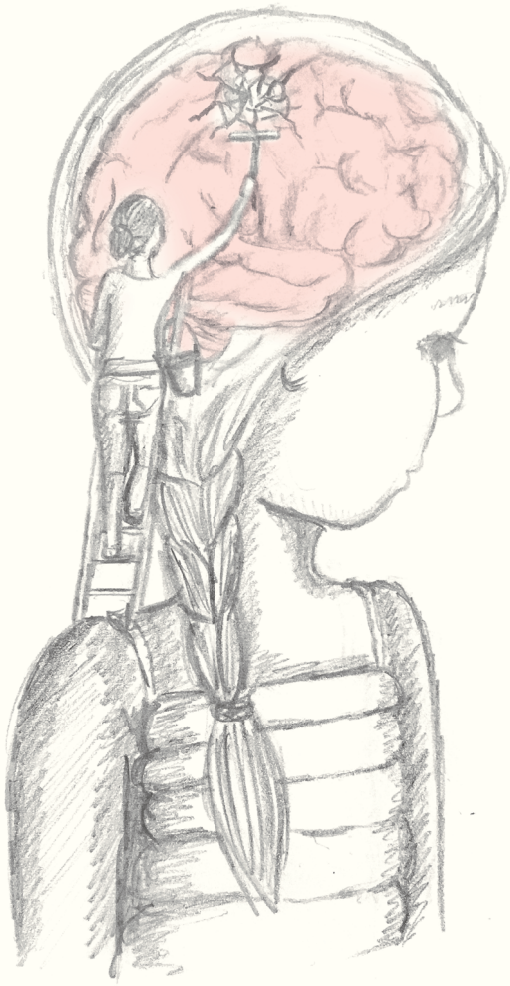

Tuberous Sclerosis Complex in children

clinical characteristics and targeted treatment

Iris Overwater

Tuberous Sclerosis Complex in children: clinical characteristics and targeted treatment

Iris Elisabeth Overwater

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CLINICAL CHARACTERISTICS AND TARGETED TREATMENT

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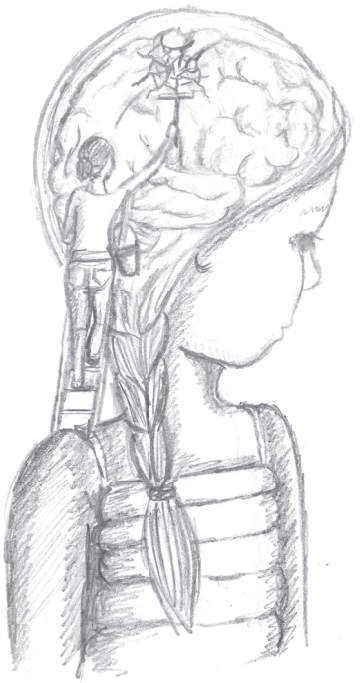
CHAPTER 1

General introduction, aims and outline



HISTORY OF TSC

Tuberous Sclerosis Complex (TSC) is a rare, genetic disorder with an incidence of 1 in 6000 births.[1] Although TSC is now considered a syndrome that consists of various clinical symptoms, these symptoms were first described as separate conditions.[2] In 1835, Rayer published a drawing of a patient with angiofibroma of the face in his atlas of skin diseases, naming it 'végétations vasculaire' (figure 1a). Von Recklinghausen described two other symptoms in 1862, after observing an infant who died moments after birth with 'myomata in the heart' and 'scleroses in the brain'. After several more descriptions of isolated symptoms, the French neurologist Désiré-Magloire Bourneville was the first to put various features together. He described a 15 year old girl with 'psychomotor retardation, epilepsy and a confluent vascular-papulous eruption of the nose, the cheeks and forehead'. The post-mortem examination showed 'hard, dense tubers in her brain' (figure 1b), and 'whitish hard masses in her kidneys'. Bourneville named this combination of features 'Sclérose tubéreuse des circonvolutions cérébrales' and published his findings in 1880. TSC was called Bourneville's disease for many years, until the term Tuberous Sclerosis Complex became widely accepted.

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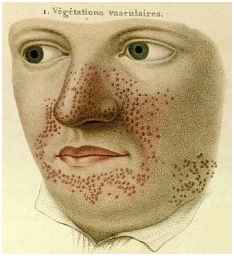

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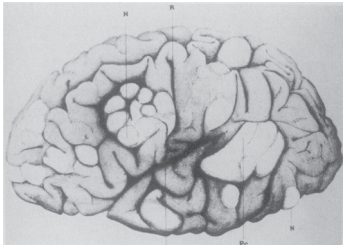

Figure 1. Images of the first descriptions of TSC. (A) Angiofibromas on the face, described at the time as 'végétations vasculaires'. (B) Brain of a TSC patient, in which 'hard, dense tubers' were found upon post-mortem examination.

MOLECULAR AND CELLULAR ASPECTS OF TSC

Decades of research into understanding TSC have led to the identification of mutations in the *TSC1*[3] or the *TSC2*[4] gene as the cause for TSC. These genes encode for the proteins hamartin and tuberin respectively, and form a complex called the TSC1-TSC2 complex. The *TSC2* gene has a GTP-ase activating protein (GAP) domain. This domain is important for the function of the TSC1-TSC2 complex. The TSC1-TSC2 complex is part of the mammalian target of rapamycin (mTOR) pathway depicted in figure 2a,[5,6] a cellular signaling pathway involved in the regulation of cell behavior. The mTOR complex 1 (mTORC1)

protein integrates signals of growth factors, energy levels and oxygen availability to regulate protein translation, cell proliferation, cell differentiation and ultimately also synaptic plasticity. This output is executed through several proteins downstream of mTORC1, including serine 6 (S6). The activated, phosphorylated S6 protein (pS6) is used as readout of mTORC1 activity in many studies.

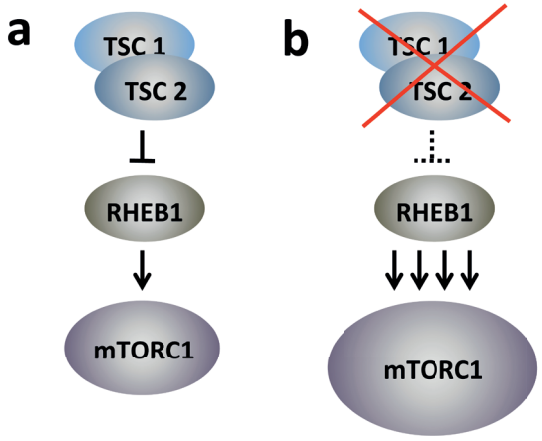

Figure 2. mTORC1 protein pathway. (A) mTORC1 pathway activation in healthy persons. The TSC1-TSC2 complex inhibits RHEB1, thereby controlling the intensity of activation of mTORC1. (B) In patients with TSC, a mutation in either *TSC1* or *TSC2* renders the TSC1-TSC2 complex inactive. This removes the inhibition of RHEB1, and allows RHEB1 to hyperactivate mTORC1.

The TSC1-TSC2 complex exerts its effects on mTORC1 via the small GTP-ase RHEB1 (RAS-homolog enriched in brain).[7] RHEB1 cycles between an active GTP bound state and an inactive GDP bound state, and is rate-limiting for mTORC1 activation.[8,9] In the active GTP bound state, RHEB1 stimulates mTORC1 activity. The GAP activity of the TSC1-TSC2 complex can catalyze the hydrolysis of RHEB1-GTP to RHEB1-GDP, inactivating RHEB1 and therefore decreasing the activated state of mTORC1.

Patients suffering from TSC have a malfunction of the mTORC1 pathway, caused by heterozygous inactivating mutations of the *TSC1* or *TSC2* gene. Using the current laboratory methods, such a mutation can be found in about 80% of patients. The inactive TSC1-TSC2 complex has reduced GAP activity, and is unable to sufficiently inactivate RHEB1. Therefore, mTORC1 is less inhibited and will stay in the activated state (figure 2b).

CLINICAL FEATURES OF TSC

TSC criteria

TSC causes morbidity in various organ systems.[10,11] The increased cell proliferation due to the hyperactivated mTORC1 pathway can lead to hamartomas in the heart (cardiac rhabdomyoma), kidneys (angiomyolipoma, AML), lungs (lymphangioleiomyomatosis, LAM) and eyes (retinal hamartoma). Patients may be recognized by TSC features on the skin, including vascular macules on the face (angiofibroma), white hypopigmented spots (hypomelanotic macules), fibromas of the nailbed (ungual fibromas) and intraoral fibromas.[12] The age of onset of these features varies highly (table 1).[10] Cardiac rhabdomyomas often develop in the fetal period and usually regress during a patient's life,[13] while renal AML develop in childhood or adulthood.[14]

Table 1. Diagnostic clinical criteria for Tuberous Sclerosis Complex, adapted from Crino[10] and Northrup[15].

Major features	**Age of onset**
Hypomelanotic macules	Infancy to adulthood
Angiofibromas or fibrous cephalic plaque	Infancy to adulthood
Ungual fibromas	Adolescence to adulthood
Shagreen patch	Childhood
Multiple retinal hamartomas	Infancy
Cortical dysplasias: tubers and radial migration lines	Fetal life
Subependymal nodules	Childhood to adolescence
Subependymal giant-cell astrocytoma	Childhood to adolescence
Cardiac rhabdomyoma	Fetal life
Lymphangioleiomyomatosis	Adolescence to adulthood
Renal angiomyolipoma	Childhood to adulthood

Minor features
'Confetti' skin lesions
Dental enamel pits
Intraoral fibromas
Retinal achromatic patch
Multiple renal cysts
Nonrenal hamartomas

The clinical diagnosis of TSC is based on clinical features (table 1).[15] Clinical criteria have been established, and have been divided in major features that are highly associated with TSC, and minor features that are less associated with TSC. A definitive diagnosis of TSC can be made if an individual exhibits two major features, or one major and two

minor features. The diagnosis of TSC can also be made if a pathogenic mutation is identified in *TSC1* or *TSC2*, regardless of any clinical features.

Neurological features

The central nervous system (CNS) is affected in over 90% of all TSC patients,[16] and morbidity of the CNS causes the majority of all TSC related health problems.[17] Several types of brain pathology can be present in TSC patients, of which most can be visualized by magnetic resonance images (MRI). Since brain pathology can worsen over a patient's lifetime, and can cause serious morbidity, it is recommended that an MRI of the brain should be made every 1-3 years in children with TSC, to monitor any progression of CNS morbidity.[18]

Cortical tubers

80-90% of patients have cortical tubers. An example of cortical tubers on MRI is shown in figure 3a. These are areas of the cerebral cortex where cells have failed to differentiate,[19] causing them to be enlarged (giant cells) and dysmorphic. The cells fail to migrate properly, and cause a loss of cortical lamination.[20] These cellular changes are caused by the increased activation of the mTORC1 pathway, as shown by elevated levels of pS6 in

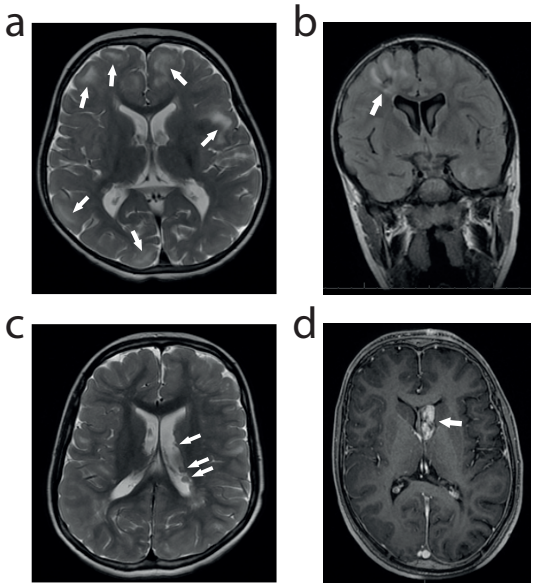

Figure 3. Brain MRI images of 4 patients with TSC. (A) Axial T2 weighted image. Cortical tubers are indicated by the white arrows. (B) Coronal FLAIR image. The white arrow indicates a RML. (C) Axial T2 weighted image. The arrows indicate three SENs in the lateral ventricle. (D) Axial T1 weighted image. The arrow indicates a SEGA in the lateral ventricle.

these cells.[21] Dysmorphic cells were also found to be present outside of cortical tubers, either solitary or in small patches of cells.[22] The specific increase in mTORC1 activation in these cells, and not in the cells surrounding the tuber, is thought to arise from a second hit on the other allele of the mutated gene. This is difficult to investigate, but has been shown in material from a number of patients that have undergone epilepsy surgery.[21]

Subependymal nodules and subependymal giant cell astrocytoma

Another type of CNS pathology is subependymal nodules (SENs), shown in figure 3c. These are hamartomas in the lateral ventricles of the brain. SENs do not cause any morbidity, as they are small and are not thought to interfere with brain function. SENs are thought to form as early as the fetal developmental period, but can also be discovered during childhood or adolescence. They can stay stable in size or grow. If a SEN grows to over 1 centimeter in diameter, it is called a subependymal giant cell astrocytoma (SEGA) (figure 3d). These are present in 10-20% of patients, and most often grow in childhood.[23] SEGAs are benign tumors and could be present throughout a patient's lifetime without causing health issues. However, if the SEGA is located near the foramen of Monro, it can cause obstruction of cerebral spinal fluid flow, and therefore cause hydrocephalus and in extreme cases even death.[24,25]

White matter

The white matter of the brain is important for signal transduction, and contains mostly myelinated axons of neurons. MRI images can show abnormalities in white matter. Recently, researchers have become more interested in areas of white matter that seem normal on traditional MRI sequences such as T1, T2 or FLAIR images, often called normal appearing white matter (NAWM). Studies have shown that this NAWM might not be entirely without abnormalities in TSC patients.[26,27] Using diffusivity MRI, it was shown that there may be a delay or impairment in myelination of axons. Another recent study showed that radial migration lines (RMLs) were present in the white matter of all 30 patients who were examined.[28] These RMLs have the same signal intensity on MRI as cortical tubers,[29] and are therefore thought to contain the same aberrant cells as cortical tubers. An example of an RML on MRI is shown in figure 3b.

Epileptic seizures

A major source of morbidity in TSC patients is epileptic seizures. Epilepsy occurs in 80-90% of TSC patients.[30] Seizures often start very early in life, and 50% of patients have their first seizure before the age of six months (figure 4).[31] More than half of all TSC patients have multiple seizure types, and two thirds of patients have refractory epilepsy that is uncontrolled despite the use of multiple anti-epileptic drugs (AEDs).[32] Epileptic seizures can be diagnosed clinically, or through observing patterns of brain activity on electro-

encephalogram (EEG). In a third of all TSC patients, the first type of seizures are infantile spasms.[33] This severe type of epileptic seizures is associated with a developmental delay and a hypsarrythmia pattern on EEG, and is called West syndrome if all three features occur. Infantile spasms are difficult to treat, and can persist after the second year of age as epileptic spasms.[34]

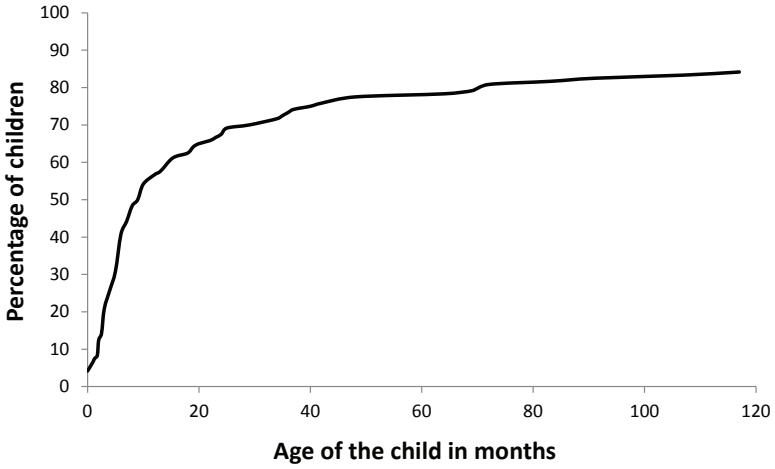

Figure 4. Age of epilepsy onset in 120 children with TSC treated in the ENCORE TSC expertise center.

Many researchers have tried to elucidate the origin of epileptic seizures in TSC. According to several groups, cortical tubers might be able to initiate seizures.[35,36] This was not a surprising finding, as cortical tubers are types of cortical dysplasia. Many different types of cortical dysplasia exist, and all patients have an increased risk of developing epilepsy, which is often intractable to AEDs.[37] The finding of the seizure initiating features of cortical tubers are supported by the fact that removing a tuber surgically can stop seizures.[38] However, it has also been suggested that not the tuber itself, but the cortex surrounding a tuber might be causing seizures, and the tuber itself is silent.[39-41] Research on animal models have shown that the increased activation of mTORC1 can also cause seizures, in the absence of any brain pathology.[42]

Intellectual disability and behavioral difficulties

Besides physical problems, TSC patients might also suffer from cognitive disabilities varying from learning difficulties to severe cognitive impairment and several behavioral problems. Even though the physical consequences of TSC cause most of the visits to medical professionals, patients and parents have indicated repeatedly that cognitive

disabilities and behavioral problems are much more debilitating to them, and have the largest impact on daily life.

