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REVIEW

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Neurobiologically-based treatments in Rett syndrome: opportunities and challenges

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ABSTRACT

Introduction: Rett syndrome (RTT) is an X-linked neurodevelopmental disorder that primarily affects females, typically resulting in a period of developmental regression in early childhood followed by stabilization and severe chronic cognitive, behavioral, and physical disability. No known treatment exists beyond symptomatic management, and while insights into the genetic cause, pathophysiology, neurobiology, and natural history of RTT have been gained, many challenges remain.

Areas covered: Based on a comprehensive survey of the primary literature on RTT, this article describes and comments upon the general and unique features of the disorder, genetic and neurobiological bases of drug development, and the history of clinical trials in RTT, with an emphasis on drug trial design, outcome measures, and implementation.

Expert opinion: Neurobiologically based drug trials are the ultimate goal in RTT, and due to the complexity and global nature of the disorder, drugs targeting both general mechanisms (e.g., growth factors) and specific systems (e.g., glutamate modulators) could be effective. Trial design should optimize data on safety and efficacy, but selection of outcome measures with adequate measurement properties, as well as innovative strategies, such as those enhancing synaptic plasticity and use of biomarkers, are essential for progress in RTT and other neurodevelopmental disorders.

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1. Introduction

During the last decade, there has been a dramatic increase in research aimed at treating neurodevelopmental disorders. These efforts have been the consequence of a better understanding of the genetic basis of several of these disorders and the subsequent development of experimental models, primarily mouse models. Although genetic treatment strategies have been successful in the laboratory setting, their clinical application is still hypothetical. Consequently, most of the emphasis on novel treatment development has focused on pharmacological approaches targeting processes downstream to the primary genetic abnormality [1,2]. Perhaps, the best-studied neurodevelopmental disorder from this targeted therapeutics perspective has been fragile X syndrome (FXS). Drug development for FXS generated considerable excitement, as a result of almost 50 publications on animal models examining several neural mechanisms and drug candidates (e.g. GABA-B agonists, mGluR5 antagonists) [3]. Despite this initial enthusiasm, the outcome of ongoing or completed trials has been at best mixed. No clinical study has replicated the dramatic effects in mouse models [3,4].

While the cause(s) for the presumed failure of the FXS trials is/are still unknown, the focus of drug development in neurodevelopmental disorders has shifted to other conditions, including Rett syndrome (RTT). RTT is a neurodevelopmental disorder with unique features (e.g. female predominance, dynamic clinical evolution, wide range of manifestations and severity; [5]), which are discussed in the next section. Nonetheless, the identification of a common genetic cause of most cases (i.e. Methyl-CpG-binding protein 2 (*MECP2)* deficit mutations), a relatively well-defined natural history, and extensive neurobiological data from postmortem and animal studies are factors that make RTT a good candidate for the development of targeted treatments [6]. Two recent comprehensive reviews cover multiple aspects of RTT treatment and drug trials [7,8]. Therefore, in this review, we focus on unique opportunities and challenges related to developing neurobiologically targeted treatments for RTT, including their implications for other neurodevelopmental disorders.

2. RTT diagnosis, clinical features, and management

2.1. Definition and diagnosis

RTT (OMIM #312750) is an X-linked neurodevelopmental disorder that affects predominantly females with an incidence of approximately 1 in 10,000 female births. It was first described by Dr. Andreas Rett in 1966, but was not widely recognized in the USA until the report in the English literature by Hagberg and colleagues in 1983 [9,10]. Despite the report of the association of RTT with mutations in the *MECP2* gene in 1999 by Amir et al., it remains a clinical diagnosis [11]. Clinical diagnostic criteria were initially published in 1988 and have been periodically updated, with the most recent revision published by Neul et al. in 2010 [5]. The diagnostic criteria for classic/typical RTT reflect the most common and characteristic presentation. In contrast with previous criteria, the 2010

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Article highlights

- RTT is a unique neurodevelopmental disorder characterized by a dynamic clinical course with complex multisystem involvement.
- Insights into the pathophysiology, neurobiology, and natural history of RTT are providing a foundation upon which to develop and test a variety of novel pharmacologic interventions.
- RTT and other neurodevelopmental disorders have overlapping therapeutic targets and face similar challenges with respect to clinical trial implementation.
- Development of biomarkers and other validated outcome measures is critically important for RTT drug trials.
- Innovative strategies in trial design will be necessary to continue to translate preclinical findings into effective treatments for persons with RTT and other rare disease populations.

This box summarizes key points contained in the article.

guidelines do not require postnatal deceleration of head growth, since it is not seen in all girls with RTT. However, when this finding is present, it should suggest consideration of the diagnosis of RTT. The essential diagnostic criterion required for both typical and atypical RTT is a history of a period of regression followed by recovery or stabilization. In addition, the 2010 guidelines include four main criteria: regression of (1) purposeful hand use and (2) spoken language, and the development of (3) gait abnormalities and (4) hand stereotypies. Two exclusionary criteria are meant to address any other primary cause of neurological dysfunction and a history of significantly abnormal development in the first 6 months of life [5].

All 4 main criteria are required for the diagnosis of typical RTT, while the diagnosis of atypical RTT requires 2 of the 4 main criteria and 5 of 11 supportive criteria. The supportive criteria capture many of the clinical features seen in RTT: breathing abnormalities when awake, bruxism when awake, sleep disturbances, abnormal muscle tone, vasomotor disturbances of the extremities, scoliosis/kyphosis, growth retardation, small cold hands and feet, unprovoked laughing/screaming, diminished pain response, and intense eye gaze [5].

2.2. Clinical features and evolution

Girls with RTT typically have a relatively normal period of development for the first 6 months of life followed by variable delay (even stagnation) and then a regression of developmental skills after the first year of life [5,12]. The regression particularly involves loss of expressive language skills and purposeful hand movements, but it can extend to gross motor and socialization skills [12]. It is typically during this regressive period when some girls may meet diagnostic criteria for autism spectrum disorder [13]. Loss of skills is variable in length in RTT; however, development commonly stabilizes by 30–36 months of life [12]. There may be further loss of motor skills in late adolescence or early adulthood, when parkinsonian features become prominent in a large proportion of individuals [14]. Following stabilization of skills, many girls with RTT develop intense eye gaze and increased social awareness. It is also in this post-regression period when most girls with RTT develop the pathognomonic stereotypic hand behavior, which includes repetitive wringing, washing, tapping, clapping, or mouthing among others [15]. Girls with RTT may partially regain some of the skills lost during the regression; however, significant loss of verbal skills and purposeful hand use remains a hallmark of the disorder.

