gross and fine motor skills. In comparison to classic RTT, a similar percentage of this poor functioning group retained fine motor skills but fewer retained gross motor skills.

Early expressive communication skills (social smile, coo, babble) were attained by nearly all classic and better functioning atypical participants, but in a smaller percentage of poorer functioning atypical individuals (Table 2). Although a large percentage of classic RTT participants gained single words, only a minority developed phrases. In contrast, nearly all better functioning atypical participants gained single-word skills and over 50% developed phrases. The atypical poorer functioning group uniformly acquired fewer expressive communication skills than the classic RTT group. Babble was lost in a significant fraction of classic RTT, and more advanced expressive communication skills (single words, phrases) were lost in nearly all. Conversely, loss of expressive language skills was not as dramatic in atypical better functioning participants, a large fraction retaining these skills. As noted for motor skills, participants with classic RTT attained expressive communication skills intermediate between individuals with better and poorer functioning atypical groups (Table 2). In general, receptive communication skills were retained far better than expressive communication skills for all groups and better for the better atypical group than for classic RTT.

An important question is how the acquisition of development skills relates to overall functioning. To assess this, we determined the effect of not gaining a skill on the MBA change during follow-up using a generalized estimating equation adjusted for age at enrollment and diagnosis. Notably, not acquiring a motor skill at the time of enrollment resulted in worse overall functioning over time, as shown by an increase in the MBA score (Table 3). This effect was seen both at the baseline visit as well as subsequent visits. When the skill was not acquired, those with classic RTT tended also to have had worse MBA scores than individuals in the better functioning atypical group, but had generally better MBA scores than the poorer functioning group. When the MBA scores were adjusted for age at enrollment and diagnosis, whether or not a motor skill was acquired was highly significant for each motor skill at baseline and for subsequent visits for classic RTT and for most skills for atypical RTT (Table 3).

For receptive and expressive communication, the MBA scores revealed a quite similar pattern, namely individuals with classic RTT had worse MBA scores than the better functioning group but better MBA scores than the poorer functioning group. Acquisition of early communication skills such as social smile, cooing, fixing and following, and quieting to voice did not affect age adjusted MBA scores (Table 4). For more complex skills such as single words, gestures, or inhibiting to ‘No’ or following commands, MBA scores were significantly better in general for those acquiring the specific skill (Table 4).

In classic RTT, acquisition of developmental skills was achieved in many participants; however, the timing of

### Table 5 MECP2 Mutations in classic and atypical RTT groups

| Mutation | Classic Rett syndrome | Atypical Rett syndrome |
|----------|-----------------------|------------------------|
|          | Better function % (N) | Poorer function % (N)  |
| None     | 2.2 (12)              | 8.0 (4)                |
| R133C (397C > T) | 4.6 (25)            | 16.0 (8)              |
| R306C (916C > T) | 7.6 (41)              | 10.0 (5)               |
| R294X (880C > T) | 4.8 (26)              | 6.0 (3)                |
| R270X (808C > T) | 5.7 (31)              | 2.0 (1)                |
| R255X (763C > T) | 11.4 (62)             | 2.0 (1)                |
| R168X (502C > T) | 12.2 (66)             | 0 (0)                  |
| T158M (473C > T) | 9.7 (53)              | 2.0 (1)                |
| R106W (316C > T) | 3.0 (16)              | 0 (0)                  |
| 3’ Truncations | 8.7 (47)              | 26.0 (13)             |
| Large deletions | 8.8 (48)             | 4.0 (2)                |
| Other point mutations | 11.6 (63) | 20.0 (10) |
| Insertions/deletions | 7.8 (42)          | 2.0 (1)                |
| Exon 1   | 0.6 (3)               | 2.0 (1)                |
| Splice site | 1.3 (7)                | 0 (0)                  |
| Total    | 100 (542)             | 100 (50)               |

Note: MBA: Motor Behavior Rating Scale.
skill acquisition was delayed compared with skill acquisition in typically developing children (Figure 1, Additional file 2: Figure S1, and Additional file 3: Figure S2). Only a minority of participants achieved most skills at the expected time. Graphs depicting the temporal pattern of skill acquisition and the respective milestone revealed definitive patterns of abnormality. Figure 1a,b,c,d demonstrates that sitting, reaching for a toy, and fixing and following were acquired by the expected age in 30% to 35% while social smile was acquired in about 75%. Other features were acquired between 30% to 60% of normal (Additional file 2: Figure S1). Graphs depicting the loss of acquired skills in classic RTT also revealed variable patterns. Gross motor and receptive communication abilities were lost by fewer participants than fine motor and expressive communication (Additional file 3: Figure S2). These data provide additional evidence for subnormal acquisition of developmental skills and greater loss of acquired skills in specific realms.

Previous studies in classic RTT have shown that individuals with R133C, R294X, R306C, and 3’ truncations have less significant involvement than individuals with T158M, R168X, R255X, and R270X [14,16,18]. Examining these mutations individually did not demonstrate significant differences, perhaps because of too few participants. When mutations were compared by two groups, severe (R106W, T158M, R168X, R255X, R270X), and large deletions versus mild (R133C, R294X, R306C, and 3’ truncations, the severe group showed a smaller proportion in acquired skills only for following commands with a gesture and a tendency for transferring objects (Additional file 1). However, a greater proportion lost skills compared to the mild group. Among the lost features, differences (Additional file 1) were noted in gross motor skills (come to sit and walking with support), fine motor skills (hold bottle, transfer, pincer grasp, and finger feeding, receptive language (fix and follow), and expressive language (babble, single words, phrases, gestures, and point for wants).