Cognitive development is described as the construction of thought processes, including remembering, problem solving and decision-making. It is often referred to as the intellectual quotient (IQ) or, in patients too young to measure intellectual abilities, as the developmental quotient (DQ). For the cognitive development of an individual to be optimal, it is important that the brain is able to make new connections between neurons. Regulating processes of these connections are called synaptic plasticity, and are thought to underlie all learning.[43] The mTOR pathway is very important in this process,[44] and many mouse models of TSC have been described with impairments in synaptic plasticity and learning.[45] Among patients with TSC, intellectual abilities vary considerably.[46,47]

Behavioral and neuropsychiatric problems are another core symptom of TSC with a wide range of severity. Recently, these cognitive and behavioral problems have been grouped as Tuberous sclerosis Associated Neuropsychiatric Disorders or TAND.[48] A TAND questionnaire was developed to aid clinicians in their consultations, to get a complete view of all cognitive and behavioral issues of a patient. constructed to aid clinicians in getting a complete view of all cognitive issues a patient may suffer from.

Neuropsychiatric symptoms include autism, and psychiatric issues such as anxiety and depression.[16,49,50] Other behavioral difficulties include aggression and tantrums, attention problems and social-emotional problems. In the general population, autism occurs 4.5 times more frequently in males than in females.[51] However in TSC, females are equally at risk for autism as males. In addition, patients with TSC are more likely to have autism or a disorder in the autism spectrum, as about 50% of TSC patients is thought to suffer from autism, compared to 1.5% in the general population.[51] The range of features within these autism patients, however, is similar to those with autism that is unrelated to TSC.

VARIABILITY OF TSC RELATED SYMPTOMS

The variability of TSC features among patients is considerable. Not only can certain features occur in some whilst never occurring in others, the severity of most features and the age of onset can also vary. Variation even occurs in family members and within monozygotic twins.[52-54] This variation occurs in features within the brain and outside of the brain. For features outside of the brain, the variation is thought to be due to loss of heterozygosity, and is most likely stochastic. For brain related factors, the cause of variation is still unknown, but is probably caused by several factors, including LOH.

One of the most striking variations is the one in cognitive development. Some patients with TSC have a normal cognitive development and attend regular schooling, while about a third of patients are severely intellectually disabled. This might be due to differences in genetics, for example the type of mutation[55-57] or the proportion of cells in the brain that have a mutated second allele.[58] It could also be because cognitive development is correlated with clinical symptoms, for example epilepsy and more specifically the age at which the first seizure occurs,[59-61] or the number of tubers.[62,63] Some studies take the description a step further, and have reported that cognitive development might be multifactorial and not depend on one specific feature related to TSC.[64-66]

Another variable symptom amongst TSC patients is the extent of brain pathology. While in some patients an MRI might show multiple cortical tubers and a SEGA requiring treatment, another patient can have an MRI with only mild abnormalities. Part of this variation might be due to a mutation in *TSC2* rather than in *TSC1*.[59,67,68]

In turn, cortical tubers in the temporal lobe and insular area[69] and cyst-like cortical tubers[70] have been associated to a higher incidence of autism spectrum disorder (ASD) in patients with TSC. In another study, a higher incidence of ASD was observed in TSC patients with epilepsy, infantile spasms, and patients with a mutation in the *TSC2* gene.[71]

As is shown by the correlations made in the mentioned studies, not only is the variability among TSC patients considerable, there is also a large number of disease related factors that could be either causing the variability, or be caused by another disease related factor. Which part of the variability can be explained by which factors has not been fully elucidated to date, especially concerning brain pathology, ASD features and IQ. In chapters 2, 3 and 4, we examined genetic mutations and TSC related brain pathology, determinants of ASD features and clinical factors associated with IQ, and studied which factors might influence the variability of these features observed in children with TSC. Identifying these factors could be very important for TSC patients, as this could help to understand what treatments may prevent other TSC related symptoms and how to manage these symptoms.

TREATMENT OF TSC SYMPTOMS

Due to its variable phenotype, no treatment exists for the full spectrum of TSC symptoms. Current treatments are mostly symptom based. These treatments traditionally do not differ from the treatment of non-TSC patients with such a symptom.

Brain pathology treatment

Tubers and RMLs are not pathogenic. They do not grow over time and do not become malignant. They can be epileptogenic, and might therefore play a role in initiating sei-

zures, however as long as they do not have a role in epilepsy, there is no reason to treat them. The situation is different if a patient has a SEGA, as this tumor can grow during life and can cause life-threatening complications.[72,73] Several studies have shown that SEGA can be excised surgically,[74,75] however other studies have shown that SEGA can regrow after it has been excised.[76]

Epilepsy treatment

Epilepsy has been estimated to occur in 0.2%-4.1% of the general population.[77] Anti-epileptic drugs reduce the number of seizures by different modes of action, in general by enhancing the effect of GABA-ergic interneurons or reducing the excitability of glutamatergic neurons.[78] Very often in TSC patients, the use of one drugs with a specific working mechanism is not sufficient for treating seizures, and several drugs are combined to attempt a reduction in seizure frequency. This combination of AEDs increases the chances of success of the therapy, however each drug has side effects, and combining different AEDs may worsen these side effects. AEDs might therefore not always be the treatment of choice, and other, more invasive treatments are also available. In patients where epileptic seizures originate from one particular brain area that can be safely surgically approached, epilepsy surgery can be curative.[79] If surgery is not an option, a ketogenic diet is another possibility.[80] By reducing the intake of carbohydrates, this diet mimics a state of fasting where the body switches to fatty acids as fuel. The liver produces ketone bodies that can be used as an energy source by the brain instead of glucose. This can reduce seizures.[81] A third option to treat epilepsy if AEDs are not sufficient is the implantation of a vagus nerve stimulator, which gives off electric pulses to the right vagus nerve in the neck to reduce the number of epileptic seizures.[82] With so many available treatments for epilepsy, it is still unclear which is the best treatment strategy for TSC related epilepsy, and whether in fact any of these treatments should be applied to TSC patients. Therefore, in chapter 5 we examined the epilepsy treatment given to patients with TSC related epilepsy, and assessed the effectiveness of these therapies on the short and long term.

Targeted treatment using mTORC1 inhibition

For a long time, management of symptoms was the only way to treat patients with TSC. However, since the discovery of the *TSC1* and *TSC2* genes and their role in the mTORC1 pathway, new targeted treatment for TSC have been developed. Elucidating the proteins involved in the pathogenesis of the TSC phenotype creates new targets for drugs that act in this pathway (figure 5). The mTORC1 protein was named after a previously identified drug called rapamycin, also known as sirolimus. Sirolimus inhibits mTORC1 by binding the cellular receptor FK-506 binding protein (FKBP12), and subsequent binding to the FKBP-rapamycin binding domain (FRB) of mTOR.[83] Sirolimus is a macrolide isolated from

a strain of *Streptomyces hygroscopius* that was found in the soil on Easter Island (also known as Rapa Nui) in 1975.[84] Decades later, biological research identified its molecular target, naming it the mammalian target of rapamycin or mTOR.[85] As it turned out, rapamycin, or sirolimus, had various qualities, such as acting as an anti-proliferative and immunosuppressive agent.[86] This triggered the use of sirolimus as a cancer treatment, and as a treatment for patient who had an organ transplant, to prevent the rejection of the donor organ.

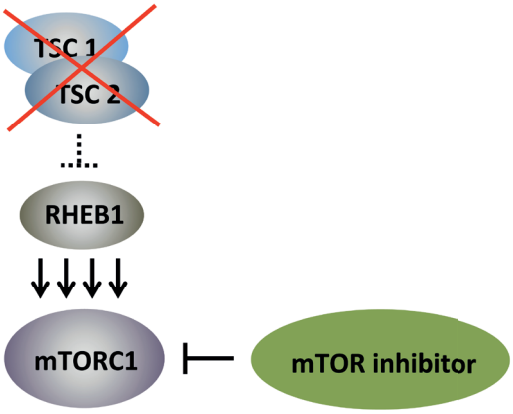

Figure 5. mTORC1 pathway when a TSC patient is treated with an mTOR inhibitor. The TSC1-TSC2 complex does not inhibit RHEB1, therefore RHEB1 still hyperstimulates mTORC1. However, due to the inhibiting effect of the mTOR inhibitor, the activation of mTORC1 decreases.

mTORC1 inhibition treatment in patients

When it was discovered that the mTORC1 protein is involved in the TSC phenotype (figure 2b), experiments focused on using sirolimus as a treatment for various TSC symptoms. Trials in patients benefitted from the experience with sirolimus treatment in the treatment of cancer and organ transplant patients. The first clinical examinations showed a decreased volume of SEGA in the brain[87] and renal AML[88] upon treatment with sirolimus. With this growing interest in sirolimus as a treatment for TSC, a pharmaceutical company developed everolimus, a drug similar to sirolimus in molecular structure and function, but with better water solubility and bioavailability.[89] Larger trials followed, and showed the effectiveness of everolimus in decreasing SEGA[90] and AML,[91] leading to the registration of everolimus as a regular treatment for SEGA and AML related to TSC.

mTORC1 inhibition treatment for epilepsy in TSC

Preclinical research has shown that sirolimus can reduce or even stop seizure frequency in several mouse models of TSC.[42,92-94] Several case series in TSC patients, both children and adults, have examined the effect of either sirolimus or everolimus on seizure frequency. Treatment frequencies, dosages and outcomes varied among these case series, and some studies only included one patient.[95-101] However, the general tone of these studies was positive towards a seizure frequency decrease due to mTORC1 inhibition treatment. In 2013, a small study of 20 TSC patients with intractable epilepsy was the first to show a beneficial effect of everolimus in a prospective manner.[102] Patients were included in the study if they had at least eight seizures in a baseline period of 30 days. All patients were treated for 12 weeks, of which the first four weeks were a titration period in which patients might not yet have reached the effective trough level of everolimus. Analysis of the number of epileptic seizures reported by parents showed that 12/20 patients had a seizure frequency decrease of 50% or more at the end of the treatment period. The authors concluded that everolimus may be a therapeutic option for intractable epilepsy in patients with TSC. This trial is indeed an important step in the assessment of the efficacy of mTOR inhibitors for TSC related epilepsy. However to conclude from this trial that mTOR inhibition may be a therapeutic option is quite a large step, as this study treated all included children with everolimus, without randomization and a comparative group or study period. As seizure frequency varies, it is important to include a control group that is either not treated or treated as usual care. We therefore conducted a randomized cross-over trial investigating the effect of sirolimus on seizure frequency in children with TSC (chapter 6).

RESEARCH INTO TREATMENT OF COGNITIVE IMPAIRMENT

Many genetic disorders are known to cause cognitive impairment. Given the severe consequences for the daily functioning of these individuals, preclinical as well as clinical research has been focused on ways to improve cognitive functioning in these patients. The possible benefit of mTORC1 inhibitors has also been investigated in relation to cognitive impairments due to TSC. Preclinical research has shown that sirolimus can improve learning in a mouse model of TSC.[103] Research in patients has been scarce so far. A study investigating the effect of sirolimus on LAM of the lung also examined neurocognitive function.[104] Four months of sirolimus treatment caused a >20% improvement of immediate and delayed recall memory in 5 out of 8 subjects. The number of treated patients in this preliminary study was small, however, and only a subset of possible cognitive outcome measures was used.

Most often, a new compound developed by for example a pharmaceutical company is first tested in cell lines, and consequently on animal models if possible. If positive results are found in these phases of research, a drug may enter trials on patients to examine pharmacokinetics and pharmacodynamics. Eventually a possible beneficial effect on disease characteristics may be discovered, leading to the registration of such a treatment as an official drug for that disorder specific symptom. The number of compounds entering the first phase of preclinical trials is many folds higher than the number of treatments that are eventually registered. In chapter 7, we performed a systematic search of current literature, to examine the cause for this discrepancy.

AIMS OF THIS THESIS

The general aim of this thesis is to study the determinants of the variability in phenotype in patients with TSC, and to investigate the optimal treatment for epilepsy in children with TSC.

The specific aims of the studies in this thesis are:

1. To obtain more knowledge about the relationships between
   - genetic mutations in *TSC1* and *TSC2* and TSC-related brain pathology,
   - TSC-related brain pathology and autistic traits,
   - and clinical factors related to TSC and IQ.
2. To investigate the efficacy of treatments that are currently used for epilepsy related to TSC.
3. To assess the effect of sirolimus on TSC related intractable epilepsy in children.
4. To give an overview of clinical trials investigating intellectual disability in genetic disorders, and identify reasons why many drugs that seem promising in animal research do not become registered treatments for the investigated condition.

OUTLINE

In chapter 2, we describe TSC-related brain pathology in children to genetic mutations found in *TSC1* and *TSC2*. We divide these genetic mutation in 3 groups: a mutation in *TSC1*, a mutation in *TSC2* that leaves an active protein, or a mutation in *TSC2* that does not leave an active protein. Based on these three mutation groups, we determine whether specific TSC related brain pathology occurs more with one mutation than another.

In chapter 3, we assess ASD signs in a group of children with TSC. To determine the relation between TSC related brain pathology and ASD, we examine whether the se-

verity of ASD is correlated with the type of brain pathology, and whether this effect is mediated by IQ.

In chapter 4, we carry out an extensive analysis on a group of 102 patients with TSC. We examine the clinical characteristics and gene mutation of these children related to their IQ, to determine whether specific genetic and clinical factors are associated with IQ in an independent manner.

Chapter 5 describes the course of epilepsy and epilepsy treatment in a group of children with TSC. We assess which epilepsy treatment was used, what the effect of the medication was, and which children had a recurrence of seizures.

In chapter 6, we describe the findings from our clinical trial, investigating the effect of the mTORC1 inhibitor sirolimus on intractable epilepsy in children with TSC. We treat23 children with sirolimus in a cross-over trial, to determine whether seizure frequency decreased after 6 months of sirolimus treatment.

Chapter 7 is a systematic review of treatments for intellectual disability due to various genetic causes, based on clinical trials. With this review, we hope to reveal how preclinical trials evolve into registered treatments for intellectual disability.

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CHAPTER 2

Genotype and brain pathology phenotype in children with Tuberous Sclerosis Complex

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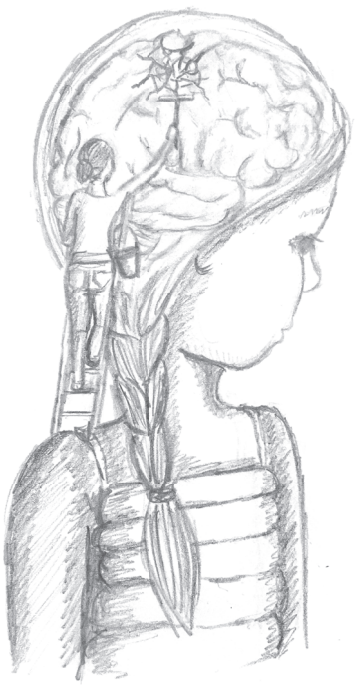

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ABSTRACT

Structural brain malformations associated with Tuberous Sclerosis Complex (TSC) are related to the severity of the clinical symptoms and can be visualized by magnetic resonance imaging (MRI). TSC is caused by inactivating *TSC1* or *TSC2* mutations. We investigated associations between TSC brain pathology and different inactivating *TSC1* and *TSC2* variants, and examined the potential prognostic value of subdivision of *TSC2* variants based on their predicted effects on *TSC2* expression. We performed genotype-phenotype associations of TSC-related brain pathology on a cohort of 64 children aged 1.4-17.9 years. Brain abnormalities were assessed using MRI. Individuals were grouped into those with an inactivating *TSC1* variant and those with an inactivating *TSC2* variant. The *TSC2* group was subdivided into changes predicted to result in TSC2 protein expression (*TSC2*p) and changes predicted to prevent expression (*TSC2*x). The *TSC2* group was associated with more and larger tubers, more radial migration lines, and more subependymal nodules than the *TSC1* group. Subependymal nodules were also more likely to be calcified. Subdivision of the *TSC2* group did not reveal additional, substantial differences, except for a larger number of tubers in the temporal lobe and a larger fraction of cystic tubers in the *TSC2*x subgroup. The severity of TSC-related brain pathology was related to the presence of an inactivating *TSC2* variant. Although larger studies might find specific *TSC2* variants that have prognostic value, in our cohort, subdivision of the *TSC2* group did not lead to better prediction.

INTRODUCTION

Tuberous Sclerosis Complex (TSC) is an autosomal dominant disorder caused by inactivating *TSC1* or *TSC2* variants.[1,2] Most TSC-associated lesions are thought to arise due to somatic second-hit mutations that inactivate the remaining wild-type *TSC1* or *TSC2* allele. The protein products of *TSC1* and *TSC2* form the TSC complex, that inhibits the mammalian Target of Rapamycin Complex 1 (mTORC1).[3] Loss or inactivation of the TSC complex results in constitutive activation of mTORC1, and mTORC1 inhibitors have been shown to be useful for treating hamartoma-related complications of TSC.[4,5]

Our aim was to investigate genotype-phenotype associations in a well-characterized cohort of TSC individuals, focusing on the relationships between specific *TSC1* and *TSC2* variants and macrostructural brain lesions detected by magnetic resonance imaging (MRI), including cortical tubers, radial migration lines (RMLs), subependymal nodules (SENs) and subependymal giant cell astrocytomas (SEGAs). In most studies, inactivating *TSC2* variants are associated with increased numbers of cortical tubers and a higher prevalence of SEGAs.[6-14] We investigated whether there was additional clinical value for subdivision of *TSC2* variants, as has been described recently for cognitive function in TSC.[15] We compared TSC-related brain pathology as assessed by MRI, in individuals with an inactivating *TSC1* variant to brain pathology in individuals with an inactivating *TSC2* variant. In addition, we compared the *TSC1* group to individuals with a *TSC2* variant predicted to prevent *TSC2* mRNA expression (*TSC2*x) and to individuals with a *TSC2* variant predicted to either alter the TSC2 amino acid sequence or result in reduced *TSC2* expression (*TSC2*p).