Among the most characteristic features of RTT is deceleration of head growth, frequently seen between 6 and 24 months of age. Acquired microcephaly occurs in 80% of girls with RTT, although 20% of patients will have a normal head circumference [16]. Approximately, 80% of girls with RTT develop ambulation but with an abnormal gait that is described as dyspraxic; among those who develop ambulation, one-third will lose this skill [12]. Patients with RTT present with many associated neurologic and medical comorbidities that complicate medical management [17]. Seizures that evolve into epilepsy develop in 60–80% of patients, typically at the end of the regression period or beginning post-regression. There is no characteristic seizure type, and in a substantial proportion of individuals, they are difficult to manage or control [18,19]. Despite the impact of seizures on RTT's quality of life (QoL), relatively little is known about antiepileptic drug efficacy, although ongoing studies attempt to address this critical issue.

Gastrointestinal problems are common and sometimes severe in RTT. They include gastroesophageal reflux, air swallowing with abdominal distention, chronic constipation, and abdominal pain due occasionally to gallbladder disease [20,21]. Problems with oral motor control frequently present with feeding issues, not uncommonly requiring G-tube placement. Growth and nutritional issues are frequently encountered in RTT and require close monitoring and aggressive support [16,20]. Orthopedic issues are also common, with scoliosis occurring in approximately 85% of affected girls and requiring surgical stabilization in 13% [22,23]. Highly prevalent related issues are bone health (i.e. increased risk of osteoporosis; [24]) and increased muscle tone (including dystonia and contractures at multiple joints; [17]). Development of rigidity over time, along with other parkinsonian features, further complicates the late stages of evolution in RTT [14].

Under the category of autonomic dysfunction, common problems include dysregulation of respiration, both hyperventilation and breath-holding, and of limb temperature, manifesting as cool/cold and purple/mottled extremities [5,25]. Whether these abnormalities have significant systemic consequences is still a matter of debate. As survival and QoL continue to improve in RTT, some manifestations are emerging as major concerns. Among them are behavioral problems, including anxiety-like and disruptive behavior, which have begun to be characterized in a more systematic way [26,27]. New technologies applied to communication therapies and rehabilitation (e.g. eye tracking-based devices; [28]) are also effective and promote better development and QoL.

2.3. Management

The multisystem involvement seen in RTT requires a coordinated multidisciplinary approach to medical care and management. There are no current therapies specific for the disorder.

Most medical management is symptomatic, targeting the aforementioned problems. In addition to traditional drug treatments (e.g. antiepileptic drugs for seizures and SSRIs for anxiety), preventive approaches are becoming standards of care. The latter include aggressive nutritional management, with particular attention to adequate caloric intake and calcium and vitamin D metabolism, prevention of gastrointestinal and orthopedic complications, as well as the entire range of rehabilitation therapies. Some of these RTT-specific approaches have been formalized as management guidelines [24,28–32], with others still under development.

3. Genetics, neurobiology, and bases for new treatments

Other reviews, such as those mentioned in the introduction [7,8], have covered many of the key issues regarding the genetics and neurobiology of RTT and how these have influenced drug treatment development. Therefore, here we will focus on data that are unique to RTT and some that are applicable to other neurodevelopmental disorders, with an emphasis on implications for drug trials. Figure 1 provides an overview of the neurobiological mechanisms underlying RTT.

3.1. MECP2 and RTT

Until the identification of *MECP2* as the gene responsible for the majority of RTT cases (i.e. the pre-*MECP2* era), most of the knowledge on the neurobiology of the disorder was based on the study of postmortem brain and other tissue samples from affected individuals and experimental paradigms modeling these abnormalities [33,34]. These studies provided some of the bases of our current understanding of RTT, which has been to some extent correlated to a deficit of MeCP2. A notable example of this is the increase in glutamate NMDA receptor density in the neocortex at early stages of the disorder [35,36]. While genetic models of disease provide highly specific information for understanding pathophysiology and have been instrumental in studies of RTT, tissue and other biosamples from patients with the disorder are still valuable and may clarify continuous discrepancies between animal and human data, including the response to drugs.

3.2. RTT is associated with MeCP2 deficiency

Regardless of type (e.g. missense, deletion), more than 200 mutations associated with the RTT phenotype lead to a deficient function of the gene product MeCP2 [12,37]. For nomenclature on *MECP2* and its products, see Neul et al. [5]. However, not all *MECP2* loss-of-function mutations lead to the RTT phenotype since other clinical presentations have been described (e.g. non-syndromic intellectual disability) [5]. Thus, MeCP2 functional deficit is required but not sufficient for RTT features. The pattern of X chromosome inactivation also influences variability of the clinical effects resulting from a given *MECP2* mutation in a female, and some mutations that result in a non-RTT phenotype in hemizygous males have little effect in heterozygous females. Other *MECP2* abnormalities that lead to gain-of-function have also been described, most commonly duplication of *MECP2*. The latter is recognized as the basis of another phenotypically different entity affecting mainly males. MECP2 duplication syndrome has a less distinctive profile than RTT, including intellectual disability, seizures, and upper respiratory tract infections, with other features still under