**Discussion**

Early development in individuals with RTT has been regarded as ‘apparently’ normal although significant questions have been raised [4-10]. The present study involving 542 participants with classic RTT and 96 with atypical RTT demonstrated that early developmental skills are acquired in most but not all participants and clear differences emerged, particularly regarding whether specific skills were acquired within the expected norms. When comparing MBA, individuals who attained specific developmental skills tended to have lower (better) scores than those who did not. With regard to specific skills, gross motor skills were retained better than fine motor skills for the most part, and receptive communication better than expressive communication. When individuals with atypical RTT were separated into two groups in terms of neurologic function (i.e., high or better and low or poorer), and compared with classic RTT, a distinctive pattern appeared. Participants with classic RTT displayed an intermediate profile between the better and poorer functioning atypical groups with respect to acquisition and loss of skills.

Analysis of atypical participants led to the first formal identification of two subtypes of individuals (i.e., better and poorer functioning), beyond the well-defined entities such as preserved speech and early seizure variants. Comparison between better and poorer functioning atypical groups showed clear differences regarding distribution of mutation types. Those with better functioning atypical RTT such as those with preserved speech patterns tended to be clustered among three specific point mutations and 3’ truncations whereas those with poorer functioning atypical RTT such as those with very early onset developmental impairment were more broadly arrayed among mutation groups and clustered among common point mutations with poorer overall CSS scores. This distribution represented a dramatic difference from either those with classic or the better functioning atypical RTT.

Phenotype-genotype correlation studies have shown that individuals with specific MECP2 mutations tend to have overall better neurologic function and developmental skill profile than others. This study reinforced the concept that individuals with R133C, R294X, R306C, and 3’ truncations acquire more gross motor skills and lose fewer skills, particularly in fine motor and expressive language. Yet, we have learned from other studies [19,20] that these individuals may have greater behavioral issues in terms of anxiety, aggressiveness, and inappropriate activities. However, as other phenotype-genotype studies have shown, specific mutations may not be the only determinant of severity within specific individuals due to the existence of other factors such as X chromosome inactivation, genetic background (the interplay of other genetic variations), and distribution of the abnormal gene in specific brain regions [14,16,18,21].

The pattern of acquisition and loss of skills supports the notion that in RTT, developmental progression predicts overall level of function. This is also reflected in the developmental profile of specific MECP2 mutations. The abnormal acquisition of early skills, specifically those acquired before 6 months, is in line with the highly prevalent head deceleration that begins in early postnatal life [11]. Both features suggest a pathogenic process that affects developmental events taking place shortly after birth. Whether these events correspond to arrest or involution in synaptic development needs to be determined. The observation that two subtypes of atypical RTT exist based on skill profiles and overall severity is a significant result. Atypical RTT may not be a milder or more severe form of RTT reflecting a greater or lesser severity of MeCP2 deficit, but instead
reflects a qualitatively different pathogenic process. We hope that this study stimulates more investigations regarding the phenotypic spectrum of atypical RTT.

The present study was based on data from a white population to a significant extent. While every effort was expended to assess a truly representative dataset, this shortcoming must be kept in mind. Another limitation is that the developmental data were obtained retrospectively. The majority of the participants was less than age 6 at the time of enrollment, but data were provided by informants up to 10 years after birth for the remainder. Review of primary care records and association with key time points were utilized to improve data retrieval. Assessments of videos taken by parents during critical developmental periods may facilitate in demonstrating early motor and communication abnormalities in RTT [6-10,22]. This approach could be used in the future for validating developmental milestone data during parental interviews more systematically. The existing video reports related to early development are based on small numbers of participants that might not cover the complete spectrum of abilities. Therefore, a more comprehensive inclusion of videotaped assessments could be a valuable approach. However, as the diagnosis is often delayed by a number of years, prospective analysis of this type might be difficult to apply.

The data presented here do reflect both cross-sectional and longitudinal perspectives in RTT and should provide critical information for clinicians and therapists as well as for stratification in future clinical trials for this very challenging neurodevelopmental disorder.

Conclusions
Early developmental skills in RTT are acquired by many, but clear differences emerge in skills expected after 6 months of age. The early developmental skills develop nearly uniformly but often the age at acquisition is delayed beyond the normal period to achieve the accepted milestone limits. Confirmation and extension of phenotype-genotype relations is provided noting that R133C, R294X, R306C, and 3' truncations are generally associated with milder delays. This large cohort of participants assessed directly by experienced clinicians for up to seven years provides a complete sequence of developmental skill or ability acquisition, loss, and retention. Furthermore, the recognition of individuals with atypical RTT as representing a mixture of two groups with quite different patterns of skill acquisition and retention suggests a qualitatively different pathogenic process may exist.

Abbreviations
BMI: body mass index; CSS: clinical severity score; MECP2: Methyl-CpG-binding protein 2; MBA: motor behavioral assessment; NICHD: National Institute of Child Health and Human Development; NHS: Natural History Study; RTT: Rett syndrome.
Twenty years of surveillance in Rett syndrome: what does this tell us?

Abstract

Background: The clinical characteristics of children diagnosed with Rett syndrome are well described. Survival and how these characteristics persist or change in adulthood are less well documented. This study aimed to describe overall survival and adult health in those with Rett syndrome.