METHODS

Patients

Children treated at the ENCORE-TSC Expertise Center of the Erasmus MC-Sophia Children's Hospital, Rotterdam, the Netherlands with a genetically confirmed TSC diagnosis and at least one brain MRI were eligible for inclusion. Inactivating *TSC1* or *TSC2* variants were identified in 108 individuals, of whom 101 had at least one MRI available. In 64 cases the quality of the MRI was suitable for analysis, based on the criteria described below.

Genetic analysis and functional assessment

Molecular testing was performed at the Department of Clinical Genetics of the Erasmus MC. All identified variants were assessed with ALAMUT mutation prediction software

(version 2.6.1 (January 2015); Interactive Biosoftware, Rouen, France). Exons were numbered according to genomic reference sequences NG_012386.1 (*TSC1*) and NG_005895.1 (*TSC2*); cDNA notation was according to transcript reference sequences NM_000368.4 (*TSC1*) and NM_000548.3 (*TSC2*).

Functional assessment was performed as described.[16] For the analysis of *TSC2* variants, HEK 293T cells in which exons 2 - 38 of *TSC2* had been deleted by CRISPR/Cas9 genome editing[17] were used. Briefly, guide oligos 5′-caccgacggagtttatcatcaccg-3′ and 5′-aaaccggtgatgataaactccgtc-3′ (exon 2), and 5′-caccggttatcgccacgcaccact-3′ and 5′-aaacagtggtgcgtggcgataacc-3′(intron 38) were cloned into the pX458 and pX459 vectors,[18] and transfected into HEK 293T cells. Following puromycin selection, GFP-positive cells were single-cell sorted and grown in 96-well plates. The resultant colonies were trypsinised, expanded and validated by PCR, sequencing and immunoblotting. A single subclone, 3H9, was used for subsequent functional assessments.

For the detection of mosaic individuals, targeted Next Generation Sequencing of the *TSC1* and *TSC2* loci was performed, as described previously.[19] Clinical, genetic and functional data from this study have been submitted to the *TSC1* and *TSC2* Leiden Open Variant Databases (LOVD) (http://www.lovd.nl/TSC2; http://www.lovd.nl/TSC1).

Magnetic resonance imaging

Brain MRIs were made at the Erasmus MC-Sophia Children's Hospital on a 1.5 Tesla General Electric scanner using a standard protocol of axial and coronal T1, T2 and fluid-attenuated inversion recovery (FLAIR) sequences. To achieve an as uniform as possible sample, the MRI made closest to 8 years of age was selected. MRIs from individuals less than 12 months of age were not used because, at that age, myelination has not progressed enough to be able to measure tuber size and detect RML reliably. MRIs were excluded if there were movement artefacts, if axial images were absent, or when secondary structural abnormalities not directly related to TSC were present.

All MRIs were assessed by two trained medical students, and re-assessed by a pediatric neuroradiologist and a pediatric neurologist, who were blinded to the genotype and clinical characteristics of the patient. Picture Archiving and Communication System (PACS) software was used for all assessments.

The numbers and locations of all TSC-related brain abnormalities were assessed and verified in all available MRI sequences. For each tuber, the largest axes parallel and perpendicular to the gyrus were measured on axial slices, and multiplied to obtain an estimate of the maximum cross-sectional area. All lesions were inspected for cystic changes or calcifications on T2, FLAIR and, if available, susceptibility weighted angiography sequences.

Statistical analysis

Univariate regression analysis was used to compare continuous outcomes. A Student's t-test was used for comparing two groups of continuous data, and a chi-square test was used for categorical data. For comparisons between multiple groups, an analysis of variance test with a Bonferroni *post-hoc* correction was used for continuous data, and a chi-square test was used for categorical data. To correct for multiple testing, a false discovery rate test was used. All the outcomes of the statistical testing are included in A-Table 3; *q* values are given in the text where the corresponding *P* value was no longer significant after correction for multiple testing.

RESULTS

Patient population and genetic variant subdivision

In total, 64 patients aged 1.4-17.9 years were included (table 1); 21 (33%) had an inactivating *TSC1* variant and 43 (67%) had an inactivating *TSC2* variant (Table 1 and A-Tables 1 and 2). We defined inactivating variants as those that were predicted to either prevent mRNA expression, truncate the open reading frame prematurely, or affect TSC complex function. We divided the variants into 3 groups: *TSC1*,*TSC2*x and *TSC2*p. The *TSC1* group consisted of 21 individuals with 20 different *TSC1* variants, including eight predicted frameshift variants, eight predicted nonsense variants, two large deletions, one predicted missense variant and one substitution predicted to affect splicing. The *TSC2*x group consisted of 26 variants that were predicted to either prevent *TSC2* mRNA expression, or render the *TSC2* mRNA subject to nonsense mediated decay (NMD). This group included seven frameshift and eight nonsense variants, four large deletions and seven variants predicted to affect splicing. The *TSC2*p group consisted of variants that were predicted to alter the TSC2 amino acid sequence or to result in reduced levels of functional *TSC2* mRNA. We defined functional mRNA as not subject to NMD and encoding the TSC2 GAP domain (amino acids 1616 - 1654[1]). Nonsense and frameshift variants in the last exon and the last 18 codons of the penultimate exon were presumed to escape NMD.[20] The *TSC2*p group consisted of 13 different variants in 16 individuals, and included 7 missense variants (1 variant in 2 individuals), an in-frame deletion, a nonsense and a frameshift variant both predicted to escape NMD, and 2 variants (1 variant in 3 individuals) that were predicted to affect splicing, but might still result in expression of functional *TSC2* mRNA.

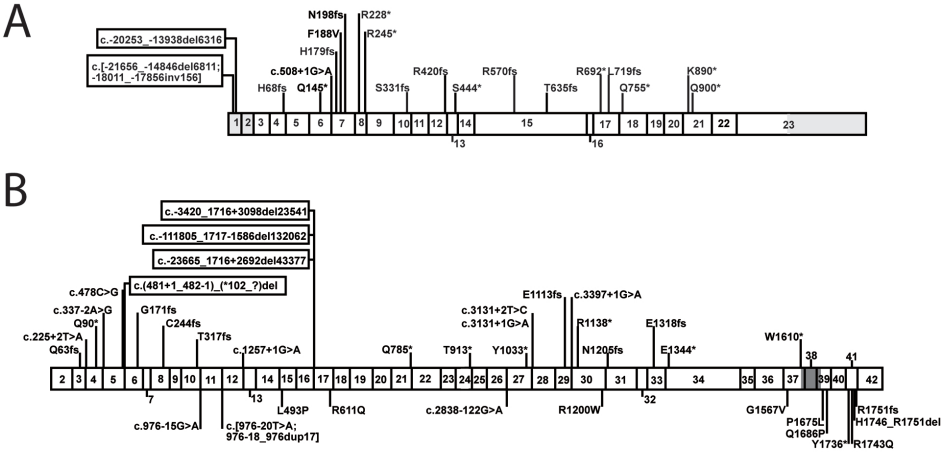

Figure 1 Schematic overview of *TSC1* and *TSC2* variants. Exon numbering is according to build GRCh37 (hg19) of the human reference sequence; cDNA numbering is according to reference transcripts NM_000368.4 (*TSC1*) and NM_000548.3 (*TSC2*).
(A) *TSC1*. Approximate positions of the *TSC1* variants identified in our TSC cohort are indicated relative to exons 1 - 23. Large deletions are boxed with the approximate position of the distal extent of the deletion, relative to the exons, indicated. Non-coding 5' and 3' untranslated regions (UTR) are shaded in grey; the 3'UTR in exon 23 is not drawn to scale. (B) *TSC2*. Approximate positions of the *TSC2* variants identified in our TSC patient cohort are indicated relative to exons 2 - 42. Large deletions are boxed and the approximate position of the distal extent of the deletions are indicated. The region encoding the TSC2 GAP domain (amino acids 1616 - 1654) is shaded grey. All variants predicted to result in the absence of TSC2 (*TSC2*x) are shown above the exons; variants where expression of a mutant form of TSC2 (*TSC2*p) was considered possible are shown below the exons (see tables in the appendix and text for details).

Table 1 Characteristics and TSC specific brain abnormalities of 64 patients with an inactivating *TSC1* or *TSC2* variant.

	TSC1 **(n=21)**	***TSC2*** **(n=43)**	***TSC2*****p** **(n=19)**	***TSC2*****x** **(n=24)**
Age at MRI in years	7.3. (2.15-15.5)	7 (1.4-17)	6.9 (1.8-9.9)	7.3 (1.4-17)
Gender male, n (%)	12 (57)	20 (47)	6 (38)	14 (52)
Inheritance, n (%)				
Familial	4 (19)	8 (19)	7 (37)	1 (4)
De novo	12 (57)	28 (65)	7 (37)	21 (88)
Cortical tubers				
Total number	8 (0-36)	41 (2-98)	21 (5-95)	45 (2-98)
Total surface area (mm^2)	304 (0-1138)	2105 (85-5552)	1089 (155-5552)	2677 (85-4435)
Number of tubers in:				
Right hemisphere	4 (0-22)	20 (1-53)	14 (2-52)	23 (1-53)
Left hemisphere	4 (0-17)	18 (0-45)	11 (0-43)	22 (1-45)
Frontal lobe	5 (0-19)	21 (2-54)	13 (3-54)	23 (2-53)

Table 1 Characteristics and TSC specific brain abnormalities of 64 patients with an inactivating *TSC1* or *TSC2* variant. (continued)

	TSC1 **(n=21)**	***TSC2*** **(n=43)**	***TSC2*****p** **(n=19)**	***TSC2*****x** **(n=24)**
Parietal lobe	2 (0-7)	7 (0-28)	5 (0-28)	8 (0-19)
Temporal lobe	1 (0-8)	5 (0-17)	3 (0-10)	6 (0-17)
Occipital lobe	0 (0-3)	2 (0-16)	2 (0-10)	3 (0-16)
Fraction of total tubers in:				
Right hemisphere	0.5 (0.0-1)	0.51 (0.17-1)	0.53 (0.28-1.0)	0.51 (0.17-0.70)
Left hemisphere	0.5 (0.0-1)	0.49 (0.0-0.83)	0.47 (0.0-0.72)	0.49 (0.30-0.83)
Frontal lobe	0.54 (0.0-1)	0.57 (0.39-1)	0.60 (0.42-1.0)	0.56 (0.39-1.0)
Parietal lobe	0.25 (0.0-1)	0.19 (0.0-0.5)	0.24 (0.0-0.50)	0.17 (0.0-0.40)
Temporal lobe	0.07 (0.0-0.4)	0.13 (0.0-0.43)	0.08 (0.0-0.23)	0.16 (0.0-0.43)
Occipital lobe	0.0 (0.0-1)	0.08 (0.0-0.26)	0.08 (0.0-0.17)	0.09 (0.0-0.26)
Cystic tubers present, n (%)	3 (14)	20 (47)	4 (25)	16 (59)
Calcified tubers present, n (%)	4 (19)	7 (16)	4 (25)	3 (11)
Radial migration lines				
Total number	11 (2-36)	16 (0-36)	11 (0-36)	24 (0-36)
Fraction associated with tuber	0.71 (0.0-1.0)	0.46 (0.0-0.8)	0.45 (0.0-0.80)	0.47 (0.0-0.71)
Cystic RMLs present, n (%)	1 (5)	8 (19)	4 (25)	4 (15)
Calcified RMLs present, n (%)	4 (19)	8 (19)	4 (25)	4 (15)
SEGA present, n (%)	0 (0)	7 (16)	2 (13)	5 (19)
Subependymal nodules				
Total number	6 (0-11)	8 (0-25)	6 (0-15)	9 (0-25)
Ventricle frontal horn	0 (0-4)	2 (0-11)	1 (0-4)	3 (0-11)
Ventricle caudothalamic groove	1 (0-5)	3 (0-7)	2 (0-7)	3 (0-7)
Ventricle posterior horn	2 (0-5)	3 (0-11)	3 (0-4)	4 (0-11)
Fraction of total SENs in:				
Ventricle frontal horn	0.06 (0.0-0.57)	0.29 (0.0-0.67)	0.25 (0.0-0.40)	0.33 (0.0-0.67)
Ventricle caudothalamic groove	0.28 (0.0-1.0)	0.27 (0.0-0.67)	0.22 (0.0-0.64)	0.29 (0.0-0.67)
Ventricle posterior horn	0.46 (0.0-1.0)	0.4 (0.0-1.0)	0.43 (0.25-1.0)	0.40 (0.0-0.75)
Cystic SENs present, n (%)	0 (0)	0 (0)	0 (0)	0 (0)
Calcified SENs present, n (%)	4 (19)	18 (42)	6 (38)	12 (44)

Numbers are median (range) unless otherwise specified. Fractions are determined in patients in whom that type of pathology is present (for example fraction of tubers in the left hemisphere is only calculated for the patients who have tubers). *TSC2*p: TSC2 protein predicted. *TSC2*x: TSC2 protein predicted to be absent. SEGA, subependymal giant cell astrocytoma. SEN, subependymal nodule.

To investigate the effects of *TSC1* and *TSC2* variants on the TSC complex-dependent inhibition of mTORC1, we expressed the variant proteins together with a S6K reporter construct and determined the T389 phosphorylation status of the S6K reporter (figures 2 and 3). First, we compared the effect of the *TSC1* c.562T>G p.(F188V) substitution to the inactivating *TSC1* c.350T>C p.(L117P) variant (figure 2). Compared to wild-type TSC1, expression of the p.F188V and p.L117P variants resulted in reduced TSC1 signals and increased S6K-T389 phosphorylation. Next, we assessed the effects of 10 *TSC2* variants on TSC complex function (figure 3). In nine cases, expression of the variant failed to inhibit S6K-T389 phosphorylation. We did not observe significant differences in S6K-T389 phosphorylation between cells completely lacking TSC2, and those expressing the TSC2 variants, indicating that in our *in vitro* assay, the variants resulted in complete inactivation of the TSC complex. The TSC2 p.L160V variant had the same effect on S6K-T389 phosphorylation as wild-type TSC2. We did not obtain evidence that the p.L160V substitution affected TSC complex function. However, splice site prediction analysis in-

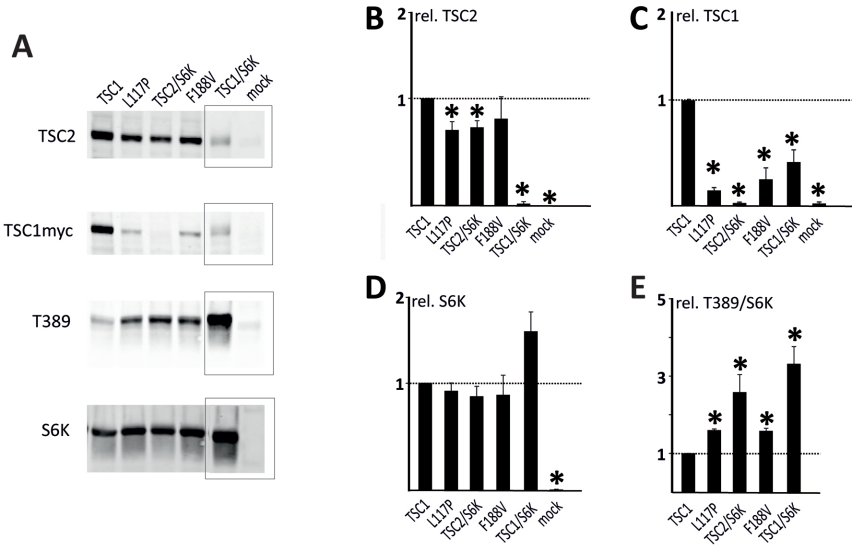

Figure 2 Functional assessment of the *TSC1* c.562T>G p.(F188V) variant.
We compared the effects of expression of the TSC1 p.F188V variant with wild-type TSC1 and the TSC1 p.L117P variant using a transfection-based immunoblot assay. Immunoblots are shown in (A); please note that for simplicity some lanes have been removed from the blot. The original, complete blots are shown in A-Figure 1 in the appendix. Signals for TSC2, TSC1, total S6K (S6K) and T389-phosphorylated S6K (T389) were determined per variant, relative to the wild-type control (TSC1) in four transfection experiments. The mean TSC2 (B), TSC1 (C) and S6K (D) signals and mean T389/S6K ratio (E) are shown for each variant. The dotted lines indicate the signal obtained upon expressing wild-type TSC1 (= 1.0). Error bars represent the standard error of the mean; variants that were significantly different from the wild-type are indicated with an asterisk ($P < 0.05$; Student's t-test). In TSC2/S6K no TSC1 protein is present. Amino acid changes are given according to the *TSC1* reference transcript NM_000368.4.