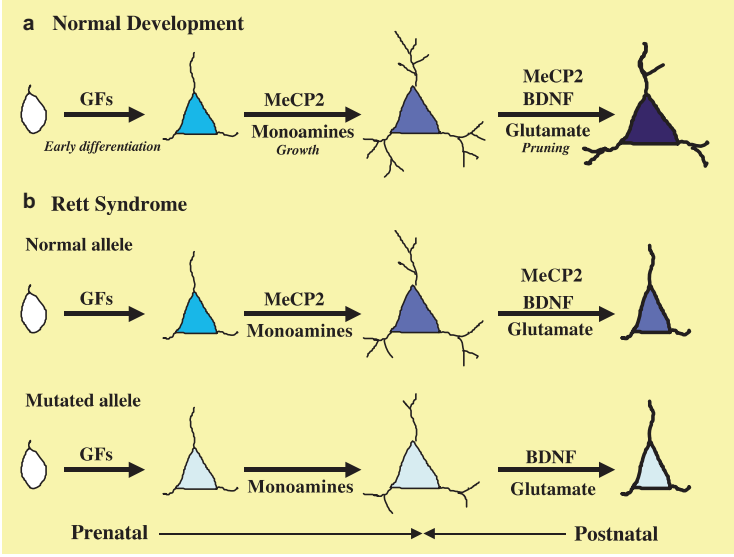

Figure 1. Model of neuronal pathology in Rett syndrome based on dendritic development in the prefrontal cortex. (a) During normal development, onset of MeCP2 expression coincides with early neuronal differentiation. Levels of MeCP2 function, depicted as intensity of blue label, increase steadily after afferents (e.g., monoamines) begin to influence cortical neuronal differentiation. Direct targets of MeCP2, such as BDNF, in conjunction with other synaptic signals have a particularly strong effect on the process of dendritic pruning. (b) Marked reduction in MeCP2 function and deficient afferent input in neurons carrying a MeCP2 mutated allele impairs appropriate dendritic expansion. The abnormality extends and worsens during dendritic pruning because of the abnormally high levels of MeCP2 targets (i.e., BDNF) and additional neurotransmitter disturbances (glutamate receptor activity). The ultimate neuronal phenotype is characterized by a smaller cell with markedly decreased MeCP2 expression and dendritic arborizations. RTT neurons carrying the normal allele are also affected. Because of decreased local (neighboring neurons with mutated allele) and distant (monoaminergic) synaptic signals, and secondary abnormalities such as increases in BDNF and glutamatergic activity, these neurons are unable to reach normal soma and dendritic size and remain as low-expressing ($MeCP2^{lo}$) cells. GFs: growth factors. Used with permission from Ref. [34].

characterization. Although a large body of information is available on genotype–phenotype correlations in RTT [37,38], and the most common specific mutations have been well characterized, efforts at correcting the gene defect have been thus far unsuccessful [39,40]. This contrasts with the success in reactivating conditional mutations in mouse models [41]. Of course, these experimental models, which use the Cre-Lox technology and in general lead to hemizygous null mutations in male mice, do not accurately reflect the human disorder that is caused by heterozygous mutations in females associated with variable but partial deficits in gene function. Nonetheless, by substantially correcting the RTT-like phenotype in adult mice, these studies have confirmed that RTT is not a degenerative disorder and that some degree of recovery is possible. Two promising strategies are the use of read-through compounds, targeting approximately 30% of nonsense *MECP2* mutations in RTT [37], and the selective activation of the X chromosome carrying the normal *MECP2* allele [42]. The latter chromosomal activation/deactivation strategy is an area of intense investigation that has already delivered exciting results in Down syndrome [43].

3.3. RTT is a global disorder of neuronal differentiation

One of the earliest findings in RTT was the demonstration of increased cell packing density and reduction in dendritic arborizations, without neuronal loss or overt gliosis [33,34,44]. These neuropathological findings from the pre-*MECP2* era have been replicated in virtually every cellular and whole-organism model of MeCP2 deficiency [34,41]. The link between *MECP2*, a gene coding for a methyl-binding protein of ubiquitous localization, and a disorder of disrupted neuronal differentiation was initially surprising. Nonetheless, the increasing body of data on the critical role of transcriptional and translational control in the fine regulation of synaptic function supports the relevance of regulators such as MeCP2 and FMRP (the protein deficient in FXS) in neuronal and synaptic development [45]. Early work supported a specific role for MeCP2 in gene silencing via recruitment of histone deacetylases (enzymatic hypothesis); however, more recent data indicate that this protein has a more complex involvement in transcriptional regulation that includes not only specific gene targeting but also global epigenetic changes [41]. Cell death and other regressive cellular processes have not been linked to RTT or MeCP2 deficiency under physiologic conditions, further highlighting the developmental bases of RTT. Nevertheless, some key cellular homeostatic processes may also be affected by MeCP2 deficiency, including mitochondrial function [46] and regulation of oxidative stress [47]. Whether the disruption of these mechanisms is a direct or secondary consequence of deficient MeCP2 function is still unknown [48]. Morphological and biochemical evidence, better exemplified by volumetric magnetic resonance imaging analyses [49], emphasize the widespread nature of the cellular abnormalities in RTT. These data confirm the need for early intervention, at least in the early postnatal period but ideally during prenatal life. The lack of neurodegeneration in RTT also supports the notion that interventions in this disorder may be effective throughout the life span of an affected individual.

3.4. Glia are also affected in RTT

A major and relatively recent shift in our thinking about RTT neurobiology originates in studies implicating astrocytes and microglia in the pathophysiology of the disorder. Although neuronal expression of MeCP2 is relatively higher than in other cells in the CNS, the brain predominant MeCP2E1 isoform is also expressed in astrocytes [50] and these cells seem to be critical for neuronal and synaptic homeostasis [51,52]. Restoration of *MECP2* expression in astrocytes [53], and even oligodendrocytes [54], ameliorates RTT features in experimental models. Time-dependent increases in myoinositol, detected by magnetic resonance spectroscopy [55], suggest a process of progressive astrocytic activation in RTT that correlates with earlier postmortem data [56]. While they are members of the monocyte/macrophage lineage and not true glial cells, microglia have also been shown to contribute to RTT pathophysiology. Although the precise mechanism mediating adverse microglial effects on neurons is still debated [57,58], excessive release of glutamate [59,60] and inflammatory cytokines have been replicated in different studies [61,62]. Elevated levels of glutamate, in conjunction with increased NMDA glutamate receptor function, have been postulated to play a key role in at least the early stages of RTT (see #3.6 below). Thus, not only global targeting of neuronal mechanisms but also astrocytic and microglial function may be an effective strategy in drug development for RTT.