Methods: Using the Kaplan-Meier method, we estimated survival of individuals registered with the Australian Rett syndrome Database (ARSD) who had been followed for up to 20 years (n = 396). We then conducted logistic and linear regression analyses investigating epilepsy, musculoskeletal, gastrointestinal, autonomic dysfunction and behaviour of individuals aged 18 years and over using cross sectional cohorts from the ARSD (n = 150) and the international database InterRett (n = 273).

Results: The likelihood of survival was 77.6% at 20 years, 71.5% at 25 years and 59.8% at 37 years. The median age of the combined cross-sectional cohort was 25 years (range 18 to 54 years), the majority (71%) were living in their parental home and the remainder being cared for in group homes or other institutions. Just over half walked either independently (18%) or with assistance (43%). The majority (86%) had scoliosis with 40% of those having undergone corrective surgery. Almost two-thirds (64%) of the women were taking anti-epileptic medications at the time of data collection. Constipation was highly prevalent (83%) and many experienced bloating (53%). Biliary dyskinesia, inflammation or infection of the gallbladder was reported for 20 women (5%) and of those 13 had undergone gallbladder surgery. Sleep disturbance was relatively common (63%), and adverse mood events and anxiety were slightly more prevalent in those aged 26-30 years in comparison to the younger and older age groups. Other frequently reported medical conditions included urinary tract infections, pneumonia and other respiratory conditions.

Conclusions: Survival in Rett syndrome has now been estimated with the most accurate follow up to date. During adulthood, continuation of multidisciplinary services and programs is necessary to optimise health and wellbeing.

Keywords: 'Rett syndrome', Survival, Ageing, Longevity, Adulthood, Wellbeing, Women

Background

The neurological disorder Rett syndrome (OMIM 312750) was first described in the English literature in 1983 [1] and later found to be associated with mutations in the methyl CpG binding protein 2 gene (MECP2) [2]. Females are mostly affected with an incidence of diagnosis of 1:9000 by the age of 32 years [3]. Diagnosis depends on clinical presentation [4] with or without a pathogenic MECP2 mutation. Genetic testing occurs commonly in current diagnostic pathways but many older women may not have undergone genetic testing. The clinical characteristics of Rett syndrome first appear in early childhood. Gradual or sudden loss of speech and hand function, loss of acquired gross motor skills and the development of stereotypic hand movements mark a period of regression between the ages of 6 and 18 months. Gastrointestinal problems, respiratory dysfunction such as hyperventilation, breath holding and apnoea, sleep disturbance, spinal curvature and epilepsy are common comorbidities. Overall severity of symptoms is highly variable across individuals and studies have identified common mutations associated with either a milder phenotype (point mutations p.Arg133Cys, p.Arg294*, and p.Arg306Cys) or a more severe clinical presentation (point mutations p.Thr158Met, p.Arg168*,...
p.Arg255*, p.Arg270* and large deletions) [5,6]. Clinical presentation in adults with Rett syndrome is less well understood.

Emerging picture of health and wellbeing in adults with Rett syndrome
Thus far only two studies conducted in the Netherlands (2007: n = 53, 2012: n = 37) [7] and Italy (n = 84) [8] have specifically examined health in adults with Rett syndrome. Overall, general health was reported to be good. However epilepsy was found to be highly prevalent in both the Dutch (93%) and the Italian cohorts (82%), and while improvement in seizure activity with age was noted for some women [8], some remained difficult to treat and the majority required medications for control of seizures. A post-adolescence decline in gross motor skills has been reported in some [7,9] but not all women with Rett syndrome [7]. Gross motor capabilities are influenced by the type of mutation present in the MECP2 gene and are generally poorer in those requiring surgical correction for scoliosis [10]. Autonomic dysfunction which may manifest as hyperventilation or breath holding, or peripheral vaso-motor disturbances is a well-recognised but poorly understood feature of Rett syndrome. Abnormal or disturbed sleep patterns and behavioural issues also appeared to persist [7,8]. Similarly, gastrointestinal issues such as constipation, reflux, and feeding difficulties remained prevalent [7,8] and may contribute to poor growth [11]. However, there is a need for replication of these studies using larger sample sizes to improve our understanding of clinical presentations beyond the growth and development of childhood.

Survival
It is possible that those adults with Rett syndrome who survive into adulthood may represent a healthier group. The most recent Australian population-based estimate indicated that ~70% would be alive at 25 years with some evidence of improved survival over calendar time when comparing with the historical Austrian cohort [12]. A study using data on 1,907 cases ascertained from a combined US and Canadian sample (n = 2,994) showed a similar result at age 25 years [13]. A recent longitudinal study conducted in The Netherlands [7] reported the death of 7 of 53 women aged between 21 and 43 years over a five-year period. However, it is difficult to measure survival accurately using small samples that are not population-based with only short follow-up time.

Our aims were to update our previous estimate of survival with now 20 years of follow-up in the Australian Rett Syndrome Database (ARSD) and to describe health status in individuals aged 18 years or older sourced from both the ARSD and the InterRett database.

Methods
Data sources
Data for this study were harnessed from two data repositories: The ARSD and the international database InterRett. The ARSD is a population-based registry established in 1993 [14] representing individuals born since 1976. Questionnaires are administered on enrolment to both families and clinicians and subsequently, follow-up questionnaires are administered approximately biennially to families. The InterRett database was first established in 2002 and invites participation from both families and clinicians on a global level [15]. Families who register are administered a family questionnaire and one of the clinicians caring for their family member with Rett syndrome is also invited to complete a clinician questionnaire. Ethics approval for both studies was obtained from the Princess Margaret Hospital for Children, Western Australia.