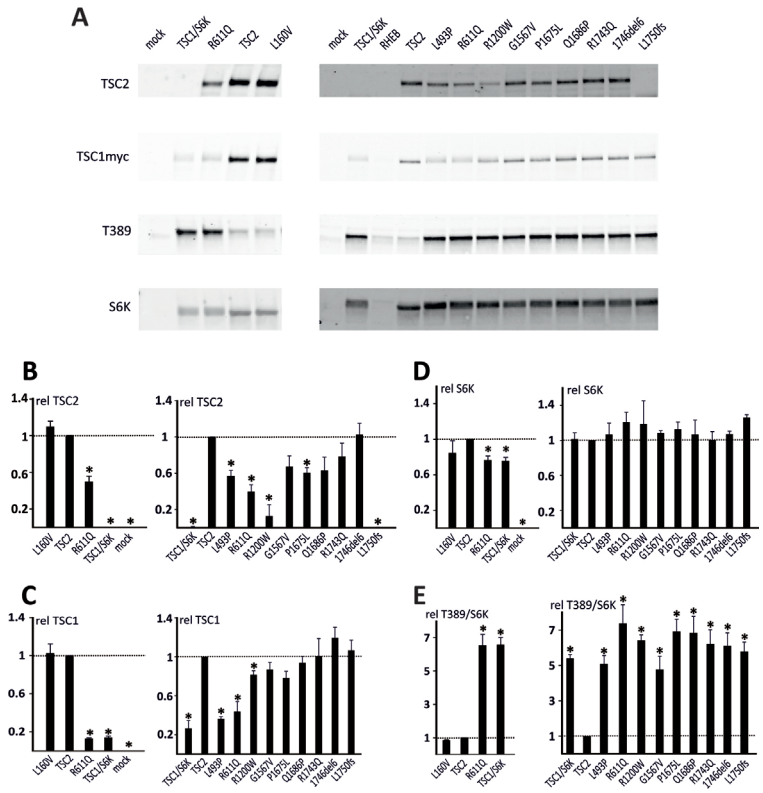

Figure 3 Functional assessment of *TSC2* variants.
We compared the effects of expression of wild-type TSC2 with 10 different TSC2 variants in HEK 293T (*TSC2* -/-; 3H9) cells using a transfection-based immunoblot assay. All the variants were identified in our patient cohort with the exception of the p.L1750fs variant. This variant is similar to the TSC2 c.5252_5259+19del27, p.(R1751Hfs*41) variant identified in our cohort. In both cases, the variant mRNA transcript is predicted to escape NMD, and the C-terminal epitope used for TSC2 protein detection is absent. Immunoblots are shown in (A). The signals for TSC2, TSC1, total S6K (S6K) and T389-phosphorylated S6K (T389) were determined per variant, relative to the wild-type control (TSC2) in four transfection experiments. The mean TSC2 (B), TSC1 (C) and S6K (D) signals and mean T389/S6K ratio (E) are shown for each variant. The dotted lines indicate the signal/ratio for TSC2 (= 1.0). Error bars represent the standard error of the mean; variants that were significantly different from TSC2 are indicated with an asterisk ($P < 0.05$; Student's t-test). Cells were cotransfected with *TSC1* and *S6K* expression constructs, except for the mock transfected cells (pcDNA3 only). TSC1/S6K refers to cells transfected with the *TSC1* and *S6K* expression constructs only; RHEB refers to cells transfected with a *RHEB* expression construct. Amino acid changes are given according to the *TSC2* reference transcript NM_000548.3

dicated that the *TSC2* c.478C>G, p.(L160V) substitution created a new 5′ splice donor site 4 nucleotides upstream of the normal splice site (*TSC2* c.478C>G, p.(A161Tfs*20)). The splicing defect was confirmed by RT-PCR and sequence analysis of RNA from cultured skin fibroblasts (A-Figure 2 in the appendix). There was no evidence that the original splice site was utilized in mRNA expressed from the variant (G) allele, indicating that the

predicted TSC2 p.L160V protein was unlikely to be expressed. Therefore, we classified the *TSC2* c.478C>G (p.A161Tfs*20) variant as *TSC2*x.

Cortical tubers

Cortical tubers were detected in 62 patients (97%); 19/21 (90%) from the *TSC1* group, and all individuals from the *TSC2* group (table 1 and A-Figure 3 in the appendix). Tubers were most often found in the frontal lobe, consistent with this lobe having the largest volume (table 1). Cystic tubers were present in 23 patients (36%; range 1-18 cystic tubers per patient; median: 6). Calcified tubers were present in 11 patients (17%; range: 1-17; median: 3). Representative MRIs of cystic and calcified tubers are shown in figures 4a and b.

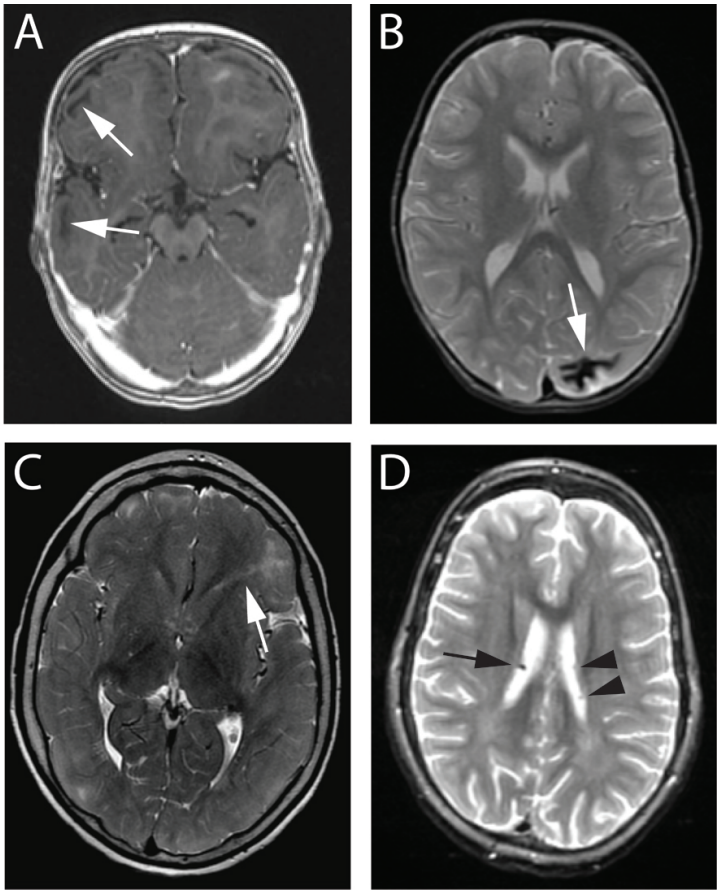

Figure 4 Examples of TSC specific brain abnormalities assessed in this study.
(A) T1 sequence showing cystic cortical tubers (arrows). (B) T2 sequence showing a calcified cortical tuber (arrow). (C) T2 sequence showing an RML in the left frontal lobe (arrow). (D) T2 dual echo sequence showing a calcified SEN (arrow). Note the SENs without calcification in the other ventricle (arrowheads).

More tubers in total ($P<0.001$) and per lobe ($P<0.001$ for all lobes) were found in the *TSC2* group compared to the *TSC1* group. The TSC2 group also had a larger total tuber surface area ($P<0.001$) but no difference was found when the percentage of tubers in each lobe was compared between these two groups. Individuals in the *TSC2* group were more likely to have cystic tubers ($P=0.012$), and the fraction of cystic tubers was higher in the *TSC2* group ($P=0.017$). Analysis of the *TSC1*, *TSC2p* and *TSC2x* groups showed similar results. The total number of tubers, number of tubers per lobe and tuber surface area were higher in the *TSC2p* and *TSC2x* groups compared to the *TSC1* group ($P\leq0.001$). The *TSC2x* group had more temporal lobe tubers than either the *TSC2p* or *TSC1* group ($P<0.001$). Cystic tubers were found most often in the *TSC2x* group ($P=0.003$), and the fraction of cystic tubers was higher in the *TSC2x* group compared to the *TSC1* group ($P=0.006$).

Radial migration lines

RMLs were present in 62 patients (97%). An example of a RML on MRI is shown in figure 4c. All individuals in the *TSC1* group, 13/16 (81%) from the *TSC2p* group, and 23/27 (85%) from the *TSC2x* group had RMLs. Roughly half of all RMLs could be traced to a tuber (Table 1 and A-Figure 3 in the appendix). Cystic RMLs were found in nine patients (14%; range: 1-9; median: 1). Calcified RMLs were present in 12 patients (19%; range: 1-8; median: 2). In two cases RMLs, but no tubers, were found. Both these individuals were from the *TSC1* group.

The total number of RMLs was significantly higher in the *TSC2* group than in the *TSC1* group, although this was no longer significant after correcting for multiple testing ($P=0.028$, $q=0.071$). No additional significant differences between the number of RMLs, or their cystic or calcified aspect were identified in the analysis of the *TSC1*, *TSC2p* and *TSC2x* groups.

Subependymal nodules

SENs were identified in 54 patients (84%). In the *TSC1* group, 18 individuals (86%) had SENs. In the *TSC2p* and *TSC2x* group, 13 (81%) and 23 (85%) individuals respectively had SENs (table 1 and A-Figure 3 in the appendix). Calcified SENs were present in 22 patients (34%; range: 1-15; median: 3). An MRI of a calcified SEN is shown in Figure 4d. Details on the location of the SENs can be found in table 1.

The *TSC2* group had a higher number of SENs ($P=0.009$), and these were more often calcified ($P=0.015$) compared to the *TSC1* group. No differences were found in the number and calcification of SENs in the analysis of the *TSC1*, *TSC2p* and *TSC2x* groups.

Subependymal Giant Cell Astrocytoma

An SEGA was identified in seven individuals from the *TSC2* group (11%) (Table 1); in 2 (13%) from the *TSC2p* group and 5 (19%) from the *TSC2x* group. No significant differences were identified.

Patients with genetic mosaicism

Two individuals from the *TSC2* group were mosaic. [14] The *TSC2* c.2838-122G>A, p.(945fs*5) and *TSC2* c.3099C>G, p.(Tyr1033*) variants were found at a frequency of 11% and 10% respectively in peripheral blood DNA. Both individuals had bilateral tubers, RMLs and SENs, none of which were cystic or calcified.

DISCUSSION

Brain pathology as assessed by MRI was compared between TSC patients with (i) a *TSC1* variant that affected function, (ii) a *TSC2* variant that affected function but was predicted to encode protein (*TSC2*p) and (iii) a *TSC2* variant that was predicted to prevent TSC2 protein expression (*TSC2*x). The added value of the results from these analyses was determined compared to analyses between the *TSC1* group and the whole *TSC2* group. Our results are consistent with previous studies[6-14,21]: the *TSC2* group was associated with more and larger tubers, more RMLs, more SEGAs, and more SENs. Subdivision of the *TSC2* group into *TSC2p* and *TSC2x* subgroups did not reveal major differences in TSC-pathology, as detected by MRI, although a higher number and fraction of tubers in the temporal lobe and a higher fraction of cystic tubers in the *TSC2*x group were observed.

Although the larger numbers of cystic tubers and tubers in the temporal lobe in the *TSC2x* group might simply be due to chance, it might be clinically relevant. Patients with more temporal tubers have a higher risk of developing autistic features[21] and cystic tubers have been associated with a higher incidence of epilepsy[22] and autism spectrum disorder.[23] The larger number of calcified SENs in the *TSC2* group could also be clinically relevant, as calcified SENs are more likely to develop into a SEGA.[24]

Two patients in our cohort were mosaic. Both had bilateral TSC-related abnormalities. It would be interesting to study genotype-phenotype associations in a larger cohort of mosaic TSC patients, to determine whether these individuals are more likely to have specific types of pathology, as has been suggested previously.[25-28]

Our cohort consisted of 41 individuals with a de novo mutation (12 *TSC1*, 29 *TSC2*), 11 individuals from 7 different families and 12 individuals (5 *TSC1*, 7 *TSC2*) for whom we did not have access to parental DNA. Familial TSC cases are reported to have a milder phenotype than sporadic TSC cases.[13] It is possible that the presence of the familial and mosaic cases in our cohort might have skewed the analysis of the relation of genotype

and the severity of the phenotype. Future studies investigating this relation could include an entire cohort of patients in which selection bias is kept to a minimum, like the Tuberous Sclerosis 2000 study.[7] Such studies could also specifically investigate familial and mosaic cases, to verify the findings of those cases having a milder phenotype. Data from various studies could be combined into a meta-analyses to increase power. As our cohort consisted of 64 individuals, we were unable to make more than two subgroups of *TSC2* variants. It should be considered however, that if such a large cohort is required to establish significant genotype-phenotype correlations, it is unlikely that making subgroups of inactivating *TSC1* or *TSC2* variants will have prognostic value in the clinic.

Although the function of the TSC complex when over-expressed in cultured cells might be different from its role *in vivo*, the similarity between the *TSC2*x and *TSC2*p groups is consistent with our *in vitro* functional assessment. S6K-T389 phosphorylation in the presence of 9 TSC2 variants was essentially the same as in the absence of TSC2 (figure 2).

Overall, more brain abnormalities were found in the *TSC2* group. *TSC2* encodes the catalytic GAP domain of the TSC complex and is therefore essential for canonical TSC complex function. Individuals with a *TSC1* variant that affects function, or a *TSC2* variant that affects function but where the GAP domain is expressed, might therefore be expected to have a less severe phenotype due to residual TSC2 GAP activity. Indeed, in our functional assessment, we observed an effect of TSC2 expression on S6K-T389 phosphorylation in the absence of co-expressed TSC1 (figure 2), but did not observe an effect of TSC1 expression on S6K-T389 phosphorylation in the absence of TSC2 (figure 3). However, we did not find strong evidence for differences between the *TSC2*x and *TSC2*p groups. Our functional study indicated that all the changes predicted to result in expression of altered TSC2 protein led to essentially complete inactivation of the TSC complex-dependent inhibition of mTORC1.

The chromosomal location, larger size and more complex structure of *TSC2*, compared to *TSC1*, might make the *TSC2* locus more susceptible to the second hit mutations that are required for TSC pathology. Indeed, there is considerable phenotypic variation between different individuals with the same *TSC1* or *TSC2* variant, even within a single family.[29] This suggests that it is highly likely that random second hit mutations are the most important cause of variation in brain pathology. This is difficult to show in patients, but may be inferred by excluding other causes for phenotypic variability. Another way to investigate the frequency of these stochastic events, is to perform histologic analyses on post mortem brains of TSC patients, to determine the presence of cells that have undergone somatic mutations, as has been done previously.[30]

A recent study showed that the length of the predicted C-terminal tails of mutant TSC1 and TSC2 proteins might be associated with intelligence.[15] We correlated the length of the predicted C-terminal tails with the number of tubers per hemisphere and per lobe, and the number of RMLs and SENs. There were no significant differences. This is in agree-

ment with the study of Wong et al,[15] suggesting that IQ is not directly related to brain abnormalities, and implies that the pathogenetic mechanisms underlying brain pathology and cognitive development in TSC are distinct. This was also reported by Goorden et al,[31] who showed that *Tsc1* mutant mice have cognitive deficits in the absence of overt brain pathology. The functional consequences of a longer or shorter C-terminal tail are unknown. It is not yet clear whether truncated TSC1 or TSC2 are expressed *in vivo*, or whether NMD prevents their synthesis.

The MRI scans used in our study were acquired during routine diagnostics of patients attending a specialist pediatric clinic at an academic hospital, which may introduce a bias towards more severe brain abnormalities. Not all MRIs were made following a standard protocol, and some abnormalities might have been missed. Nonetheless, the numbers of abnormalities identified in our cohort were mostly similar or higher than those reported in previous studies.[9,10,24] The number of RMLs in our cohort was lower than that reported in another cohort, possibly because we did not use diffusion tensor imaging or three-directional scans.[32]

In summary, we compared TSC brain pathology to genotype. *TSC2* variants were associated with more tubers, RMLs and SENs than *TSC1* variants, and although larger studies might identify clinically relevant subdivisions of *TSC1* and *TSC2* variants, we found little additional value for the subdivision of *TSC2* variants. Our study is consistent with the hypothesis that the frequency of second hit events is the most important driver of the variability in TSC-associated brain lesions, as detected by MRI.