3.5. RTT involves multiple neural pathways and neurotransmitters

To date, anatomical studies have demonstrated variable involvement of virtually every neural pathway [34]. Likewise, every neurotransmitter system has been implicated in the pathophysiology of RTT. Although in the pre-*MECP2* era the study of cerebrospinal fluid (CSF) and brain postmortem samples mainly revealed abnormalities in monoamine and opioid levels [33,63], the current view, expanded by mouse models of RTT, is that all major pathways and neurotransmitter systems are involved in the disorder. It is not difficult to understand the complexity of neurochemical abnormalities in RTT if one considers the ontogeny of MeCP2 expression in humans. Early expression is found in monoaminergic brainstem nuclei, followed by basal forebrain cholinergic nuclei, eventually reaching glutamatergic and gamma-aminobutyric acid (GABA)-ergic cortical neurons [64]. Whether a particular neurotransmitter plays a greater role in RTT pathogenesis and symptomatology appears to be rather time dependent, although this is still an issue under active discussion. In the next subsection, we provide more details about glutamatergic dysfunction, probably the best characterized neurotransmitter abnormality in RTT, and its potential role in the developmental regression that characterizes RTT [65]. More recently, MeCP2-related deficits in GABAergic neurons have been linked to a variety of clinical features in RTT [66–68]. MeCP2 deficiency in GABAergic neurons and abnormalities in GABA receptors may

also lead to impairments in other GABA-dependent regulatory processes [69], excitatory-inhibitory imbalance [70], and epileptogenesis [71]. The potential role of GABAergic abnormalities in disrupting critical periods of cortical plasticity [69] emphasizes the time dependency of neurotransmitter dysfunction in RTT. Although little is known about RTT in adulthood, the prevalent parkinsonian features during this period [14] suggest a prominent dopaminergic deficit. In addition to the relatively global neurotransmitter changes summarized above, there are complex patterns of interrelated abnormalities involving specific brain regions. The best examples are the changes in noradrenergic, serotoninergic, glutamatergic, and GABAergic components involving brainstem nuclei that regulate breathing [68,72,73]. The multiple disruptions in neurotransmitter systems suggest that, unless targeting the period in which the specific neurotransmitter abnormality plays the greatest role, the effects of treatment may be limited in range and duration. Moreover, the complexity of neurotransmitter balances, such as those involving glutamate and GABA, encourage a cautious approach since drug treatments may lead to worsening of symptoms or significant side effects.

3.6. Excessive glutamatergic activity may be linked to developmental regression in RTT

In the pre-*MECP2* era, most of the data on CSF and postmortem samples demonstrated abnormalities, primarily decreases in monoamine levels [63]. A few studies also reported glutamatergic abnormalities, including changes in density or levels of glutamate receptors. Specifically, reductions in AMPA glutamate receptors and increases in NMDA glutamate receptors with relative preservation of GABA receptors in the cerebral cortex were observed [36]. Interestingly, the glutamate receptor changes, in particular those involving NMDA receptors, were age dependent with higher levels in younger individuals that decreased below normal levels in late childhood. Some of these abnormalities were also found in other brain regions, such as the basal ganglia, although data are more limited [63,74]. Extension of this work to mouse models has confirmed the age-dependent change in NMDA receptors and further characterized the regional selectivity of these patterns (neocortex and striatum but not hippocampus or thalamus) [35], as well as delineated an evolution that mimics the regression period in affected patients [75]. Glutamate levels per se also appear to be elevated during early childhood in RTT, as revealed by CSF [76] and magnetic resonance spectroscopy [55] studies. This combination of high levels of free glutamate with elevated density of NMDA receptors, particularly during early childhood, supports specific windows and pharmacological agents for intervention in RTT [77]. Dopaminergic agonists may provide the counterpart to NMDA receptor antagonists at later stages of the disease, as discussed above.

3.7. RTT is a complex and dynamic disorder with multiple potential drug targets

Based on the genetics and neurobiology of RTT reviewed here, there are multiple potential therapeutic targets. Gene and protein replacement have been difficult and present the additional challenge of providing the correct dosage of MeCP2. The fact that *MECP2* duplications are associated with a severe neurological phenotype underscores the importance of a balanced amount of MeCP2 [41]. In a widespread CNS disorder such as RTT, the use of drugs that can target multiple neural networks and processes (e.g. growth factors) is particularly appealing. Nonetheless, even generalized processes may change over time. An example is the level of brain-derived neurotropic factor (Bdnf) in Mecp2-deficient mice; while Bdnf levels are normal to elevated at early stages due to reduced transcriptional silencing, the decrease in synaptic complexity present in adult animals is associated with reduced levels [78]. Thus, the use of BDNF and the related protein insulin-like growth factor-1 (IGF-1) in patients with RTT needs to be carefully monitored to address these changes. As general pharmacological agents, drugs that target specific neurotransmitter systems may be more effective at specific periods. Moreover, during these time windows, their actions could extend beyond a set of pathways and become more generalized. Consequently, it is critical to develop methods for detecting the stages of RTT and monitoring neuronal and glial abnormalities in a noninvasive manner. These biomarkers are becoming available; examples include blood-based assays of monocyte/microglia glutamate release [59] and neurophysiologic indices of disease progression [79]. However, more work needs to be done in this area in order to more accurately match pathophysiology and treatment in RTT. The next section reviews the range of drugs tested in clinical trials in RTT. Additional information about therapeutic targets and details on specific trials can be found in Pozzo-Miller et al. [7] and Katz et al. [8].

4. Drugs tested in RTT trials

As discussed in the previous section, the most feasible strategy for treating RTT is to target events downstream to the primary gene abnormality and MeCP2 deficit. Multiple global and cell- and pathway-specific targets have been identified. The *MECP2* era has provided mouse models revealing that, even late in disease progression, it is possible to significantly improve symptoms, leading to enthusiasm from researchers, patients, and advocates. Table 1 lists the various drugs that have been used in clinical trials for patients with RTT, including those that address general cellular processes, growth factors, and neurotransmitter modulators.

Prior to the discovery that pathogenic alterations in *MECP2* are the primary cause of RTT [11], drug trials were limited by a lack of understanding of the genetic basis of RTT and an inherent inability to perform preclinical studies using an appropriate animal model. Researchers used clinical findings, particularly metabolic disturbances, to guide treatment strategies. Patients with RTT were noted to have elevated blood lactate and pyruvate levels and low plasma carnitine levels, leading to clinical trials of the ketogenic diet and L-carnitine [88,89,100]. The study of naltrexone was based on the observation of increased CSF β-endorphin levels in the CSF of multiple RTT patients and in specific regions of a postmortem brain specimen from a single RTT patient, as well as animal studies in which intraventricular endorphin administration led to an

Table 1. Drugs used in clinical trials for Rett syndrome by mechanism.