Survival
Female cases with a confirmed diagnosis of Rett syndrome registered with the ARSD (n = 396) were included. Data on deceased status and cause of death (excluding cases that were subject to a coroner’s investigation) were obtained from the Australian Institute of Health and Welfare (AIHW) National Death Index (NDI) database and were valid as at 31st January 2014. Age at first contact with the ARSD was used as entry points and age at death or age at last contact date if alive was used as exit points. Time at risk was defined as the difference between the entry and exit points, reflecting the delayed entry nature of the study. As such, the interpretation of the survival time is that of survival to exit age conditional on survival up to the age at entry. The censor status of each case was set as 1 if the individual was deceased and 0 if the individual was known to be alive.

Health status
Females aged 18 years or older at their most recent data collection were sourced from both the ARSD (n = 150) and InterRett (n = 273) databases providing a total of 423 unique cases. Rett syndrome diagnosis was confirmed at ascertainment based on consensus diagnostic criteria [4,16,17] or on the presence of a pathogenic mutation in the MECP2 gene. Demographic information and variables relevant to health status that had been harmoniously ascertained across the two databases were selected for analysis. Clinical comorbidities included epilepsy, musculoskeletal aspects (mobility, scoliosis), gastrointestinal health (weight, constipation, reflux, bloating and gall bladder problems), autonomic dysfunction, sleep disturbance, and mood and anxiety. Where more detailed information on a particular health area was available in one data source but not the other, this information was analysed and reported for the
relevant subset of the cohort. Families had also been asked to describe any medical problems (current or present) other than those usually associated with Rett syndrome or that had required day admissions or surgery in hospital. This information was reviewed to determine whether or not any particular medical problems were more represented.

Ability to walk was categorised as one of the following: no assistance required, a little assistance (eg, one hand held), a moderate amount of assistance (eg, needing trunk support) or unable to walk. Epilepsy status was categorised according to frequency of seizures and the number of anti-epileptic medications. Active epilepsy represents those with seizure frequency of at least monthly at the time the questionnaire was completed, and drug resistant epilepsy was defined as having active epilepsy and taking two or more anti-epileptic drugs at the time of data collection [18].

Responses to the Rett Syndrome Behaviour Questionnaire (RSBQ) [19], which has been administered for the Australian cohort, were used to calculate scores for the mood and anxiety subscales. The mood subscale comprised eight questions describing behaviors such as screaming spells and irritability with 3 response options (0-2) giving a possible total score of 16. The anxiety subscale score comprised four questions describing behaviors such as panic spells and fear with 3 options (0-2) giving a possible total score of 8. Higher scores indicated more problematic behaviors.

Also for the Australian cohort, weight z-scores for age were calculated using the LMS method based on data from the 2000 US Centers for Disease Control and Prevention Growth [20]. The reference value of age 20 years was applied to any individual over this age.

Mutations were grouped as large deletions (LD), C-terminal deletions (CT), early truncating (ET) or one of the common eight mutations. All others were grouped as “other” mutations. Females who were mutation negative or with unknown mutation status were included in the “Negative/Unknown” (Neg/Unk) group. Age was grouped as 25 years and younger, between 26 and 30 years, and older than 30 years for health status comparisons.

Statistical analysis
The Kaplan-Meier method was used for estimating the conditional probability of survival. Cox proportional hazards models were fitted to investigate the influence of genotype on survival and logistic regression was used to estimate the associations of mutation type and age with the presence of comorbidity. The p.Arg133Cys group, a mutation associated with an overall milder phenotype [21], was used as a baseline. The effects of mutation type on continuous independent variables such as weight, RSBQ mood score and RSBQ anxiety score were assessed using simple linear regression. Predicted values of these explanatory variables were then estimated based on the recycled predictions approach using the Stata margins command.

All statistical analyses were conducted using Stata software version 13 [22].

Results
Survival
As in January 2014 the ARSD contained birth and death information for 396 females (median age 18.3, interquartile range (IQR) 12.1-25.4 years). Information on cause of death was available for 57 (82.6%) out of 69 females who were deceased. Lower respiratory tract infection (36.8% 21/57) was the most common cause of death, followed by aspiration/asphyxiation (31.6% 18/57), respiratory failure (14.0% 8/57) and seizure related illness (5.3% 3/57). The conditional probability of survival was 77.6% (95% confidence interval (CI) 71.3, 82.8) at 20 years, 71.5% (95% CI 64.6, 77.3) at 25 years and 59.8% (95% CI 49.3,68.8) at 37 years (Figure 1). There were 300 females in the ARSD with a pathogenic mutation in the MECP2 gene (Table 1) and 96 females with negative MECP2 testing or unknown genotype. Using the Cox proportional hazard model, those with large deletions had, at any time during the observation period, slightly more than 3 times (HR 3.11, 95% CI 0.60, 16.10) the risk of death than those with the p.Arg133Cys mutation. High mortality risk was also observed in those with p.Arg270* (HR 3.05, 95% CI 0.63, 14.73), p.Arg106Trp (HR 2.24, 95% CI 0.45, 11.11) and p.Arg306Cys mutations (HR 2.66, 95% CI 0.49, 14.53) (Figure 2). The risk was lowest for those with the p.Arg255* mutation (HR 0.52, 95% CI 0.05, 5.76).