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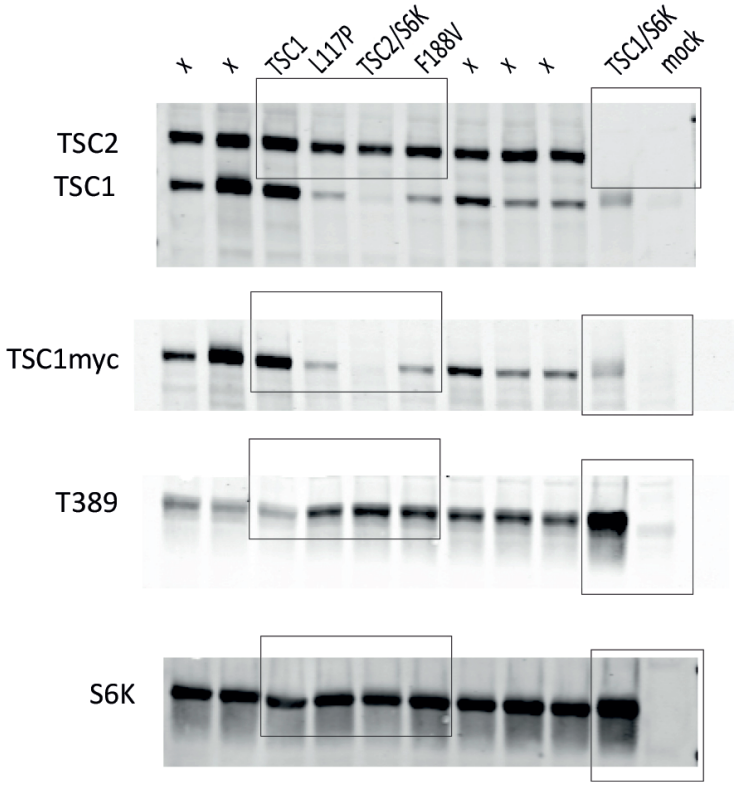
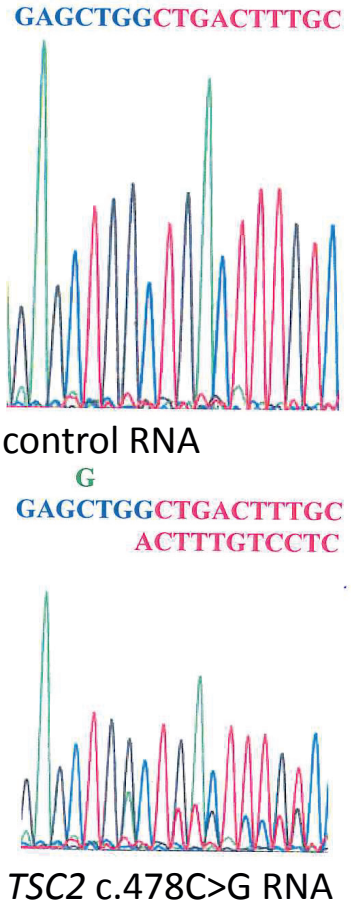
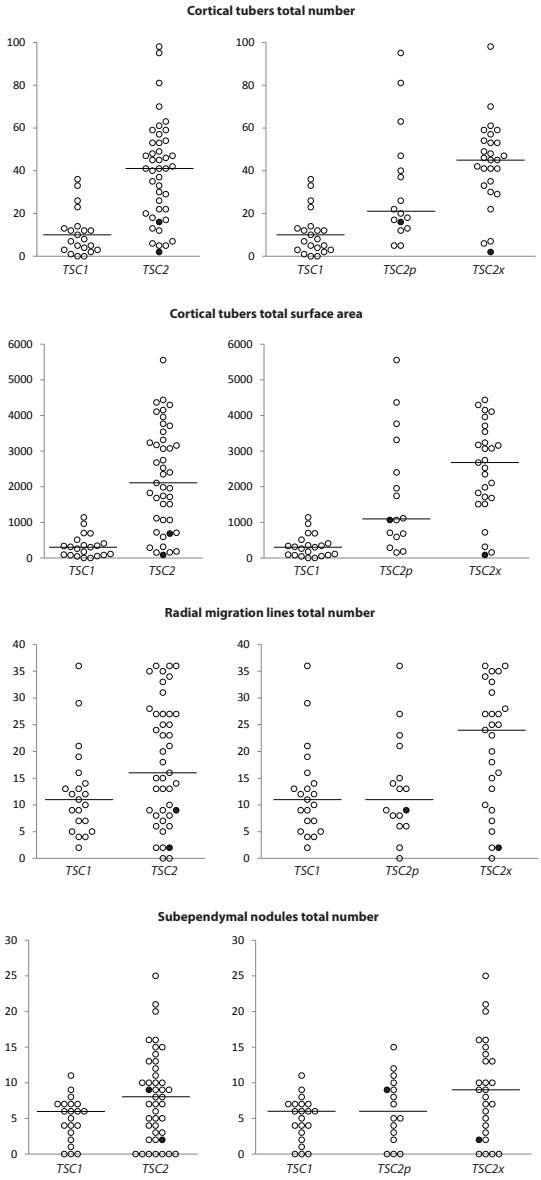
APPENDIX



A-Figure 1. Functional assessment of the *TSC1* c.562T>G p.(F188V) variant. We compared the effects of expression of the TSC1 p.F188V variant with wild-type TSC1 and the TSC1 p.L117P variant using a transfection-based immunoblot assay. Please refer to Figure 2 in the main manuscript. Unedited immunoblots are shown. Portions of the blots used in Figure 2 are boxed. 'x' refers to variants not related to those described in the manuscript.



A-Figure 2. Analysis of RNA from the individual with the *TSC2* c.478C>G variant. Sequence encoded by exon 5 is shown in blue; sequence encoded by exon 6 is shown in red; variant G allele is indicated in green. Note that the last 4 nucleotides of exon 5 (GTGG) are absent from the mutant RNA. No normally spliced RNA encoded by the variant G allele was detected.



A-Figure 3. Scatter plots of brain pathology observed in our cohort. Each dot represents one TSC patient. For every characteristic, the left graph shows *TSC1* and *TSC2* patients, the right graph shows *TSC1*, *TSC2*p and *TSC2*x. Mosaic patients are indicated by the black dots. The horizontal lines indicate the median value.

A-Table 1. *TSC1* variants identified in our study cohort.

Variant	Variant description and *TSC1* classification
TSC1 c.[-21656_-14846del6811;-18011_-17856inv156], p.? (exon 1)	de novo deletion; unlikely to lead to functional transcript due to deletion of the promoter and transcription start site (TSS)
TSC1 c.-20253_-13938del6316, p.? (exon 1)	de novo deletion; unlikely to lead to functional transcript due to deletion of the promoter and TSS
TSC1 c.203_204delAT, p.(H68Rfs*9) (exon 4)	de novo frameshift; NMD
TSC1 c.433C>T, p.(Q145*) (exon 6)	nonsense; NMD
TSC1 c.508+1G>A, p.? (exon 6)	de novo loss of 5′ donor site; unlikely to lead to functional transcript
TSC1 c.533dupT, p.(H179Pfs*39) (exon 7)	2 individuals; familial frameshift; NMD
TSC1 c.562T>G, p.(F188V) (exon 7)	de novo missense; affects TSC complex function (Figure 2)
TSC1 c.591dupC, p.(N198Qfs*20) (exon 7)	frameshift; NMD
TSC1 c.682C>T, p.(R228*) (exon 8)	nonsense; NMD
TSC1 c.733C>T, p.(R245*) (exon 8)	de novo nonsense; NMD
TSC1 c.989dupT, p.(S331Efs*10) (exon 10)	de novo frameshift; NMD
TSC1 c.1257delC, p.(R420Gfs*20) (exon 12)	familial frameshift; NMD
TSC1 c.1331C>G, p.(S444*) (exon 13)	familial nonsense; NMD
TSC1 c.1708_1709delAG, p.(R570Gfs*17) (exon 15)	de novo frameshift; NMD
TSC1 c.1904_1905delCA, p.(T635Rfs*52) (exon 15)	de novo frameshift; NMD
TSC1 c.2074C>T, p.(R692*) (exon 17)	de novo nonsense; NMD
TSC1 c.2155delC, p.(L719Sfs*5) (exon 17)	de novo frameshift; NMD
TSC1 c.2263C>T, p.(Q755*) (exon 18)	nonsense; NMD
TSC1 c.2668A>T, p.(K890*) (exon 21)	de novo nonsense; NMD
TSC1 c.2698C>T, p.(Q900*) (exon 21)	nonsense; NMD

Exon numbering is according to genomic reference sequence NG_012386.1; cDNA notation is according to reference transcript NM_000368.4. Variants found in more than one patient are only shown once. All variants were classified as inactivating.[33] NMD nonsense-mediated decay.

A-Table 2. *TSC2* variants identified in our study cohort.

Variant	Variant description and *TSC2* classification
TSC2 c.187delC, p.(Q63Rfs*43) (exon 3)	de novo frameshift; NMD; (*TSC2*x)
TSC2 c.225+2T>A, p.? (intron 3)	de novo loss of 5'donor site; unlikely to lead to functional transcript; (*TSC2*x)
TSC2 c.268C>T, p.(Q90*) (exon 4)	de novo nonsense; NMD; (*TSC2*x)
TSC2 c.337-2A>G, p.? (intron 4)	de novo loss of 3'acceptor site; unlikely to lead to functional transcript; (*TSC2*x)
TSC2 c.478C>G, p.(L160V)/p.A161Tfs*20 (exon 5)	de novo creation of a new 5'donor site; normal splice site is retained, but no evidence that it is utilised (Supplementary Figure 2); predicted p.L160V missense variant is active but not expressed; (*TSC2*x)
TSC2 c.(481+1_482-1)_(*102_?)del, p.? (exons 6 -42)	familial; large deletion, only exons 1 - 5 retained; unlikely to lead to functional transcript; (*TSC2*x)
TSC2 c.509_510insAT, p.(G171Lfs*12) (exon 6)	de novo frameshift; NMD; (*TSC2*x)
TSC2 c.729_730delCT, p.(C244Sfs*93) (exon 8)	de novo frameshift; NMD; (*TSC2*x)
TSC2 c.949_950insTA, p.(T317Ifs*47) (exon 10)	de novo frameshift; NMD; (*TSC2*x)
TSC2 c.976-15G>A, p.? (exon 11)	creates new 3'acceptor site; normal splice site is retained, possible expression of normal transcript; (*TSC2*p)
TSC2 c.[976-20T>A; 976-18_976-2dup17], p.? (intron 11)	3 individuals; familial; duplication that creates a new 3' acceptor site; existing 3'acceptor site is retained, possible expression of normal transcript; (*TSC2*p)
TSC2 c.1257+1G>A, p.? (exon 12)	de novo loss of 5'donor site; unlikely to lead to functional transcript; (*TSC2*x)
TSC2 c.1478T>C, p.(L493P) (exon 15)	de novo in affected mother; missense, affects TSC complex function (Figure 3); (*TSC2*p)
TSC2 c.-3420_1716+3098del23541, p.? (exons 1 - 16)	de novo deletion; unlikely to lead to functional transcript; (*TSC2*x)
TSC2 c.-111805_1717-1586del132062, p.? (exons 1 - 16)	deletion; unlikely to lead to functional transcript; (*TSC2*x)
TSC2 c.-23665_1716 +2692del43377, p.? (exons 1 - 16)	deletion; unlikely to lead to functional transcript; (*TSC2*x)
TSC2 c.1832G>A, p.(R611Q) (exon 17)	de novo, missense affects TSC complex function (Figure 3); (*TSC2*p)
TSC2 c.2353C>T, p.(Q785*) (exon 21)	de novo nonsense; NMD; (*TSC2*x)
TSC2 c.2737_2738delAC, p.(T913*) (exon 24)	de novo nonsense; NMD; (*TSC2*x)
TSC2 c.2838-122G>A, p.? (intron 26)	de novo (mosaic), new 3'acceptor site created; possible expression of normal transcript; (*TSC2*p)
TSC2 c.3098dupA, p.(Y1033*) (exon 27)	nonsense; NMD; (*TSC2*x)
*TSC2*c.3099C>G, p.(Y1033*) (exon 27)	de novo (mosaic), nonsense; NMD; (*TSC2*x)
TSC2 c.3131+1G>A, p.? (intron 27)	de novo, loss of 5'donor site; unlikely to lead to functional transcript; (*TSC2*x)

A-Table 2. *TSC2* variants identified in our study cohort. (continued)

Variant	Variant description and TSC2 classification
TSC2 c.3131+2T>C, p.? (intron 27)	de novo, loss of 5'donor site; unlikely to lead to functional transcript; (*TSC2*x)
TSC2 c.3337dupG, p.(E1113Gfs*55) (exon 29)	frameshift; NMD; (*TSC2*x)
TSC2 c.3397+1G>A, p.? (intron 29)	de novo, loss of 5'donor site; unlikely to lead to functional transcript; (*TSC2*x)
TSC2 c.3412C>T, p.(R1138*) (exon 30)	de novo (1 case), nonsense; NMD; (*TSC2*x)
TSC2 c.3598C>T, p.(R1200W) (exon 30)	2 individuals; familial, missense, affects TSC complex function (Figure 3); (*TSC2*p)
TSC2 c.3612delG, p.(N1205Tfs*5) (exon 31)	de novo, frameshift; NMD; (*TSC2*x)
TSC2 c.3952_3961del10, p.(E1318Rfs*4) (exon 33)	de novo, frameshift; NMD; (*TSC2*x)
TSC2 c.4030G>T, p.(E1344*) (exon 34)	de novo, nonsense; NMD; (*TSC2*x)
TSC2 c.4700G>T, p.(G1567V) (exon 37)	de novo, missense affects TSC complex function (Figure 3); (*TSC2*p)
TSC2 c.4829G>A, p.(W1610*) (exon 37)	de novo, nonsense; NMD; (*TSC2*x)
TSC2 c.5024C>T, p.(P1675L) (exon 39)	missense, affects TSC complex function (Figure 3); (*TSC2*p)
TSC2 c.5057A>C, p.(Q1686P) (exon 39)	de novo, missense, affects TSC complex function (Figure 3); (*TSC2*p)
TSC2 c.5208C>A, p.(Y1736*) (exon 41)	de novo, nonsense; predicted to escape NMD; possible expression of *TSC2* transcript; (*TSC2*p)
TSC2 c.5228G>A, p.(R1743Q) (exon 41)	de novo, missense, affects TSC complex function (Figure 3); (*TSC2*p)
TSC2 c.5238_5255del18, p.(H1746_R1751del6) (exon 41)	de novo, in-frame deletion, affects TSC complex function (Figure 3); (*TSC2*p)
TSC2 c.5252_5259+19del27, p.(R1751Hfs*41) (exon 41)	de novo, frameshift; predicted to escape NMD; (*TSC2*p)

Exon numbering is according to genomic reference sequence NG_005895.1; cDNA notation is according to reference transcript NM_000548.3. Variants found in more than one patient are only shown once. All variants were classified as inactivating.[1] NMD nonsense-mediated decay.

References

1. Plon SE, Eccles DM, Easton D *et al*: Sequence Variant Classification and Reporting: Recommendations for Improving the Interpretation of Cancer Susceptibility Genetic Test Results. *Hum Mutat* 2008; 29: 1282-1291.

A-Table 3. Outcomes of statistical comparisons made between *TSC1* and *TSC2*, and between *TSC1*, *TSC2*p and *TSC2*x.

	TSC1* vs *TSC2		***TSC1* vs *TSC2p* vs *TSC2x***	
	p value	**q value**	**p value**	**q value**
Cortical tubers				
Total number	<0.001	<0.001	<0.001	<0.001
			TSC2x vs *TSC2p* 0.232	
			TSC2x vs *TSC1* <0.001	
			TSC2p vs *TSC1* 0.005	
Total surface area (mm^2)	<0.001	<0.001	<0.001	<0.001
			TSC2x vs *TSC2p* 0.192	
			TSC2x vs *TSC1* <0.001	
			TSC2p vs *TSC1* 0.001	
Numbers of tubers in:				
Right hemisphere	<0.001	<0.001	<0.001	<0.001
			TSC2x vs *TSC2p* 0.353	
			TSC2x vs *TSC1* <0.001	
			TSC2p vs *TSC1* 0.005	
Left hemisphere	<0.001	<0.001	<0.001	<0.001
			TSC2x vs *TSC2p* 0.180	
			TSC2x vs *TSC1* <0.001	
			TSC2p vs *TSC1* 0.009	
Frontal lobe	<0.001	<0.001	<0.001	<0.001
			TSC2x vs *TSC2p* 0.491	
			TSC2x vs *TSC1* <0.001	
			TSC2p vs *TSC1* 0.005	
Parietal lobe	<0.001	<0.001	0.001	0.003
			TSC2x vs *TSC2p* 1.000	
			TSC2x vs *TSC1* 0.001	
			TSC2p vs *TSC1* 0.009	
Temporal lobe	<0.001	<0.001	<0.001	<0.001
			TSC2x vs *TSC2p* 0.002	
			TSC2x vs *TSC1* <0.001	
			TSC2p vs *TSC1* 0.480	

A-Table 3. Outcomes of statistical comparisons made between *TSC1* and *TSC2*, and between *TSC1*, *TSC2*p and *TSC2*x. (continued)

	TSC1* vs *TSC2		***TSC1* vs *TSC2p* vs *TSC2x***	
	p value	**q value**	**p value**	**q value**
Occipital lobe	<0.001	<0.001	0.001	0.002
			TSC2x vs *TSC2p* 0.365	
			TSC2x vs *TSC1* 0.000	
			TSC2p vs *TSC1* 0.082	
Fraction of tubers in:				
Right hemisphere	0.609	0.707	0.670	0.754
Left hemisphere	0.609	0.731	0.670	0.778
Frontal lobe	0.723	0.813	0.778	0.824
Parietal lobe	0.294	0.424	0.120	0.227
Temporal lobe	0.167	0.251	0.008	0.022
			TSC2x vs *TSC2p* 0.016	
			TSC2x vs *TSC1* 0.046	
			TSC2p vs *TSC1* 1.000	
Occipital lobe	0.877	0.928	0.890	0.915
Cystic tubers present	0.012	0.038	0.003	0.012
Fraction of tubers that are cystic	0.017	0.048	0.006	0.018
Calcified tubers present	0.783	0.854	0.487	0.585
Fraction of tubers that are calcified	0.396	0.509	0.252	0.378
Radial migration lines				
Total number	0.028	0.073	0.009	0.022
			TSC2x vs *TSC2p* 0.056	
			TSC2x vs *TSC1* 0.015	
			TSC2p vs *TSC1* 1.000	
Fraction associated with tuber	0.073	0.131	0.080	0.169
Cystic RMLs present	0.135	0.220	0.212	0.332
Fraction of RMLs that are cystic	0.050	0.105	0.293	0.405
Calcified RMLs present	0.966	0.994	0.710	0.774
Fraction of RMLs that are calcified	0.442	0.549	0.299	0.398

A-Table 3. Outcomes of statistical comparisons made between *TSC1* and *TSC2*, and between *TSC1*, *TSC2*p and *TSC2*x. (continued)

	TSC1* vs *TSC2		***TSC1* vs *TSC2p* vs *TSC2x***	
	p value	**q value**	**p value**	**q value**
SEGA	0.050	0.100	0.122	0.219
SEN				
Total number	0.009	0.034	0.028	0.066
			TSC2x vs *TSC2p* 0.296	
			TSC2x vs *TSC1* 0.028	
			TSC2p vs *TSC1* 1.000	
Ventricle frontal horn	0.002	0.009	0.003	0.011
			TSC2x vs *TSC2p* 0.063	
			TSC2x vs *TSC1* 0.003	
			TSC2p vs *TSC1* 1.000	
Ventricle caudothalamic groove	0.036	0.081	0.037	0.083
			TSC2x vs *TSC2p* 0.406	
			TSC2x vs *TSC1* 0.036	
			TSC2p vs *TSC1* 1.000	
Ventricle posterior horn	0.031	0.075	0.006	0.019
			TSC2x vs *TSC2p* 0.056	
			TSC2x vs *TSC1* 0.010	
			TSC2p vs *TSC1* 1.000	
Fraction of SENs in:				
Ventricle frontal horn	0.108	0.186	0.144	0.246
Ventricle caudothalamic groove	0.980	0.980	0.889	0.915
Ventricle posterior horn	0.327	0.453	0.258	0.371
Cystic SENs present	0.149	0.234	0.353	0.454
Fraction of SENs that are cystic	0.331	0.442	0.375	0.465
Calcified SENs present	0.071	0.135	0.176	0.289
Fraction of SENs that are calcified	0.015	0.044	0.109	0.217

TSC1 and *TSC2* were compared using a *t*-test, *TSC1*, *TSC2*p and *TSC2*x were compared using ANOVA with post-hoc test. A multiple testing correction was used by calculating q values according to the false discovery rate method.