Drug	References
General effects	
Creatine	[80]
EPI-743	[81] and ClinicalTrials.gov identifier NCT01822249
Folate/Betaine	[82]
Folinic acid	[83–87]
Ketogenic diet	[88]
L-carnitine	[89]
Lovastatin	ClinicalTrials.gov identifier NCT02563860
Naltrexone	[90]
Omega-3 fatty acids	[91–93]
Triheptanoin	ClinicalTrials.gov identifier NCT02696044
General synaptic regulators	
Cerebrolysin	[94]
Fingolimod	ClinicalTrials.gov identifier NCT02061137
Glatiramer acetate	[95] and ClinicalTrials.gov identifiers NCT02153723, NCT02023424
IGF-1	[25,96] and ClinicalTrials.gov identifier NCT01777542
Trofinetide (NNZ-2566)	[97] ClinicalTrials.gov identifiers NCT01703533, NCT02715115
Neurotransmitter modulators	
Bromocriptine	[98]
Desipramine	ClinicalTrials.gov identifier NCT00990691
Dextromethorphan	[99] and ClinicalTrials.gov identifier NCT01520363
Ketamine	ClinicalTrials.gov identifier NCT02562820

RTT-like phenotype (including motor dysfunction, seizures, breathing abnormalities, and stereotypic behaviors) that was reversible with naloxone [101]. Efforts were also made to investigate treatments that ameliorate other apparently similar human disorders, such as akinetic mutism (bromocriptine; [98]) and refractory seizures (ketogenic diet; [88]).

Even after the discovery of the molecular basis of RTT (e.g. the *MECP2*-targeted trial era), many studies have continued to test the effectiveness of treatments with global effects that are not specifically targeted to MeCP2 but to changes presumably secondary to the deficient function of this protein. Trials of omega-3 fatty acids have been designed to address increased markers of oxidative stress [91,92,102]. Methyl donors such as folate/betaine, folinic acid, and creatine have been used in trials based on clinical observations (low levels of folate in CSF) as well as knowledge of MeCP2 function (DNA methylation) [80,82–84,103]. Some recent and current trials continue to employ a general therapeutic approach targeting mitochondrial function with drugs such as EPI-743 and triheptanoin [81] (ClinicalTrials.gov identifier NCT02696044). Individuals with RTT have altered cholesterol metabolism [25], and lovastatin is currently under clinical trial evaluation (ClinicalTrials.gov identifier NCT02563860). In addition to these metabolic and homeostatic processes, other potential general targets are astrocytic- and microglial-specific mechanisms.

General synaptic regulators, such as BDNF or IGF-1, have the advantage of impacting multiple CNS regions and the promise of restoring balance by 'cooperating' with ongoing compensatory mechanisms. Their downside is the obvious potential for excessive synaptic modulation and for disrupting regions with preserved function. In 2001, there was one published open-label trial of cerebrolysin, a neuropeptide preparation that includes nerve growth factors [94]. Recently, there have been several trials of full-length recombinant IGF-1 and an active tripeptide component of IGF-1 (trofinetide) that may have a greater effect on glial processes [104]. There are also ongoing trials of two BDNF-boosters, glatiramer acetate and fingolimod (ClinicalTrials.gov identifiers NCT02153723, NCT02061137).

Restoring neurotransmitter balance is another approach based on solid research. However, in this case the challenge is to determine which neurotransmitter abnormality to target and when to intervene. While some neurotransmitter disturbances seem to be linked to specific periods of the disorder (e.g. enhanced NMDA receptor activity and regression), whether a particular individual is experiencing such an abnormality in neurotransmission is unclear. Data from mouse models are not easy to translate to patient populations; availability of biomarkers reflecting neurotransmitter or synaptic abnormalities is essential and some progress has been made in this area [59,79]. Another challenge is to determine which neurotransmitter(s) to target when more than one is presumably involved. This issue is most likely to be raised when trying to correct a specific phenotype; success has been achieved in Mecp2 mouse models in improving breathing abnormalities by simultaneously modulating GABAergic and serotoninergic neurotransmission [105]. Whether this can be accomplished in patients with RTT, without substantial side effects, is still unknown. Clinical findings of impaired dopaminergic activity led to a trial of bromocriptine even before the discovery of *MECP2*'s relationship to RTT [98]. Another drug with effects on monoamine neurotransmitters is desipramine, a tricyclic antidepressant that blocks the uptake of norepinephrine (ClinicalTrials.gov identifier NCT00990691). Dextromethorphan (DM) and ketamine have been studied due to their NMDA receptor antagonist activity [99] (ClinicalTrials.gov identifiers NCT01520363, NCT02562820). This is an area of active research and subunit-specific modulators of NMDA receptors are under development [106].

5. Outcome measures and biomarkers in RTT trials

In both the pre-*MECP2* and *MECP2* eras, a combination of ad hoc and standardized measures have been used as trial endpoints. Most of these are clinical evaluations (i.e. based in medical history or physical examination); however, a few laboratory/diagnostic tests and other objective measures have also been used. The following paragraphs summarize and comment on the application of outcome measures in RTT drug trials, which are also listed in Table 2. A detailed list of end points employed in each RTT trial is provided in the review by Katz and colleagues [8].

5.1. Distinctive RTT symptoms and signs

RTT is characterized by a unique combination of features. Some of these specific symptoms and signs have been evaluated as individual end points in many clinical trials (e.g. bruxism, breathing abnormalities), using nonstandardized assessments in most cases. In the *MECP2* era, several trials have employed multidimensional instruments covering a range of cognitive (including communication), motor,

Table 2. Most common outcome measures used in clinical trials for RTT.