Health status
The combined study population represented 24 countries with the greatest numbers residing in the USA (40.4%,
171/423), Australia (38.3%, 162/423), Canada (5.9%, 25/423) and the UK (5.7%, 24/423). Age at the time of data collection ranged from 18.0 to 54.3 years (median 24.9 years, IQR 21.5-30.7 years). Over two thirds of the women (71.2%, 301/423) were living in their parental home or with other family members and 29% (122/423) were being cared for in facilities such as group homes and other institutions. The characteristics of the women are shown in Table 2.

**Epilepsy**

Almost two-thirds (64.0%, 263/411) of the women were taking anti-epileptic medications at the time of data collection with only 17.3% (71/411) having never experienced seizures. Of those receiving anti-epileptic medication, seizures were completely controlled in 38.0% (100/263), partially controlled in 25.9% (68/263), and were classified as drug resistant in 36.1% (95/263). A small proportion (5.6%, 23/411) was not taking anti-epileptic medication despite some level of seizure activity and 13.1% (54/411) who had previously been diagnosed with epilepsy, were currently seizure free. Some families reported a reduction in severity or the number of seizures with increasing age while others reported increased seizure activity. The p.Thr158Met mutation group had the highest odds of individuals having active epilepsy (OR 2.77, 95% CI 0.66, 11.67) (Figure 3). There were no significant differences in prevalence of active epilepsy across mutation types.

### Table 1 Number (%) of girls and women in the survival analysis for each mutation type (n = 300)

| Mutation type, n (%) | C-terminal | Early truncating | Large deletion | p.Arg106Trp | p.Arg133Cys | p.Arg168* | p.Arg255* | p.Arg270* | p.Arg294* | p.Arg306Cys | p.Thr158Met | Other |
|---------------------|------------|-----------------|---------------|-------------|-------------|-----------|-----------|-----------|-----------|-------------|------------|-------|
|                     | 27 (9.0)   | 21 (7.0)        | 21 (7.0)      | 14 (4.7)    | 23 (7.7)    | 34 (11.3) | 18 (6.0)  | 25 (8.3)  | 24 (8.0)  | 19 (6.3)    | 32 (10.7)  | 42 (14.0) |

*p*stop codon.

### Table 2 Number (%) for each mutation type and medical condition in the health status analyses (n = 423)

| Mutation type, n (%) | ARSD* | InterRett | Overall |
|---------------------|-------|-----------|---------|
| C-terminal          | 11 (9.9) | 8 (6.6) | 19 (8.2) |
| Early truncating    | 7 (6.3)  | 6 (4.9)  | 13 (5.6) |
| Large deletion      | 6 (5.4)  | 5 (4.1)  | 11 (4.7) |
| p.Arg106Trp         | 4 (3.6)  | 6 (4.9)  | 10 (4.3) |
| p.Arg133Cys         | 10 (9.0) | 7 (5.7)  | 17 (7.3) |
| p.Arg168*           | 12 (10.8)| 9 (7.4)  | 21 (9.0) |
| p.Arg255*           | 9 (8.1)  | 10 (8.2) | 19 (8.2) |
| p.Arg270*           | 9 (8.1)  | 8 (6.6)  | 17 (7.3) |
| p.Arg294*           | 9 (8.1)  | 7 (5.7)  | 16 (6.9) |
| p.Arg306Cys         | 6 (5.4)  | 6 (4.9)  | 12 (5.2) |
| p.Thr158Met         | 12 (10.8)| 4 (3.3)  | 16 (6.9) |
| Other               | 16 (14.4)| 46 (37.7)| 62 (26.6) |

Total: 111d 122e 233

| Medical conditions, n/N (%)| Seizures | Unable to walk | Scoliosis | Constipation | Bloating | Gallbladder problems | Gastro-oesophageal reflux diseases | Gastrosstomy | Altered breathing patterns | Sleep disturbances |
|----------------------------|----------|----------------|-----------|--------------|----------|----------------------|----------------------------------|--------------|--------------------------|-------------------|
|                            | 64/144   | 70/150         | 122/150   | 111/146      | 85/147   | 3/144                | 22/144                           | 41/150       | 97/146                   | 92/142            |
|                            | 44.4%    | 46.7%          | 81.3%     | 76.0%        | 57.8%    | 2.1%                 | 15.3%                            | 27.3%        | 66.4%                    | 64.8%             |
|                            | 122/267  | 97/272         | 237/270   | 221/255      | 121/244  | 17/233               | -                                | 41/150       | 97/146                   | 154/249           |
|                            | (45.7%)  | (35.7%)        | (87.8%)   | (86.7%)      | (49.6%)  | (7.3%)               |                                  | (27.3%)      | (66.4%)                  | (61.9%)           |
|                            | 186/411  | 167/422        | 359/420   | 332/401      | 206/391  | 20/377               |                                  | 246/391      | 246/391                  |                   |
|                            | (45.3%)  | (39.6%)        | (85.5%)   | (82.8%)      | (52.7%)  | (5.3%)               |                                  | (62.9%)      |                         |                   |

*a*Australian Rett Syndrome Study; bthe denominator is different for each medical condition because of some families did not complete all questionnaire sections; *c*stop codon; *d*negative or unknown mutation (n = 39); *e*negative or unknown mutation (n = 151).