SEGA subependymal giant cell astrocytoma, SEN subependymal nodule.

CHAPTER 3

Brain pathology and quantitative autistic traits in children with Tuberous Sclerosis Complex – a clinical epidemiological study

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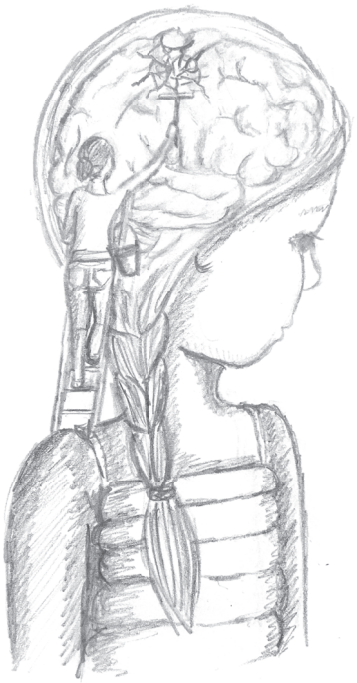

Manuscript in preparation

ABSTRACT

Background: Tuberous Sclerosis Complex (TSC) is characterized by brain pathology and high prevalence rates of autism spectrum disorders (ASD). Little is known about the association between brain pathology and ASD in TSC. Previous studies have suggested tuber burden to be an important predictor for an ASD diagnosis. To date, no studies have investigated the association between tuber count and quantitative measures of ASD severity and studies investigating the relation with radial migration lines (RMLs) are scarce. Finally, although TSC is characterized by cognitive impairment, the role of cognitive functioning in the association between brain pathology and ASD has been insufficiently studied.

Methods: In a clinical epidemiological sample of 52 TSC patients (24 boys, 0-17 years) cognitive functioning (intelligence/developmental quotient; IQ/DQ) and ASD severity (Autism Diagnostic Observation Scale; ADOS) were assessed. Tuber and RML count and location were manually recorded, using FLAIR or T2-weighted images from a 1.5T Siemens scanner. Regression and mediation analyses were performed.

Results: Tuber and RML count was strongly positively related to ASD severity. When IQ/DQ was added to the analyses only total (β=0.29, p=0.046) and frontal (β=0.30, p=0.042) tuber count remained significantly associated to the severity of restricted and repetitive behaviors. For RML count, only the number of RMLs in the occipital lobes remained associated with total ASD (β=0.28, p=0.013) and social communication and interaction deficits (β=0.30, p=0.013) severity. Formal mediation analyses confirmed these findings and showed that all other initial associations were fully mediated by IQ/DQ.

Conclusions: Clear associations were found between tuber and RML count and ASD severity. Cognitive functioning was identified as an important explanatory factor in the associations, highlighting the importance of taking cognitive functioning into account when studying the relation between brain pathology and ASD. Furthermore, our study underlines the relevance of separately studying difficulties in social communication and interaction on the one hand, and restricted and repetitive behaviors on the other hand.

INTRODUCTION

Tuberous Sclerosis Complex (TSC) is an autosomal dominant disorder affecting 1 in 6,000 people. The disorder is caused by a mutation in the TSC1 (chromosome 9) or TSC2 (chromosome 16) genes, which are responsible for the encoding of the proteins hamartin and tuberin respectively. Hamartin and tuberin form the intracellular TSC1-TSC2 protein complex, which serves as a regulator of the mammalian target of rapamycin (mTOR) pathway. Mutations in the TSC1 or TSC2 gene lead to a dysregulation of the mTOR pathway, causing uncontrolled cell progression and the proliferation of benign overgrowths of cells and tissue in many organ systems including the brain, skin, kidneys, heart, eyes, lungs and bones.[1] In the brain, the disruption of neuronal proliferation, migration and differentiation of brain cells during development may lead to a wide range of structural abnormalities, of which the most common are cortical tubers, affecting over 80% of all TSC patients. Tubers are benign tumors that develop during gestation and can be detected by neuroimaging from 20 weeks of gestation onwards.[1] Other abnormalities include white matter pathology, such as the presence of radial migration lines (RMLs). RMLs are linear abnormalities that extend from the ventricles to the cortex, representing areas of hypomyelination and white matter heterotopia.[2] RMLs are a marker of abnormal neural migration and cortical organization. In figure 1, an example of a Magnetic Resonance Imaging (MRI) scan showing cortical tubers and RMLs is provided.

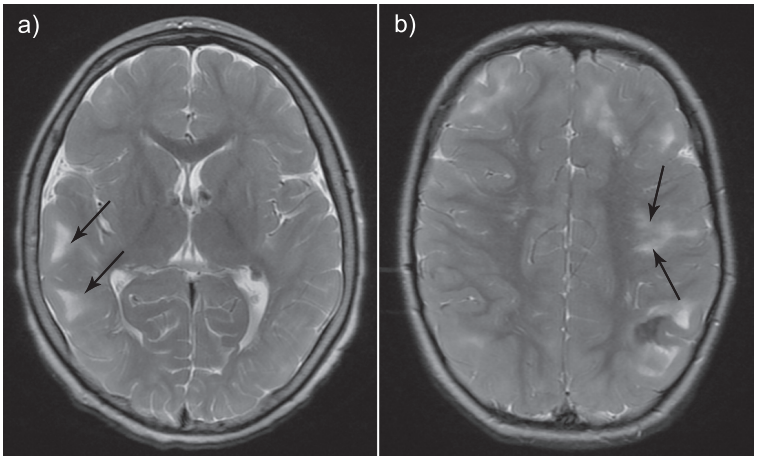

Figure 1. Example of T2-weighted images with arrows indicating (A) cortical tubers, and (B) radial migration lines.

Other features associated with brain pathology in TSC include cognitive impairment, with about 50% of patients having intellectual disability (IQ<70), and a range of be-

havioral or psychiatric symptoms. Commonly reported behavioral problems include aggression, anxiety, inattention and social problems. Autism spectrum disorder (ASD) is highly prevalent in children with TSC, with prevalence rates estimated around 40-50%.[3]

Previous studies have suggested the total number of cortical tubers to be an important predictor for an ASD diagnosis in TSC, and the temporal lobes were suggested to be specifically implicated.[4,5] Other studies found the presence (yes/no) of temporal tubers to be associated with a higher likelihood of an ASD diagnosis,[6] specifically found the number of cyst-like tubers to be related to ASD diagnostic status[7] or found a diagnosis of autism to be related to frontal and posterior tubers.[8] Still others did not find an association between the occurrence of cortical tubers and an autism diagnosis,[9] or found the number of tubers to be equally prevalent in mentally retarded non-autistic and mentally retarded autistic children and thus non-specific for ASD.[10] These inconsistencies in findings point out that the association between tuber burden and ASD is still poorly understood, most likely due to the use of different methodologies and varying diagnostic criteria.

Although these previous studies have experimented with different ways of defining cortical tuber involvement (for example by studying tuber presence (yes/no), tuber count, or the size of tubers), in these studies ASD has always only been categorically defined as the presence or absence (yes/no) of an ASD diagnosis. Research in the past several years has shown that child psychopathology, such as ASD, might be better described within a quantitative, or dimensional, framework. Within this framework of continuous symptom levels, the entire spectrum of severity is covered. Previous studies have shown that the symptoms and etiology of ASD indeed form such a spectrum, even extending into the general population.[11,12] Studying ASD as a quantitative trait rather than as a categorically defined disorder can contribute to a better understanding of the disorder and the potentially contributing biological pathways. In addition to being a more naturalistic representation of symptoms, the use of quantitative severity scores provides more statistical power and allows the application of advanced statistical methods in research.[11] To our knowledge, only one previous study has investigated the correlation between a quantitative measure of ASD severity (Childhood Autism Rating Scale, CARS) and tuber count, and did not find an association between cortical tuber count or location and overall ASD severity.[13]

Importantly, ASD is characterized by various difficulties that, according to the latest edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5),[14] can be divided in two main domains of functioning; 1) deficits in social communication and interaction and 2) restricted or repetitive patterns of behavior, interests or activities. The nature of the symptoms in these domains is substantially different, making it highly plausible that distinct neural mechanisms in different brain regions may underlie these two different domains of ASD symptoms. To our knowledge, no previous studies have

investigated these two different domains of ASD symptomatology in relation to tuber burden in TSC.

It is known that TSC is characterized by cognitive impairment and previous studies have shown that the degree of cognitive impairment is clearly related to tuber burden.[6,15–17] Similarly, an association between intellectual (dis)ability and autism severity has been demonstrated.[18] It is of interest, but still unclear, how cognitive functioning affects the association between tuber burden and ASD severity, and whether it could possibly be a mediator in this association.

Most previous neuroimaging studies in TSC have focused specifically on tuber characteristics and the association with cognitive or behavioral problems. To our knowledge, only two studies exist that have focused on the contribution of RMLs to the neurocognitive phenotype in TSC. The first study demonstrated that RMLs were more common in children with intellectual disability.[19] In a more recent study, RML frequency was found to be strongly associated with intelligence, as well as with the severity of autistic traits.[20]

In the present study, we aim to investigate the association between a clinical observational quantitative measure of ASD severity and the number and location of cortical tubers and RMLs, and study the role of cognitive functioning in this association. Moreover, we will investigate the specific association between ASD severity within the two main subdomains of ASD symptomatology (deficits in social communication and interaction, and restricted or repetitive behaviors) and tuber and RML count.

METHODS

Participants

The medical records of all TSC patients within the expertise center ENCORE (Erasmus MC-Sophia Children's Hospital, Rotterdam, the Netherlands) were retrospectively reviewed. In a sample of 75 patients, MRI scans were available. Of this sample, 55 patients also visited the department of Child and Adolescent Psychiatry/Psychology for behavioral assessment. In 3 patients, no ASD severity scores could be calculated due to an age falling outside the norm age range for severity scores (n=1) or incomplete data (n=2). This led to a total sample of 52 patients (24 boys, 28 girls) between 2 and 17 years of age.

Since all data was collected as part of regular patient care and was analyzed retrospectively and coded, this study was declared to not fall under the scope of the Medical Research Involving Human Subjects Act (WMO) by the Medical Ethics Committee of the Erasmus MC, the Netherlands.

Measures

Autism spectrum symptoms

The presence and severity of ASD was assessed using the Autism Diagnostic Observation Scale (ADOS).[21,22] The ADOS is a semi-structured and standardized observational procedure to evaluate social interaction, imagination/creativity, and stereotyped behaviors and restricted interests. The ADOS consists of four different modules, depending on the developmental and expressive language level of the child. In the current study all modules were used.

Children were classified as having ASD (autism or the broader ASD phenotype) or not according to the revised ADOS-2 algorithms.[21] For the main analyses, a total standardized calibrated severity score (CSS) was calculated, as well as a CSS for the two separate subdomains of the ADOS; social affect (SA) and restricted and repetitive behaviors (RRB).[23–25] The use of these standardized calibrated severity scores allows the comparison of ASD severity over the different ADOS modules used, and gives an indication of ASD severity relative to the child's age and expressive language level.[25] All ADOSes were administered and scored by a trained and certified psychologist or psychiatrist.

Cognitive functioning

Cognitive functioning was assessed using different intelligence measures according to best practice standards; in the majority of children (n=32, 62%) this was either the Wechsler Intelligence Scale for Children-III (WISC-III) or the Wechsler Preschool and Primary Scale of Intelligence-III (WPPSI-III).[26,27] In some children (n=7, 13%) a non-verbal intelligence test was used, which was either the Wechsler Non Verbal scale of ability (WNV)[28] or the Snijders-Oomen Nonverbal Intelligence Test (SON-R).[29] From all intelligence tests full-scale intelligence quotients (TIQs) were used. For children who were at the floor of their age-appropriate standardized scores, a developmental quotient (DQ) was calculated (developmental age / chronological age x 100).[30] The developmental age used in this formula was based on the mean age equivalent of test scores. Like IQ scores, a DQ of 100 is considered the mean.

In a part of the children (n=13, 25.0%) no formal intelligence test could be performed due to a young (developmental) age. In the majority of these children one of the Bayley Scales of Infant and Toddler Development (BSID-II or Bayley-III) was used[31,32] to evaluate the cognitive developmental age. In a couple of children, the developmental level was assessed using the Vineland Screener[33]. For these children, we again used the estimated cognitive developmental age to calculate a DQ, according to the formula provided above.

Neuroimaging

All MRI scans were made at the Erasmus MC-Sophia Children's Hospital on a 1.5 Tesla Siemens MRI scanner. For children with more than one available MRI, the MRI closest in time to the ADOS assessment was selected. All MRI scans were visually inspected by two trained research assistants, and re-assessed by a pediatric neuroradiologist and a pediatric neurologist, using the Picture Archiving and Communication System (PACS) software. A protocol for data collection was developed in which FLAIR images were used to assess the number and location of tubers and RMLs. If FLAIR images were not present, or for clarification purposes, T2-weighted images were used.

Statistical analysis

Data were analyzed in IBM SPSS Statistics version 21.[34] Associations between the various variables of interest were studied calculating Pearson correlation coefficients. To investigate the association between ASD severity and tuber and RML count, linear regression analyses were performed. Post-hoc analyses were performed, assessing the associations in the separate lobes of the brain. A Bonferroni correction was applied to correct for multiple testing. Because of the considerable intercorrelations between the number of tubers or RMLs in the separate lobes (ranging between 0.63-0.78 and 0.32-0.51 respectively), we first calculated the effective number of tests and adjusted the Bonferroni correction accordingly to account for this lack of independence.[35] The calculation yielded an effective number of 2.98 tests for the tuber analyses and 3.57 tests for the RML analyses. In all tables, both uncorrected as well as Bonferroni corrected (p_{corr}) are provided, as well as b and adjusted R^2 effect size measures.

To assess whether IQ/DQ was a mediator in the association between ASD severity and tuber or RML count, formal mediation analyses were performed using the 'PROCESS' macro for SPSS, version 2.15 (http://www.afhayes.com/) with bias-corrected bootstrapping using 1,000 replications.[36] For the mediation analyses, effect sizes are reported as k^2, with values of 0.01, 0.09 and 0.25 considered as small, medium and large respectively.[37]

RESULTS

Patient characteristics

In total, the data of 52 patients (24 boys, 28 girls) were included. The mean age at time of the MRI was 7.0 years (range 0-17) and the mean age at time of the ADOS assessment was 8.8 years (range 2-17). Of the patients, 26.9% had a TSC1 mutation and 69.2% a TSC2 mutation. Epilepsy was present in 88.5% of all children. The total number of tubers ranged between 0 and 81 and the total number of RMLs between 0 and 37. The largest number of tubers and RMLs were located in the frontal lobes. According to the ADOS,

a total number of 24 children (46.2%) met the criteria for an ASD diagnosis. Additional patient characteristics are shown in table 1.
In A-Table 1 in the appendix, the Pearson correlations between all variables of interest are shown.