Type of outcome measure	Examples [original or RTT-specific reference]	Trials utilizing the outcome measure
Distinctive RTT symptoms and signs	Motor Behavioral Assessment (MBA) [107]	[25,80,82,89,90,97,103] ClinicalTrials.gov identifier NCT02715115
	Clinical Severity Scale [108,109]	[25,81,91–93,97,102] ClinicalTrials.gov identifiers NCT01253317, NCT01822249, NCT02023424
General neurologic and physical measures	Nonstandardized assessments of motor function and seizure frequency/severity [see trials]	[83,84,88,96,103] ClinicalTrials.gov identifiers NCT02023424, NCT02563860, NCT02696044
	Growth parameters [16]	[81,82] and ClinicalTrials.gov identifier NCT02023424
Specialized neurological measures	Vineland Adaptive Behavior Scales [110]	[90,97,99] and ClinicalTrials.gov identifiers NCT00593957, NCT01253317
	Hand Apraxia Scale [111]	[89,100,103]
Behavioral Instruments	Rett Syndrome Behavioral Questionnaire (RSBQ) [112]	[25,81] and ClinicalTrials.gov identifiers NCT01253317, NCT01777542, NCT01822249, NCT02562820,
	Anxiety, Depression, and Mood Scale (ADAMS) [26]	[25] and ClinicalTrials.gov identifier NCT01253317
CGI and VAS	Clinical Global Impression (CGI) Scale [113]	[96]
		[97,114] and ClinicalTrials.gov identifiers NCT01253317, NCT02715115
	Visual Analog Scale (VAS) [115]	[97] and ClinicalTrials.gov identifiers NCT01253317, NCT02715115
Laboratory and neurophysiologic measures	EEG and other neurophysiological measures [116]	[25,83,88,90,94,96,97,99,103,117] and ClinicalTrials.gov identifiers NCT01253317, NCT02023424, NCT02562820, NCT02563860, NCT01777542
	Plethysmography [118,119]	[25,88,90,97] and ClinicalTrials.gov identifiers NCT00990691, NCT02023424, NCT02562820, NCT02563860, NCT01822249, NCT01777542
	Blood-based oxidative stress markers [see trials]	[91–93,102] and ClinicalTrials.gov identifier NCT01822249
Quality of life measures	Pediatric Quality of Life Inventory (PedsQL) [120]	ClinicalTrials.gov identifiers NCT01822249, NCT02563860
	Overall Well-Being Index [121]	[89,103]

behavioral, and other RTT-specific parameters. The most widely used has been the Motor Behavioral Assessment (MBA), a comprehensive neurologic evaluation of individuals with RTT, initially described in 1990 [107], but more widely applied to RTT research since its incorporation into the NIH-funded natural history study (current: U54 HD061222). Other clinical instruments more recently used in drug trials include the Clinical Severity Scale (CSS; [108]) and the International Scoring System (ISS; [109]). Although the MBA, CSS, and ISS provide information predominantly about current status by direct observation, these measures were developed as phenotyping tools with face validity. Their measurement properties in terms of sensitivity (i.e. change with intervention), reliability, and other validity parameters are unknown.

5.2. General neurological and physical measures

These include nonstandardized assessments of motor function and seizure frequency and severity. Growth parameters, mainly head circumference but also height and weight, represent more objective and standardized parameters [16]. Recently disorder-specific published growth curves allow appropriate interpretation of these parameters for patients with RTT.

5.3. Specialized neurological measures

A variety of standardized or structured measures of motor and cognitive function have also been employed. These include the Bayley Scales, Vineland Scales, and Hand Apraxia Scale. Despite their at least partially validated nature, these instruments have not been adapted to RTT or tested in terms of sensitivity to change. Careful interpretation of the data is, therefore, needed [27].

5.4. Behavioral instruments

Behavioral problems, other than autistic features, have only been recently recognized. The only available comprehensive and disorder-specific measure, the Rett Syndrome Behavioral Questionnaire (RSBQ), has been applied to clinical trials in the last 5 years. Another standardized instrument, the Anxiety, Depression, and Mood Scale (ADAMS), has been used in two recent IGF-1 trials [25]. As reported by us [26], the RSBQ and the ADAMS, a scale developed for general use in individuals with intellectual disability, have less than optimal measurement properties. Nonetheless, the ADAMS appears to be better than other instruments in evaluating social anxiety. Despite the extensive use of the RSBQ, there is a need for an RTT-specific instrument with strong psychometric properties for assessing a wide range of abnormal behaviors.

5.5. Clinician and caregiver assessments (CGI, VAS)

In contrast to their use in clinical trials for FXS and autism spectrum disorder, the Clinical Global Impression-Severity (CGI-S), the Clinical Global Impression-Change/Improvement (CGI-C/CGI-I), and the Visual Analog Scale (VAS) have only occasionally been employed as inclusion criteria or measures of efficacy in trials for RTT. This is probably a reflection of the complex clinical manifestations of RTT, including cognitive, motor, and behavioral features, which are better defined by disorder-specific scales. However, the recent introduction of the CGI-S/CGI-I and VAS to several trials using IGF-1-like compounds in RTT represents both a change in study design and standards and a new emphasis on behavioral symptoms, traditionally assessed by CGIs. From secondary outcome measures in the IGF-1 trials in Boston [25] to primary end points in the IGF-1 trial in Italy [96] and the adolescent/adult trofinetide trial [97], the refinement of CGI definition and anchors [113] have certainly encouraged the application of the CGI in RTT trials. Use of the VAS in RTT and other

neurodevelopmental disorders is driven by the interest in capturing clinical improvements of significance for individual patients, given the phenotypic diversity of these conditions.

5.6. Laboratory and neurophysiological measures

A variety of EEG parameters, frequently spike counts, have been used as endpoints since the pre-*MECP2* era [25,83,122]. More recently, other biomarkers have been employed. These include plethysmography-derived parameters (e.g. apnea index), autonomic measures, and blood-based oxidative stress markers [25,92]. Most of these outcome measures are of unknown clinical or functional significance and have thus remained secondary or exploratory endpoints.

5.7. QoL measures

A relatively new category of outcome measure, QoL assessments, is critical for demonstrating the clinical impact of virtually all of the aforementioned parameters. These measures themselves can also be adequate end points. Those employed in RTT include the Pediatric Quality of Life Inventory (PedsQL; [120]), the Overall Well-Being Index [83], and the short form 36 (items) health status questionnaire (SF-36; [100]).

Overall, the vast majority of outcome measures used in RTT drug trials have not been disorder validated according to regulatory standards. This represents an area of great need since imprecise measurements may lead to over- or underestimation of drug efficacy. Another important issue is the dynamic nature of neurodevelopmental disorders such as RTT. Therefore, selection of adequate measures for a specific stage in the development or evolution of the disorder is also critical. For example, head circumference is an objective, quantitative measure; however, its use beyond the first few years of life is not adequate. There is great interest in developing biomarkers that can detect drug effects and, consequently, be used as endpoints. Although some progress has been made in this area in FXS and autism spectrum disorder [123,124], limited efforts have been reported in RTT [25,59,79]. Objective outcome measures may not only impact clinical trial design but also clinical care.