**Figure 2** Risk of death during the observation period in the Australian cohort (n = 396) by common mutation groups in comparison to the p.Arg133Cys mutation group. The error bars denote 95% confidence intervals. CT, C-terminal deletion; ET, early truncating; LD, large deletion; Neg/Unk, mutation negative or unknown.
Musculoskeletal aspects
Many of the women continued to walk independently (17.8%, 75/422) or with a little (22.5%, 95/422) or moderate amounts of assistance (20.1%, 85/422). Slightly fewer than half (39.6%, 167/422) were unable to walk at all at the time of data collection. Those with a large deletion (OR 0.11, 95% CI 0.02, 0.65), or a mutation that resulted in early truncation of the protein (e.g. p.Gly269fs) (OR 0.21, 95% CI 0.04, 1.03) appeared to be more severely affected when compared to those with the p.Arg133Cys mutation (Figure 3).

Scoliosis
Scoliosis had developed in the majority (85.5% 359/420). The proportion of women who could not walk was greater in those with scoliosis (45.3%, 162/358), in comparison to those with no spinal curvature (8.2%, 5/61). Across mutation types the odds of having scoliosis was lowest in women with the p.Arg294* mutation (OR 0.24, 95% CI 0.05, 1.10) and highest with a large deletion (OR 3.34, 95% CI 0.32, 35.00) (Figure 3). Information on scoliosis treatment was provided by 337 of the families whose daughter had scoliosis and in 40.0% (135/337) had undergone spinal fusion, mostly between the ages of 11 and 16 years.

Growth and gastrointestinal disorders
Information on body weight, method of feeding and presence of reflux was only available for cases ascertained from the ARSD. The mean weight-for-age z-score was -3.04 (SD 3.39). The majority of women (52.8%, 65/123) were underweight (z-score ≤ -2.0) including some (8/65) who were markedly underweight (z-score ≤ -10). Slightly fewer than half (46.3%, 57/123) were in the normal weight range and one was overweight (z-score ≥ 2.0). Weight-for-age z-scores were lowest in those with large deletions (Predicted value -5.25, 95% CI -8.64, -1.86) and those with p.Arg106Trp (Predicted value -5.18, 95% CI -8.59, -1.77) mutations and highest in those with C-terminal mutations (Predicted value -1.32, 95% CI -3.58, 0.93) (Figure 4).

Also for the Australian cohort, 27.3% (41/150) had had a gastrostomy feeding tube inserted to: improve food/fluid intake; prevent aspiration; or to provide relief from bloating. Almost half (46.2%, 18/39) of those receiving nutrition through gastrostomy also had some oral intake, 15.4% (6/39) had all food orally and 38.5% (15/39) had all food/fluid intake by gastrostomy. Four women had had a gastrostomy for a short period during childhood and were now feeding orally.

Reflux was reported for 15.3% (22/144) of women in the Australian cohort. For the combined Australian and Inter-Rett cohorts, constipation was highly prevalent (82.8%, 332/401), and many were experiencing bloating (52.7%, 206/391). Biliary dyskinesia, inflammation or infection of the gallbladder was reported for 20 women (5.3%, 20/377) and of these 13 had undergone gallbladder surgery.

Breathing patterns
Information on breathing patterns was available for the Australian cohort. Abnormal breathing patterns were reported for two thirds of women (66.4%, 97/146) including 74.2% (72/97) who hyperventilated and 88.7% (86/97) with breath-holding or apnoeic episodes. Within this group, 62.9% (61/97) both hyperventilated and breath-held. These breathing patterns varied little across mutation types (Figure 3).

Sleep disturbances, mood and anxiety
Sleep disturbances were common (62.9%, 246/391) and almost half the women (47.7%, 178/373) experienced

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**Figure 3** Influence of mutation type, adjusted for age, on selected health conditions. The error bars denote 95% confidence intervals. Seizure (n = 411) was defined as having active epilepsy. Walking (n = 422) was defined as able to walk with or without assistance. Scoliosis (n = 420) was defined as ever had scoliosis. Breathing problems (n = 146) were defined as having a breathing problem including breath holding and hyperventilation. Sleep problems (n = 391) were defined as having sleep disturbances. CT, C-terminal deletion; ET, early truncating; LD, large deletion; Neg/Unk, mutation negative or unknown.
night laughing. Night screaming occurred often for 13.2% (18/136), occasionally for 20.6% (28/136) and not at all for 66.2% (90/136) of ARSD women. In comparison to p.Arg133Cys, sleep disturbance was more prevalent in those with p.Arg294* (OR 1.39, 95% CI 0.30, 6.55) and less common in those with p.Arg106Trp mutations (OR 0.29, 95% CI 0.06, 1.52) and those with p.Arg255* (OR 0.41, 95% CI 0.09, 1.79) (Figure 3).

Families participating in the InterRett study reported that 46.0% (109/237) experienced screaming spells during the day. Otherwise, information on mood and anxiety was only available for those in the ARSD (n = 137). Information pertinent to mood and behaviour, obtained using the RSBQ, indicated that adverse behaviour such as screaming spells and irritability occurred often (RSBQ score >=11) in 19% of women, occasionally (RSBQ score 6-10) in 41% and rarely (RSBQ score <=5) in the remainder (40%). Anxiety, as determined by response to four related RSBQ questions was experienced often (RSBQ score >=7) for 11%, occasionally (RSBQ score 3-6) for 58% and rarely if ever (RSBQ score <=2) for 31%.