Table 1. Patient characteristics

		n (%)	Mean (SD)	Min-Max
Gender, male		24 (46.2)		
Age in years				
	During MRI		7.0 (3.9)	0-17
	During ADOS		8.8 (4.2)	2-17
Age difference in years between ADOS-MRI			1.8 (2.9)	0-14
Mutation				
	TSC1	14 (26.9)		
	TSC2	36 (69.2)		
	No mutation identified	1 (1.9)		
	Clinical diagnosis	1 (1.9)		
Epilepsy, yes		46 (88.5)		
Number of tubers				
	Total		27.5 (20.2)	0-81
	Frontal lobe		16.0 (12.1)	0-54
	Parietal lobe		5.5 (4.6)	0-19
	Temporal lobe		3.7 (3.3)	0-12
	Occipital lobe		2.4 (2.6)	0-9
Cystic tubers				
	Present, yes	19 (36.5)		
	Number		1.85 (3.88)	0-18
Calcified tubers				
	Present, yes	9 (17.3)		
	Number		0.71 (2.52)	0-17
SENs				
	Present, yes	47 (90.4)		
	Number		7.5 (5.2)	0-21
SEGAs				
	Present, yes	6 (11.5)		
	Number		0.2 (0.51)	0-2
Number of radial migration lines				
	Total		16.0 (10.2)	0-37
	Frontal lobe		8.2 (5.6)	0-23
	Parietal lobe		3.9 (3.3)	0-15

Table 1. Patient characteristics (continued)

		n (%)	Mean (SD)	Min-Max
	Temporal lobe		3.0 (3.0)	0-12
	Occipital lobe		0.9 (1.2)	0-4
IQ/DQ			59.7 (24.5)	8-114
ADOS module				
	Module 1	15 (28.8)		
	Module 2	11 (21.2)		
	Module 3	18 (34.6)		
	Module 4	8 (15.4)		
ADOS ASD diagnosis				
	Non-spectrum	28 (53.8)		
	Autism spectrum disorder	7 (13.5)		
	Autism	17 (32.7)		
ADOS calibrated severity score				
	Total		4.0 (2.7)	1-10
	Social affect domain		4.3 (2.6)	1-10
	Restricted and repetitive behaviors domain		4.8 (2.8)	1-10

Note: *n*=52. ADOS=Autism Diagnostic Observation Scale, DQ=developmental quotient, IQ=intelligence quotient, MRI=magnetic resonance imaging, SEGA=subependymal giant cell astrocytoma, SEN=subependymal nodule.

Association ASD severity and tuber count

A highly significant association was found between the ADOS total severity score and total tuber count (β=0.46, $p<0.001$), and about 20% of the variance in the ADOS total severity score could be explained by the total number of cortical tubers. Post-hoc analyses assessing the separate lobes of the brain indicated similar results for all lobes (table 2).

Table 2. Association ADOS total calibrated severity score and tuber count

	Model I						Model I + IQ/DQ					
	B	95% CI	β	*p*	p_{corr}[a]	R^2_{adj}	B	95% CI	β	*p*	p_{corr}[a]	R^2_{adj}
Total number of tubers	0.06	0.03;0.09	0.46	**<0.001**	-	0.195	0.02	-0.01;0.06	0.18	0.188	-	0.356
Frontal lobes	0.09	0.03;0.15	0.41	**0.002**	**0.007**	0.155	0.03	-0.04;0.09	0.11	0.414	1	0.341
Parietal lobes	0.24	0.10;0.39	0.43	**0.002**	**0.005**	0.166	0.11	-0.04;0.25	0.19	0.150	0.447	0.360
Temporal lobes	0.31	0.10;0.52	0.38	**0.005**	**0.016**	0.129	0.11	-0.09;0.31	0.14	0.278	0.829	0.348
Occipital lobes	0.40	0.13;0.67	0.39	**0.004**	**0.012**	0.136	0.24	-0.00;0.47	0.23	0.054	0.160	0.382

Note: n=52. ADOS=Autism Diagnostic Observation Scale, IQ=intelligence quotient, DQ=developmental quotient. R^2_{adj}=Adjusted R squared model. [a]Multiple testing correction (2.98 effective tests) applied.

Table 3. Association ADOS subdomain calibrated severity scores and tuber count

		Model I						Model I + IQ/DQ					
		B	95% CI	β	*p*	p_{corr}[a]	R^2_{adj}	B	95% CI	β	*p*	p_{corr}[a]	R^2_{adj}
Total number of tubers	SA domain CSS	0.05	0.01;0.08	0.37	**0.008**	-	0.117	0.01	-0.02;0.05	0.10	0.497	-	0.262
	RRB domain CSS	0.07	0.03;0.10	0.49	**<0.001**	-	0.224	0.04	0.00;0.08	0.29	**0.046**	-	0.299
Frontal lobes	SA domain CSS	0.07	0.01;0.12	0.32	**0.023**	0.069	0.081	0.00	-0.06;0.07	0.02	0.882	1	0.255
	RRB domain CSS	0.12	0.06;0.17	0.49	**<0.001**	**0.001**	0.229	0.07	0.00;0.14	0.30	**0.042**	0.124	0.301
Parietal lobes	SA domain CSS	0.22	0.08;0.36	0.40	**0.003**	**0.010**	0.141	0.10	-0.05;0.25	0.19	0.169	0.503	0.283
	RRB domain CSS	0.22	0.06;0.38	0.36	**0.008**	**0.025**	0.114	0.09	-0.08;0.26	0.15	0.275	0.821	0.257
Temporal lobes	SA domain CSS	0.20	-0.01;0.41	0.26	0.064	0.192	0.048	0.02	-0.19;0.23	0.02	0.876	1	0.255
	RRB domain CSS	0.37	0.15;0.58	0.43	**0.001**	**0.004**	0.168	0.21	-0.02;0.43	0.25	0.071	0.211	0.288
Occipital lobes	SA domain CSS	0.34	0.08;0.61	0.35	**0.012**	**0.036**	0.102	0.20	-0.05;0.45	0.20	0.111	0.330	0.293
	RRB domain CSS	0.34	0.04;0.63	0.31	**0.026**	0.079	0.077	0.18	-0.10;0.46	0.16	0.201	0.598	0.264

Note: n=52. ADOS=Autism Diagnostic Observation Scale, CSS=calibrated severity score, SA=Social Affect, RRB=Restricted and Repetitive Behaviors, IQ=intelligence quotient, DQ=developmental quotient. R^2_{adj}=Adjusted R squared model. [a]Multiple testing correction (2.98 effective tests) applied.

Because IQ/DQ was significantly related to both the ADOS total severity score and the total number of tubers (as well as to the two separate ADOS subdomain scores and the number of tubers in all separate lobes) (A-Table 1 in the appendix), the analyses were repeated with IQ/DQ added as a covariate. The results of these analyses show that this correction rendered all associations insignificant, indicating that IQ/DQ was an important explanatory variable in the associations (table 2).

Next, we studied the association between total tuber count and ASD severity in the two subdomains of the ADOS; social affect (SA) and restricted and repetitive behaviors (RRB) (table 3). Again, strong associations were found between the total number of tubers and ADOS SA and RRB severity scores (β=0.37, p=0.008 and β=0.49, p<0.001), and 12% and 22% of the variance in respectively SA and RRB severity score explained by total tuber count.

After adding IQ/DQ as a covariate, the total number of tubers only remained significantly associated with the RRB severity score (β=0.29, p=0.046). Post-hoc analyses studying the separate lobes of the brain indicated that this association was mainly driven by tuber count in the frontal lobes (β=0.30, p=0.042, p_{corr}=0.124). An (uncorrected) trend was visible for the association between temporal tuber count and RRB severity (β=0.25, p=0.071, p_{corr}=0.211).

To formally assess whether IQ/DQ was a mediator in the association between the total number of tubers and the ADOS total severity score, a mediation analysis was performed (figure 2, panel A). The mediation analysis showed that the direct effect (c' path) of total tuber count on the total severity score was insignificant. The indirect (a*b path)

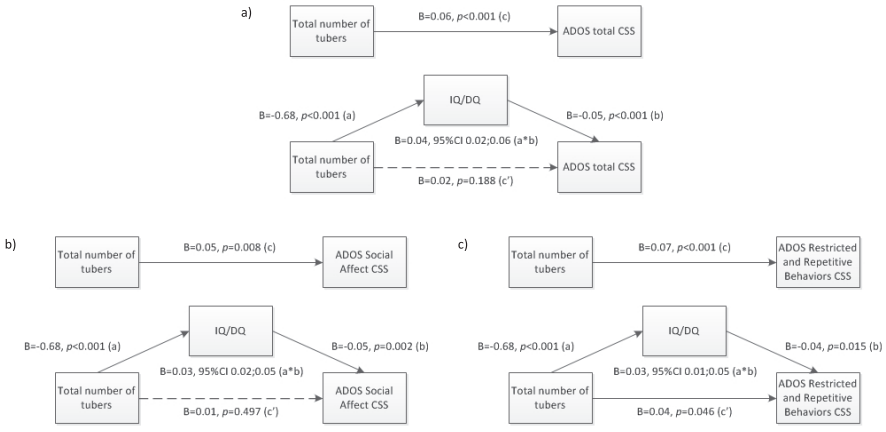

Figure 2. Mediation analyses tuber count, ASD severity score and IQ/DQ. (A) ADOS total severity score, (B) ADOS Social Affect (SA) domain severity score, (C) ADOS Restricted and Repetitive Behaviors (RRB) domain severity score.

effect through IQ/DQ was large and statistically significant (B=0.04, 95%CI=0.02;0.06, k^2=0.263). This implies that the total effect (c path) between total tuber count and the total severity score was fully mediated by IQ/DQ. Post-hoc mediation analyses for the separate lobes were also performed (figures not shown), also showing full mediation by IQ/DQ for all lobes.

Next, mediation analyses were performed studying the role of IQ/DQ in the association between total tuber count and the severity score of the two separate ADOS domains. For the SA domain (figure 2, panel B), the direct effect (c' path) of total tuber count on the SA severity score was insignificant. The indirect (a*b path) effect through IQ/DQ was medium to large and statistically significant (B=0.03, 95%CI=0.02;0.05, k^2=0.242). Again, this means that the total effect (c path) between total tuber count and the SA severity score was fully mediated by IQ/DQ. However, in line with the regression analyses, the mediation analysis performed with the RRB domain (figure 2, panel C) showed that although the indirect (a*b path) through IQ/DQ was medium to large and significant (B=0.03, 95%CI=0.01;0.05, k^2=0.189), the direct effect (c' path) of total tuber count on the RRB severity score also remained significant (B=0.04, *p*=0.046). This means that a direct effect of total tuber count on the RRB severity score was present and that the total effect (c path) was only partly mediated by IQ/DQ. Again, in line with the regression analyses, post-hoc analyses of the separate lobes (figures not shown) indicated full mediation by IQ/DQ, with the exception of the frontal lobes. For the frontal lobe association with the RRB score, the indirect (a*b path) through IQ/DQ was medium to large and significant (B=0.05, 95%CI=0.01;0.09, k^2=0.188), but the direct effect (c' path) between frontal lobe tuber count and the RRB severity score remained significant as well (B=0.07, *p*=0.042), again implying only partial mediation by IQ/DQ.

To study whether specific associations were found between the ADOS severity scores and the total number of cystic and calcified cortical tubers, additional supplementary analyses were performed (A-Table 2 in the appendix). The analyses showed that, after correction for IQ/DQ, only the number of calcified tubers was significantly associated with the ADOS total severity score (β=0.27, *p*=0.020) and the RRB subdomain severity score (β=0.36, *p*=0.010). A trend-level association was found between the number of calcified tubers and the SA subdomain severity score (β=0.21, *p*=0.084).

Association ASD severity and radial migration line count

A significant association was found between the ADOS total severity score and the total number of radial migration lines (RMLs) (β=0.40, *p*=0.003), and 15% of the variance in ADOS total severity score was accounted for by the total number of radial migration lines. Post-hoc analyses assessing the separate lobes of the brain indicated significant associations for the parietal lobes and occipital lobes specifically (table 4).

Table 4. Association ADOS total calibrated severity score (CSS) and radial migration lines (RMLs)

	Model I						Model I + IQ/DQ					
	B	**95% CI**	**β**	***p***	p_{corr} [a]	R^2_{adj}	**B**	**95% CI**	**β**	***p***	p_{corr} [a]	R^2_{adj}
Total number of RMLs	0.11	0.04;0.17	0.40	**0.003**	-	0.146	0.05	-0.02;0.11	0.19	0.141	-	0.362
Frontal lobes	0.14	0.02;0.27	0.31	**0.025**	0.090	0.078	0.04	-0.08;0.15	0.08	0.505	1	0.338
Parietal lobes	0.28	0.07;0.49	0.35	**0.011**	**0.038**	0.106	0.14	-0.05;0.33	0.18	0.140	0.501	0.362
Temporal lobes	0.21	-0.04;0.46	0.24	0.094	0.336	0.036	0.10	-0.11;0.31	0.11	0.364	1	0.344
Occipital lobes	0.90	0.30;1.50	0.39	**0.004**	**0.015**	0.135	0.65	0.14;1.16	0.28	**0.013**	**0.047**	0.412

Note: n=52. ADOS=Autism Diagnostic Observation Scale, IQ=intelligence quotient, DQ=developmental quotient. R^2_{adj}=Adjusted R squared model. [a]Multiple testing correction (3.57 effective tests) applied.

Table 5. Association ADOS subdomain calibrated severity scores (CSS) and radial migration lines (RMLs)

		Model I						Model I + IQ/DQ					
		B	95% CI	β	*p*	p_{corr}[a]	R^2_{adj}	B	95% CI	β	*p*	p_{corr}[a]	R^2_{adj}
Total number of RMLs	SA domain CSS	0.10	0.04;0.17	0.41	**0.002**	-	0.155	0.06	-0.01;0.12	0.23	0.080	-	0.300
	RRB domain CSS	0.09	0.02;0.17	0.33	**0.016**	-	0.093	0.04	-0.04;0.11	0.14	0.300	-	0.255
Frontal lobes	SA domain CSS	0.15	0.03;0.27	0.33	**0.017**	0.061	0.091	0.06	-0.06;0.18	0.14	0.296	1	0.271
	RRB domain CSS	0.13	-0.00;0.27	0.27	0.057	0.203	0.052	0.03	-0.10;0.17	0.07	0.608	1	0.243
Parietal lobes	SA domain CSS	0.27	0.07;0.47	0.35	**0.010**	**0.037**	0.107	0.15	-0.04;0.35	0.20	0.110	0.394	0.293
	RRB domain CSS	0.21	-0.03;0.44	0.25	0.081	0.288	0.041	0.07	-0.14;0.29	0.09	0.504	1	0.246
Temporal lobes	SA domain CSS	0.20	-0.04;0.44	0.23	0.101	0.359	0.034	0.10	-0.11;0.32	0.12	0.343	1	0.268
	RRB domain CSS	0.24	-0.03;0.50	0.25	0.075	0.269	0.043	0.13	-0.10;0.37	0.14	0.262	0.935	0.258
Occipital lobes	SA domain CSS	0.87	0.29;1.45	0.39	**0.004**	**0.015**	0.137	0.66	0.14;1.18	0.30	**0.013**	**0.047**	0.343
	RRB domain CSS	0.64	-0.03;1.31	0.26	0.062	0.222	0.049	0.40	-0.20;1.00	0.16	0.186	0.665	0.266

Note: n=52. ADOS=Autism Diagnostic Observation Scale, SA=Social Affect, RRB=Restricted and Repetitive Behaviors, IQ=intelligence quotient, DQ=developmental quotient. R^2_{adj}=Adjusted R squared model. [a]Multiple testing correction (3.57 effective tests) applied.

Because IQ/DQ was significantly related to both the ADOS total severity score and the total number of RMLs (as well as to the two separate ADOS subdomain scores and the number of RMLs in the frontal and parietal lobes) (A-Table 1 in the appendix), the analyses were repeated with IQ/DQ added as a covariate. The results of these analyses show that after the correction for IQ/DQ only the association for the occipital lobe remained significant (β=0.28, p=0.013, p_{corr}=0.047) (table 4).

Next, we studied the association between RML count and ASD severity in the two subdomains of the ADOS; social affect (SA) and restricted and repetitive behaviors (RRB) (table 5). Again, highly significant associations were found between total RML count and ADOS SA and RRB severity (β=0.41, p=0.002 and β=0.33, p=0.016), and 16% and 9% of the variance in respectively SA and RRB severity score explained by total RML count.

After adding IQ/DQ as a covariate, only the number of RMLs in the occipital lobes remained significantly associated with the SA severity score (β=0.30, p=0.013, p_{corr}=0.047).

Again, a formal mediation analysis was performed, assessing whether IQ/DQ was a mediator in the association between the total number of RMLs and the ADOS total severity score (figure 3, panel A). The mediation analysis showed that the direct effect (c' path) of total RML count on the total severity score was insignificant. The indirect (a*b path) effect through IQ/DQ was medium to large and statistically significant (B=0.06, 95%CI=0.03;0.10, k^2=0.223). This implies that the total effect (c path) between total RML count and the total severity score was fully mediated by IQ/DQ. Post-hoc mediation analyses for the frontal and parietal lobes were performed as well (figures not shown),

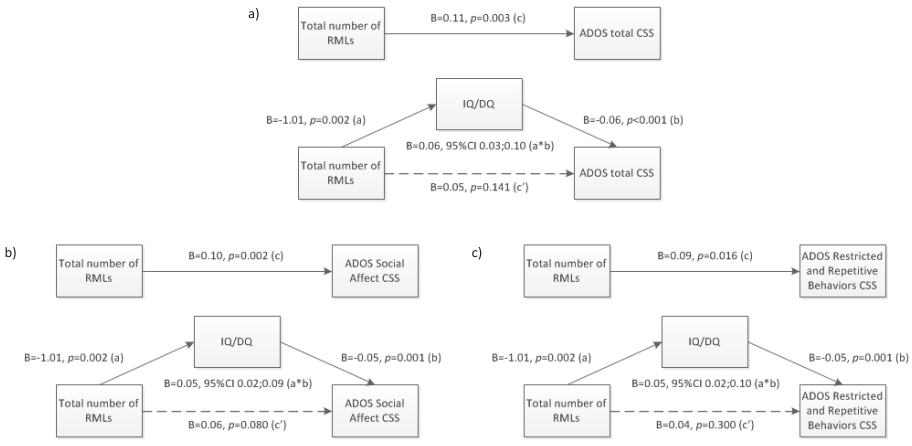

Figure 3. Mediation analyses RML count, ASD severity score and IQ/DQ. (A) ADOS total severity score, (B) ADOS Social Affect (SA) domain severity score, (C) ADOS Restricted and Repetitive Behaviors (RRB) domain severity score.

also showing full mediation by IQ/DQ. Since IQ/DQ was not significantly related to RML count in the temporal and occipital lobes, the earlier identified association between total number of RMLs in the occipital lobes and the ADOS total severity score could not be mediated by IQ/DQ, implying a direct effect of occipital lobe RML count on the total ADOS severity score.