6. Expert opinion: the unique and common challenges in developing drug trials for RTT

As summarized in the preceding sections, a wide range of clinical trials using a variety of drugs and outcome measures has been already implemented in RTT. Some trials have focused on specific clinical manifestations or mechanisms, while others have intended to modify the course of the disorder and, therefore, have targeted multiple symptoms. Only a few of these studies have been designed on the basis of neurobiological mechanisms. Overall, of the 9 positive trials in RTT (4 in the pre-*MECP2* era; [8]), several have been open label (including one phase I study) and none has been replicated. What is the reason for these inconsistent results and lack of clear positive effects? An analysis of the presumably better 'targeted' trials in RTT identifies unique challenges linked to the symptoms and evolution of the disorder. However, other issues seem to be shared with most neurodevelopmental and pediatric neuropsychiatric disorders. We review here major issues that may impact drug trial design and implementation in RTT.

6.1. Transition from animal to human studies: sooner rather than later

One of the most difficult issues is to determine when animal data are sufficient for transitioning into phase I human trials. The experience from FXS indicates that correction in mice, sometimes almost complete, does not guarantee a successful application of the new treatment in humans [3]. Conversely, the lack of marked improvement of a particular symptom in mice does not mean that this is not possible in humans (e.g. minimal change in abnormal behaviors including anxiety in Fmrp-deficient mice). We postulate that the best use of mouse and other animal models is to demonstrate 'proof of principle' efficacy of a drug, regardless of the range of positive effects. Of course, replication of efficacy and use of best practices in preclinical models is essential, as recommended by the NIH [125] and specifically discussed in the context of RTT [126]. In the case of RTT, it is also ideal if work on hemizygous *Mecp2* null mice can be complemented with data from heterozygous mouse models. Animal work is crucial for other purposes, such as toxicology. Because animal model research is essential for drug discovery, it is a necessary first step. Nonetheless, ultimately the study of affected individuals is the only final demonstration of safety and efficacy. Consequently, we recommend that the animal–human transition occurs as soon as possible.

6.2. Trial design: optimizing data collection while addressing unique features of RTT

The fact that preclinical studies do not guarantee the success of human drug trials is only one of the factors that make trial design a key issue. The experience with FXS and the initial targeted trials in RTT demonstrates that range of symptoms and magnitude of effect in mouse studies do not translate directly into clinical trials. For instance, the mouse trial with IGF-1 (mecasermin) resulted in marked improvements in locomotor activity, social behavior, heart rate, breathing patterns, and anxiety [127], while the phase I human trial revealed changes only in the latter two parameters, and the effects on breathing were restricted to apnea [25]. Similarly, administration of trofinetide to Mecp2-deficient mice resulted in marked improvements [128] that, in contrast, were wide ranging but very mild in the first trial with RTT patients [97]. Consequently, adaptive response and other dynamic trial designs become critical to advancing the field. These approaches allow changes in outcome measures and drug administration schedules within a single study, combining the features of phase I (safety, tolerability) and phase II (efficacy) trials and increasing the probability of a successful outcome. The importance of dosing and schedule of administration, though a general issue in drug trials, takes a unique dimension in a medically vulnerable population affected by a disorder such as RTT [17]. Both adolescent/

adult trofinetide trials, for RTT and FXS, showed a dose-dependent effect [97,129]. These trials also reported linear pharmacokinetics; however, the related IGF-1 phase I trial showed a complex nonlinear pharmacokinetics in line with the receptor-binding features of the drug [25]. Dosage and administration issues have to be tested in patients, since it is difficult to model in mice the variable polypharmacology needed to manage individuals with RTT. Drug interactions and side effects are not only considerations in a trial's inclusion criteria but also in the potential application of a drug to the target population (i.e. avoiding superselection in trials).

6.3. Considering heterogeneity and evolution of RTT in the design of drug trials

Phenotypic heterogeneity is not unique to RTT but is also present in many neurodevelopmental disorders. This needs to be taken into consideration for the appropriate balance of statistical power and feasibility; clinical severity indices such as the Clinical Severity Scale (CSS) and the CGI, or a focus on specific symptoms, are adequate solutions. A unique RTT feature is, however, its evolution. Although most neurodevelopmental disorders are dynamic, the distinctive and diagnostic regression period and the recently recognized late decline are challenges to trial design in RTT [5,65]. To our knowledge, the IGF-1 (mecasermin) trials are the first pediatric RTT studies to define inclusion criteria on the basis of the period of developmental regression and not age. Distinguishing between lack of efficacy and the 'natural decline' of the disorder makes data interpretation very difficult for interventions during the regression period with the potential for false-negative results. Paradoxically, reversal of regression is definitively one of the major goals of drug trials in RTT. Although there are not yet solutions to this conundrum, use of objective measures (e.g. head circumference) is even more critical at this stage. Nevertheless, if efficacy is demonstrated at older ages (i.e. using 12 months after the loss of the last skill as the operational definition of post-regression) [79], optimizing dosage and administration may demonstrate unquestionably a drug's positive effects. Similarly, although the decline at the other end of the age spectrum is less pronounced, strategies applied to neurodegenerative disorders could assist with trial design and analysis for studies of adults with RTT. Unfortunately, the easiest methodological solution to both heterogeneity and evolution (i.e. different developmental slopes) is large cohort size, which is both logistically and financially undesirable.

6.4. Combining drugs with cognitive stimulation for optimizing response

Like many neurodevelopmental disorders, RTT is considered to be a disorder of synaptic plasticity and most drugs used in trials target synaptic activity. For this reason, enhancing synaptic activation during drug action is a new strategy for optimizing trial outcome. While this approach has been employed in anxiety trials, no drug study to date has applied it to neurodevelopmental disorders. However, an upcoming trial in young children with FXS will combine the mGluR5 antagonist mavoglurant with a language learning paradigm (U01 NS096767-01. Effects of AFQ056 on Language Learning in Young Children with Fragile X Syndrome, P.I.: E. Berry-Kravis). Theoretically, these combined trials need to be implemented at the earliest possible ages, when synaptic plasticity is greatest. This is a particular challenge in RTT given that diagnosis, the earliest time point for intervention, typically coincides with the period of regression [130].