The average score for mood was 6.86 (SD 4.07) out of a possible total of 16. After adjusting for age group, predicted mood scores above the average (indicating difficult behaviour) were observed for mutation groups considered to have a milder phenotype, p.Arg133Cys (Predicted score 9.07, 95% CI 6.36, 11.78) and p.Arg294* (Predicted score 8.76, 95% CI 6.09, 11.44) and also those with a large deletion (Predicted score 8.95, 95% CI 5.35, 12.56) who generally have a more severe overall phenotype (Figure 4). Individuals within the 26-30 years age group were likely to have a slightly higher mood scores (Predicted score 8.54, 95% CI 7.00, 10.09) compared to younger women (Predicted score 6.50, 95% CI 5.59, 7.41) and those over 30 years of age (Predicted score 6.16, 95% CI 4.60, 7.72) after adjusting for mutation type.

The average score for anxiety was 3.66 (SD 2.14) out of a possible total of 8. In comparison to those with p.Arg133Cys mutations (Predicted score 5.09, 95% CI 3.69, 6.48), women with p.Thr158Met had reduced anxiety (Predicted score 2.45, 95% CI 1.14, 3.76) as did those with p.Arg168* mutations (Predicted score 3.23, 95% CI 1.97, 4.49) after adjusting for age group (Figure 4). As with mood, those aged 26-30 years were likely to experience slightly more episodes of anxious behaviour (Predicted score 4.21, 95% CI 3.41, 5.01) compared to younger women (Predicted score 3.62, 95% CI 3.15, 4.08) or those over 30 years (Predicted score 3.20, 95% CI 2.40, 4.01) after adjusting for mutation type.

Other health issues
Responses to questions regarding hospitalisations, investigations and treatments for health issues other than those normally associated with Rett syndrome revealed 11 conditions reported for at least 4 individuals (Table 3). Of these, urinary tract infections and respiratory conditions, including pneumonia, were the most commonly reported.

Discussion
Using a population-based cohort we have shown that current survival to the age of 20 years is 77.6% with 59.8%...
surviving to early middle age. In women aged 18 years and older we found that epilepsy, breathing and sleep problems persisted, low weight and gastrointestinal issues were prevalent and although most have scoliosis loss of walking is not inevitable. The influence of genotype, while moderate, is in agreement with previous findings.

A major strength of this study is the rich data sources available for analyses. Our population-based ARSD with follow-up of individuals up to 20 years was coupled with up-to-date death information has allowed us to make an accurate estimate of survival in Rett syndrome. Our cross-sectional analysis of a large multi-country study population, over half of whom have a confirmed mutation in the MECP2 gene has allowed us to more fully investigate the clinical profile, and influence of genotype, in ageing women. Limitations of this study include lack of representation of older women not living in the parental home in the international community since, unlike those who live in their parental home, their family members would be less likely to be active participants in online initiatives such as the InterRett database. Despite the survival analysis being undertaken on Australian population-based data collected over 20 years the capacity to detect differences in mortality risk across mutation groups was limited by the small number of deaths within specific mutation groups.

Importantly, we can now report survival in the ARSD cohort of slightly less than 60% at 38 years of age although the current estimate of survival to 25 years (71.5%) is lower compared to our 2006 analysis (77.8%) [23]. Death was most commonly caused by respiratory illnesses. Increased ascertainment and follow-up of individuals in the more recent analyses likely explains the difference in the survival estimate over time. A higher estimate of survival to 25 years (~80%) was reported for a cohort of US and Canadian females (n = 1,907) [13]. The authors of this study reported that many known individuals who had been identified through the family association were not included in their analysis due to failure to make contact (52%, n = 1,555) and it is likely that individuals who have not registered with the family association or clinics also exist within the US and Canadian populations. The survival estimate is likely over-estimated because it is quite plausible that the proportion of deceased compared to living cases would have been higher in the group whose families were not able to be contacted in comparison to the group whose families were able to be contacted and on whose data the analysis was based. As recommended by the authors of the study, a population-based national registry, similar to that in Australia, with links to national death registry and other pertinent data sources might be advantageous, as mortality in such cohort will yield more precise survival rates as case numbers and periods of follow-up increase over time.

Compared to those with the p.Arg133Cys mutation, the risk of death was high in those with a p.Arg270* mutation consistent with our previous findings [24]. Our new findings indicate that survival in those with the p.Arg106Trp mutation or large deletion is similar to that of the p.Arg270* group and that those with p.Arg255* mutations demonstrate better survival. A proportion of those who were older had not been tested for a MECP2 mutation because of its relative recent availability and some families do not now want to revisit diagnostic processes. Nevertheless, genetic testing for women with a clinical diagnosis of Rett syndrome has important roles to play. Some families do value more specific diagnostic information [25] and the data contributes to greater accuracy in future estimations of the effects of genotype on phenotype including survival.

Our cross-sectional analyses used the largest sample size to date for investigating health and wellbeing in adulthood. Seizures are common in Rett syndrome and not surprisingly, almost two-thirds (263/411) of the women continued to take anti-epileptic medications at the time of data collection. Previous studies have reported stabilisation or improvement in epilepsy in older women [7]. While some parents in the current study noted that the frequency or severity of seizures had diminished with age, the majority had active seizures, and of these, a third was considered to be refractory. An investigation of epilepsy in the InterRett study showed similar results for those aged 17 years or older [18]. These findings indicate that management of epilepsy remains a serious challenge in ageing women and this picture was consistent across all mutation types.