6.5. Implementing trials in a rare disorder like RTT

Implementing drug trials in rare diseases is challenging because of their low prevalence. In the case of RTT, additional factors that impact trial design are the severity of the disorder and the medical fragility of affected patients. Randomization to placebo and parallel design are not well accepted by many caretakers. This leads to blinded crossover designs that increase length and potential complications of the study, since it becomes difficult to differentiate a drug's adverse effects from expected medical complications of RTT. On the other hand, the perception of a poor prognosis with current treatments increases interest in drug trial participation. A new variable in recruitment and retention of subjects in clinical trials is social media, which could be an important modality for recruitment and dissemination of both useful and harmful information. Examples of the latter include sharing test answers for inclusion criteria, inaccurate data on adverse events, and attempts to unblind a trial.

6.6. Limited availability of adequate outcome measures

In a previous section, we summarized outcome measures employed throughout the history of drug trials in RTT. We also commented on their unique features and shortcomings. We concluded that most outcome measures used in RTT have not been disorder-validated according to regulatory standards. This means that the measures have not demonstrated adequate reliability, validity, and other measurement properties, such as sensitivity to change, according to the Food and Drug Administration (FDA) and the European Medicines Agency (EMA). More important than satisfying regulations is the need for high sensitivity endpoints, since early phase trials tend to use relatively lower doses because of concerns about adverse events. The experience from the trofinetide trials emphasizes the importance of attempting higher dosages in RTT, but doing so cautiously with the help of sensitive outcome measures. As reported in FXS and autism spectrum disorder, the limited availability of outcome measures with adequate measurement properties has been one of the main obstacles for progress in the field [123,131]. An additional challenge in RTT is the need to measure neurological outcomes in nonverbal, motor-impaired, commonly nonambulatory subjects. This affects not only instruments that directly evaluate cognition, communication, or motor function but also tests that require interaction with the subject. Objective endpoints and tests, usually termed biomarkers, are therefore desirable for RTT trials. Their development is still in early stages [79] and should be accelerated.

6.7. Demonstrating improvements in function and QoL

A recent emphasis of regulatory agencies is that efficacious drugs should also demonstrate improvements in levels of functioning and particularly QoL. In neurodevelopmental disorders, functioning is typically captured by adaptive behavior scales [27] or instruments measuring activities of daily living [132], both providing a more direct view of the actual capacity and level of independence of the patients. QoL has been studied at the level of individuals with RTT [133] and their caretakers [134] using the Child Health Questionnaire 50 and the Optum SF-36v2 Health Survey, respectively. Interestingly, while in children and adolescents with RTT clinical severity was directly correlated with poor physical QoL, motor function was inversely correlated with psychosocial QoL. These QoL instruments and data had previously been useful for assessing potential behavioral outcome measures [26]. Although already applied in some clinical trials, we expect the use of QoL measures to become standard in RTT drug studies.

6.8. From the clinic to drug trial: repurposing drugs and pragmatic trials

While designing drug trials on the basis of neurobiological mechanisms downstream to the genetic defect is the gold standard, this could be a very lengthy process. Alternatives include repurposing drugs, which allows omitting steps focused on safety and tolerability, and pragmatic trials that utilize clinical care as the setting for comparing the efficacy of common treatments. Of these, repurposing drugs has been a strategy applied multiple times in RTT with medications as different as bromocriptine, glatiramer acetate, and IGF-1. Most likely, the field of RTT clinical trials will continue to pragmatically combine drug repurposing with the testing of experimental medications.

With only a few completed mainly early phase clinical trials, it is difficult to predict the future of neurobiologically targeted treatments in RTT. The pre-*MECP2* era, in which drug development was based on a limited body of knowledge on disrupted neural mechanisms in RTT, was certainly disappointing. However, it did set the stage for the current trials based on preclinical work in mouse models. Despite the modest positive effects of most trials, which could be the result of low dosages (e.g. adolescent/adult trofinetide trial), there is considerable enthusiasm and several trials are at different stages of planning (see Katz et al. [8] for a systematic review). This active pipeline is driven in part by a continuous process of drug development in mouse models. The addition of other animal models, such as rats with *Mecp2* mutations, is likely to further accelerate the identification of new candidate drugs. As for FXS, the initiation of new drug trials is slowed by the clinical team bottleneck (i.e. limited number of qualified clinical investigators designing and implementing trials). Patient availability is not a limiting factor at this point; however, trial fatigue and disappointment may become issues if outcomes are not clearly positive.

The RTT field faces some of the same challenges posed by other neurodevelopmental disorders. One such challenge relates to multiple investigator-initiated trials with design shortcomings and/or lack of industry support. Underpowered, nonrandomized controlled studies lead to quick dismissal of potentially useful drugs. Trials that are lengthy because of recruitment and other protocol issues result in disengagement and confusion. Approval of drugs by regulatory agencies should be a goal per se, since drug availability and affordability are essential for medical impact in any clinical population. A limitation in the development of treatments specifically for RTT thus far includes a primary focus on two mechanisms or targets, namely IGF-1 compounds and glutamate modulators. The GABAergic system remains largely unexplored clinically, to some extent because of lack of clarity on the type and timing of abnormalities. The dynamic evolution of RTT, with its identification only after loss of skills is evident, represents another challenge for trial design that is further exacerbated by heterogeneity in its clinical course and severity. Targeting developmental regression is a logical goal that requires a better understanding of this phenomenon, and NMDA receptor-mediated toxicity seems the most plausible mechanism thus far. Timing of intervention and adequacy of outcome measures and biomarkers for selecting cohorts and detecting effects continue to be the main issues in trial design. Unfortunately, most trials have been launched without sufficient knowledge of the measurement properties of outcome measures that have been developed without systematic validation. There has been some progress in this area (e.g. modification of the Motor Behavioral Assessment scale for the trofinetide adult trial), but this is still insufficient. Promising biomarkers, such as the plethysmography-based apnea and hyperventilation indices for the IGF-1 trials, are objective and have face validity that makes them ideal endpoints. However, their acceptability by regulatory agencies is still an issue.

In conclusion, we are at the beginning of a difficult but potentially highly rewarding stage in drug trials for RTT. The suggestion of effectiveness of trofinetide in adults with RTT raises the possibility of successful treatments throughout the life span of an individual with the disorder. Strategies applied in other neurodevelopmental disorders, such as synaptic plasticity enhancement, may increase the prospects of clinical trials in RTT.

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Declaration of interest

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