Over half (60%) of the study cohort had some walking skills at the time of data collection. The proportions of women who could walk independently, with assistance, or not at all, were similar to that previously reported for an Italian cohort of women and adolescents aged 14 or more years [8]. A longitudinal study of adult women with Rett syndrome conducted in the Netherlands [7] indicated that age-related deterioration of gross motor function is slow. This observation concurs with findings from a video study which investigated general stability in gross motor function over a 3- to 4-year period: girls younger than 13 years with walking skills were more likely than teenagers and women to lose the more complex motor skills that enabled better negotiation of the environment [26]. Together these findings add strength to the thesis that loss of walking is not inevitable as women with Rett syndrome age. It is likely, however, that these women represent the milder range of the severity spectrum and that those who could not walk from an early age, being more severely affected, may have died before reaching adolescence or adulthood. Those with spinal curvature were less likely to be ambulant and
encouragingly, many were well following previous surgical management.

The proportion of those who could walk in the p.Arg133Cys mutation baseline group (76%) was similar to those with a p.Arg294* mutation (81%) and significantly better than those with large deletions (27%). Improved gross motor skills for those with a p.Arg133Cys and p.Arg294* has been previously observed in a video study of 99 girls and women from the ARSD registry, 29 of whom were 19 years or older, possibly reflecting some survival bias [10]. A 2008 study (n = 272) found that the overall phenotype of those with p.Arg133Cys and p.Arg294* mutations is mild [5] and Cuddapah et al [6] found significant differences in ambulation, not adjusted for age, between those with p.Arg133Cys and nine other mutation groups (p.Thr158Met, p.Arg168*, p.Arg255*, p.Arg270*, Deletions, Insertions, Large Deletions, Splice Sites and No mutations). These consistent findings across and within age groups help to provide a clearer clinical picture of mobility outcomes in those affected by these common mutations.

We found, as had others, that sleep disturbance [7,8] and gastrointestinal problems [7] were highly prevalent in older women. We recently found the rate of gall bladder disease in a Rett syndrome population-based study to be 2.3 per 1000 person years (ie. two new cases if 100 individuals are followed for 10 years) [27]. Available population-based data on typical paediatric populations suggested a lower prevalence than this [28,29]. Although gall bladder disease was identified in 5% of adults in our current study it is unclear how this compares with the general population. We suggest that gall bladder disease should be considered in the differential diagnosis of abdominal pain in Rett syndrome.

Unusual breathing patterns including hyperventilation and breath holding were highly prevalent yet there has been a paucity of research in this area. Women with a p.Arg255* mutation were least likely to hyperventilate and also had a lower mortality. The influence of breathing irregularities on overall health and survival is not well understood and further research in this area is warranted. Similarly, the frequency of respiratory type infections indicates that these health issues may also need to be closely monitored, including attention to upright postures during daytime activities and a low threshold to the prescription of antibiotics.

Fortunately, behavioural difficulties and anxiety only appeared to affect a relatively small proportion of women although these issues would likely have a major impact on the daily quality of life of those with Rett syndrome and their carers. Small increases in the mood and anxiety scores were observed for those aged 26-30 years in comparison to the older and younger age groups. In the Dutch cohort, behaviour was assessed using the Observational Questionnaire Elderly Residents with Intellectual Disabilities (OOB). Based on this instrument, two thirds of Dutch women showed anxiety and during the five year study period improvements were mainly seen in those age 30 years or more (2007-2012) [7]. It might be expected that those with a milder phenotype can better communicate their feelings, however, we observed higher predicted scores for mood and anxiety in mutations associated with both mild and severe phenotypes.

The change in composite health status by mutation type over time was outside the scope of the current study. However, a previous study of health trajectories over time in our Australian cohort [30] found that for most mutations, including p.Arg133Cys where health status at a younger age was better, health status deteriorated over time. For a few others, which tended to be more severe mutations, health status either remained unchanged (p.Arg106Trp) or improved slightly (p.Arg255* and p.Arg168*) with age [30]. The authors of the US longitudinal study [6] reported that severity of symptoms increased by age for most mutation types in both cross-sectional analysis across five age groups and longitudinal analysis within individuals. The severity score for an individual was determined by a protocol developed by the authors who acknowledged that the inclusion of some items, which cannot change with time, may have led to an underestimation of age-related changes in their longitudinal analyses. Given that there is considerable variance in clinical outcomes within mutation types, consistent ongoing follow-up of existing cohorts is required to obtain a clearer picture of mutation-specific severity in women with Rett syndrome.

Conclusion

The data contained in this paper represents twenty years of surveillance and highlights the consistent research effort required to understand rare disorders. Individuals with Rett syndrome have potential for prolonged survival with approximately 60% surviving to early middle age. Many women continue to have some level of ambulation and it is therefore critical to develop and advocate for care plans that help to maintain and build on these skills. Further investigation into mood and anxiety could help to better define needs and inform care strategies. We also recommend that the maintenance of clinical care services for adults with Rett syndrome including close monitoring of respiratory and gastrointestinal function. The impact of breathing irregularities on survival is poorly understood and is an important topic for future investigation. Ongoing monitoring of the ARSD and other population-based cohorts has a vital role in providing accurate estimates of survival in Rett syndrome.
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