As with tuber count, the mediation analyses were also performed studying the role of IQ/DQ in the association between total RML count and the severity score of the two separate ADOS subdomains (figure 3, panel B and C). For both subdomains, the direct effect (c' path) of total RML count on the subdomain severity score was insignificant. The indirect (a*b path) effect through IQ/DQ was medium to large and statistically significant (B=0.05, 95%CI=0.02;0.09, k^2=0.184 for SA domain, and B=0.05, 95%CI=0.02;0.10, k^2=0.190 for RRB domain). This implies that the total effect (c path) between total RML count and the SA and RRB severity scores was fully mediated by IQ/DQ. Post-hoc analyses of the separate lobes (figures not shown) again showed full mediation by IQ/DQ for the frontal and parietal lobes for both the SA and RRB subdomains. Because IQ/DQ was not significantly related to RML count in the temporal and occipital lobes, the earlier found association between RML count in the occipital lobes and the SA severity score could not be mediated by IQ/DQ, implying a direct effect of occipital lobe RML count on the SA severity score.

DISCUSSION

In the current clinical epidemiological study, the association between the number and location of cortical tubers and RMLs and a quantitative observational measure of ASD severity was studied in a clinical sample of children with TSC. The specificity of the association with the two main subdomains of ASD symptomatology (deficits in social communication and interaction, and restricted or repetitive behaviors) was studied as well. Finally, we focused on the role of cognitive functioning in these associations.

Total cortical tuber count, as well as tuber count in the separate lobes of the brain, was strongly related to the severity of ASD. The associations were strongest for the severity of restricted and repetitive behaviors, but also present for difficulties in social affect. When IQ/DQ was added as a covariate to the analyses, only total and frontal tuber count remained related to the severity of restricted and repetitive behaviors, although it must be noted that the frontal association did not survive correction for multiple testing. When studying the association between RML count and ASD severity, we found the total number of RMLs and RML count in the parietal and occipital lobes to be related to ASD severity. RML count was specifically related to the severity of problems in social affect,

and not to the severity of restricted and repetitive behaviors. When IQ/DQ was added to the models, only the association between RML count in the occipital lobes and the total ASD severity score, as well as the severity of problems in social affect, remained. The formal mediation analyses confirmed all results and showed that, indeed, most initial findings were fully mediated by IQ/DQ.

Our results emphasize the importance of taking cognitive functioning into account when studying the relation between brain pathology and ASD in patients with TSC. TSC is characterized by cognitive impairment, and previous studies have shown that cognitive impairment is strongly related to both brain pathology [6,15–17] and ASD severity,[18] thereby acting as an important confounding (or rather explanatory) factor in this association. However, one should also realize that regardless of the explanatory role of cognitive functioning, ASD symptoms remain a significant problem in patients with TSC, which should be managed and/or treated accordingly.

We found a direct association, regardless of cognitive functioning, between frontal lobe tuber count and the severity of restricted and repetitive behaviors in children with TSC. This is an interesting finding, based on the fact that impulse inhibition (or behavioral control) is one of the main functions of the frontal lobes, of which structural abnormalities have often been reported in (developmental) disorders characterized by poor behavioral and inhibitory control such as attention-deficit/hyperactivity disorder,(38) obsessive-compulsive disorder[39] and ASD.[40] Furthermore, a direct association was found between RML count in the occipital lobes and the severity of problems in social communication and interaction. The main function of the occipital lobes is processing visual stimuli, and structural and functional abnormalities in the occipital and occipito-temporal regions have been frequently reported in ASD.[41]

A strength and novel aspect of our study is the use of a quantitative measure of ASD severity. Not only does this provide a more naturalistic representation of ASD symptoms, it also provides more statistical power and allows the application of advanced statistical methods.[11] Furthermore, next to studying overall ASD severity, this approach allowed us to study the two different domains of ASD symptomatology; difficulties in social communication and interaction, and restricted and repetitive behaviors. According to the latest edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5)[14] ASD symptoms should be divided in these two domains, and the severity of difficulties in these domains should be evaluated separately. Because the nature of the symptoms in these domains is substantially different, it is highly plausible that distinct neural mechanisms in different brain regions may underlie the two different domains of symptoms, which indeed seems to be the case based on the results of our study. Another strength of this study is the use of a standardized observational measure of ASD, thereby reducing reporter bias. However, a disadvantage of this measure and a weakness of this study is the lack of information from other informants, such as parents

or teachers. Also, the relatively small sample size of our study might have reduced the power to reveal relatively subtle effects, potentially resulting in an underestimation of effects. Finally, even though all TSC patients within our expertise center ENCORE are being referred for a developmental and psychiatric evaluation (regardless of whether or not the child experiences cognitive or behavioral difficulties) which reduces the risk of selection bias (and improves the generalizability of findings to the pediatric TSC population as a whole), the risk of residual selection bias remains.

CONCLUSIONS

To conclude, our study showed strong associations between brain pathology and ASD severity, with children with more cortical tubers and RMLs having more severe ASD symptoms. Cognitive functioning was identified as an important confounding, or rather explanatory, factor in this association, highlighting the importance of taking cognitive functioning into account when studying the relation between brain pathology and ASD symptomatology. Furthermore, our study underlines the relevance of separately studying problems in social communication and interaction on the one hand, and restricted and repetitive behaviors on the other hand. Regardless of cognitive functioning, children with more frontal lobe tubers displayed more severe restrictive and repetitive behaviors and children with more RMLs in the occipital lobes specifically showed more difficulties in social communication and interaction.

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APPENDIX

A-Table 1. Pearson correlation coefficients main measures.

		1	2	3	4	5	6	7	8	9	10	11	12	13
1	No. of tubers	-												
2	No. of tubers frontal	0.97**	-											
3	No. of tubers parietal	0.84**	0.73**	-										
4	No. of tubers temporal	0.84**	0.77**	0.63**	-									
5	No. of tubers occipital	0.71**	0.61**	0.55**	0.55**	-								
6	No. of RMLs	0.71**	0.69**	0.61**	0.52**	0.54**	-							
7	No. of RMLs frontal	0.54**	0.61**	0.37**	0.35*	0.29*	0.90**	-						
8	No. of RMLs parietal	0.59**	0.48**	0.80**	0.37**	0.46**	0.70**	0.48**	-					
9	No. of RMLs temporal	0.51**	0.49**	0.30*	0.54**	0.46**	0.67**	0.49**	0.20	-				
10	No. of RMLs occipital	0.51**	0.41**	0.45**	0.40**	0.74**	0.58**	0.35*	0.46**	0.41**	-			
11	ADOS total CSS	0.46**	0.41**	0.43**	0.38**	0.39**	0.40**	0.31*	0.35*	0.24	0.39**	-		
12	ADOS SA domain CSS	0.37**	0.32*	0.40**	0.26	0.35*	0.41**	0.33*	0.35*	0.23	0.39**	0.92**	-	
13	ADOS RRB domain CSS	0.49**	0.49**	0.36**	0.43**	0.31*	0.33*	0.27	0.25	0.25	0.26	0.64**	0.39**	-
14	IQ / DQ	-0.56**	-0.56**	-0.47**	-0.45**	-0.31*	-0.42**	-0.40**	-0.322*	-0.22	-0.20	-0.60**	-0.53**	-0.52**

Note: n=52. ADOS=Autism Diagnostic Observation Scale, CSS=calibrated severity score, SA=Social Affect, RMLs=Radial migration lines, RRB=Restricted and Repetitive Behaviors, IQ=intelligence quotient, DQ=developmental quotient. **$p<0.01$, *$p<0.05$

A-Table 2. Association ADOS calibrated severity scores and cystic/calcified tuber count

		Model I				Model I + IQ/DQ			
		B	**95% CI**	**β**	***p***	**B**	**95% CI**	**β**	***p***
Number of cystic tubers	Total CSS	0.10	-0.09;0.30	0.15	0.281	-0.00	-0.16;0.16	-0.00	0.994
	SA domain CSS	0.09	-0.10;0.27	0.13	0.354	-0.00	-0.17;0.16	-0.01	0.965
	RRB domain CSS	0.21	0.01;0.40	0.28	**0.043**	0.12	-0.07;0.30	0.16	0.207
Number of calcified tubers	Total CSS	0.10	-0.20;0.39	0.09	0.523	0.28	0.05;0.52	0.27	**0.020**
	SA domain CSS	0.06	-0.23;0.35	0.06	0.691	0.22	-0.03;0.47	0.21	0.084
	RRB domain CSS	0.17	-0.14;0.49	0.16	0.271	0.36	0.09;0.62	0.32	**0.010**

Note: n=52. ADOS=Autism Diagnostic Observation Scale, CSS=calibrated severity score, SA=Social Affect, RRB=Restricted and Repetitive Behaviors, IQ=intelligence quotient, DQ=developmental quotient.

CHAPTER 4

Interdependence of clinical factors predicting cognition in children with Tuberous Sclerosis Complex

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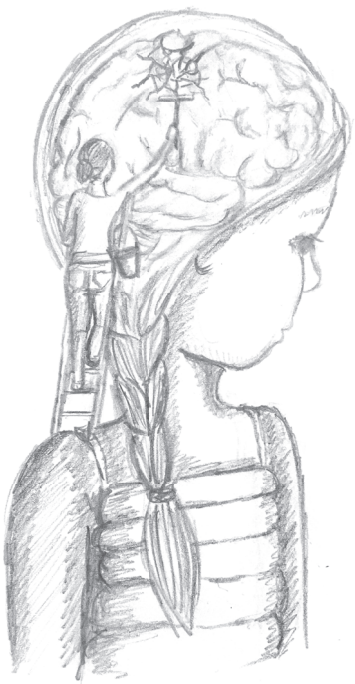

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ABSTRACT

Objective: Cognitive development in patients with Tuberous Sclerosis Complex is highly variable. Predictors in the infant years would be valuable to counsel parents and to support development. The aim of this study was to confirm factors that have been reported to be independently correlated with cognitive development.

Methods: 102 patients included in this study were treated at the ENCORE-TSC expertise center of the Erasmus Medical Center-Sophia Children's Hospital. Data from the first 24 months of life were used, including details on epilepsy, motor development and mutation status. Outcome was defined as cognitive development (intellectual equivalent, IE) as measured using tests appropriate to the patients age and cognitive abilities (median age at testing 8.2 years, IQR 4.7-12.0). Univariable and multivariable regression analyses were used.

Results: In a univariable analysis, predictors of lower IE were: presence of infantile spasms (β=-18.3, p=0.000), a larger number of antiepileptic drugs used (β=-6.3, p=0.000), vigabatrin not used as first drug (β=-14.6, p=0.020), corticosteroid treatment (β=-33.2, p=0.005), and a later age at which the child could walk independently (β=-2.1, p=0.000). An older age at seizure onset predicted higher IE (β=1.7, p=0.000). In a multivariable analysis, only age at seizure onset was significantly correlated to IE (β=1.2, p=0.005), contributing to 28% of the variation in IE.

Conclusions: In our cohort, age at seizure onset was the only variable that independently predicted IE. Factors predicting cognitive development could aid parents and physicians in finding the appropriate support and schooling for these patients.

INTRODUCTION

Tuberous Sclerosis Complex (TSC) is a genetic disorder, with a large variation in symptoms between patients. Up to 90% of patients suffer from epilepsy, often starting in infancy.[1] Two thirds of patients have refractory seizures.[1,2] Cognitive function is highly variable in patients with TSC. One third of patients suffer from profound disability, while others have normal cognitive abilities.[3-5]

Several studies have attempted to unravel the causes of this variation in cognitive development. Many studies have shown that epilepsy is associated with delayed development and impaired cognitive functioning.[6] In previous studies, refractory seizures and infantile spasms were correlated with lower cognitive functioning, while older age at seizure onset and early treatment correlated with better cognition.[4,7-10] Lesion burden on brain MRI has also been correlated with cognition, including the presence and number of cortical tubers.[11,12]

Most of these correlations were identified using a univariable analysis, between the factor of interest and the cognitive abilities of the patient. While it is useful to explore these correlations, it remains unclear what the actual contribution of a specific variable to cognitive functioning is, if the statistical method does not control for other possible influencing factors. This is further complicated by the rare nature of TSC; multivariable regression models can only hold multiple variables if sufficient patients are available to include in the analyses. In a previous study where a multivariable model was applied, the age at seizure onset was found to be the only independent predictor of IE.[9]

In a cohort of 102 children with TSC, we aimed to confirm previously studied and identify new unstudied characteristics collected in the first 24 months of life, that are independent predictors for cognitive development later in life.

METHODS

Patients were selected from a retrospective follow-up clinical database at the ENCORE-TSC expertise center at the Erasmus Medical Center-Sophia Children's Hospital. Missing data were completed through correspondence with physicians and other care providers. Of the total population of 121 children treated at our clinic, data on cognitive development were available for 102 children.

For all variables except intelligence equivalent (IE), data from the first 24 months of life were used. Mutation analysis was performed at the Department of Clinical Genetics of the Erasmus Medical Center according to standard procedures, using DNA extracted from peripheral blood. Success of the first AED was categorized as 'yes' if the first AED caused a seizure frequency decrease of 50% or more.

For all variables concerning epilepsy, children without epilepsy were given the most beneficial outcome, since previous studies have shown that the absence of epilepsy is beneficial for cognitive development.[6] The variables adjusted in patients without epilepsy are listed in the appendix. In addition, some values were adjusted in children with epilepsy in order to include data from the first twenty-four months of life only; the variable 'age at seizure onset ' was adjusted to twenty-four months ('24') in children with age at onset after twenty-four months of age (16 children), and for the variable 'age of walking independently', children who were not able to walk were assigned the value of twenty-four months ('24') (17 children).

The outcome of all analyses was IE, measured by the most reliable cognitive development testing based on the calendar age and cognitive abilities of the child. The Dutch versions of the following neuropsychological tests were used: Wechsler Intelligence Scale for Children (WISC; n=45), Bayley Scales of Infant Development (BSID; n= 36), Wechsler Preschool and Primary Scale of Intelligence (WPPSI; n=14), Snijders-Oomen Non-verbal Intelligence assessment (SON-R; n=5), Attachment, Interaction, Mastery, Support (AIMS; n=1) and Vineland Adaptive Behavior Scales (VABS; n=1). Since some of these psychological tests measure development and not IQ, the value obtained from all tests was called intelligence equivalent, IE.[13]

Statistical analysis

For all continuous variables except IE, outliers were removed by only allowing data values within the 5^{th} and 95^{th} percentile, and adjusting values outside this range to the values of the 5^{th} and 95^{th} percentile. This adjusted three data points for the variable 'age of walking independently' and two for the 'number of AEDs used' variable. Multiple imputation was used to eliminate missing values. Missing values were present for the following variables: 'number of AEDs used' (6), 'treatment success of first AED' (7) and 'age of walking independently' (6).

IE values between children with and without epilepsy were compared using an independent samples T test.

Univariable and multivariable regression models were fitted with IE as outcome. Predictor variables in the regression model were selected from previous studies investigating the association between cognitive development and disease characteristics in patients with TSC. Variables with a Pearson correlation of >0.8 with other variables were left out of the model to prevent colinearity. This excluded the variable measuring the total number of months in which seizures were present during the first 24 months of life. Beta's with 95% confidence intervals and corresponding p values were reported from the models. A variable was considered to contribute significantly to IE if the p value was below 0.05. The total amount of variance in IE explained by the models was expressed with the R^2. IBM SPSS statistics version 22 was used for all analyses.

RESULTS

Characteristics of 102 children included in this study are shown in table 1. IE was measured at a median age of 8.2 years. The median IE of all children was 59 (IQR 39-78) and the median IE of children with epilepsy was 55 (IQR 30-73). IE values are shown in Fig. 1, for children with epilepsy and children without epilepsy. Distribution of IE values of the total study population was shifted towards lower values compared to values in the general population. Median IE in children with epilepsy was 55, and in children without epilepsy 81. Children with epilepsy had lower IE values ($p<0.001$).

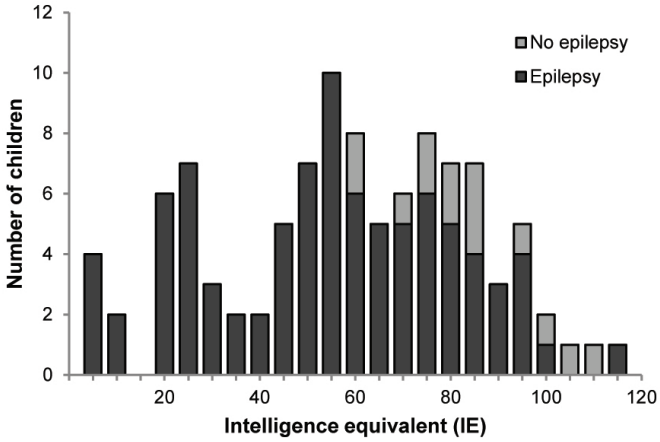

Figure 1. Intelligence equivalent (IE) measured by standard cognitive tests (no epilepsy n=14, epilepsy n=88).

Variables included in our regression models and their effect on IE are shown in table 2 for all included children and in table 3 for the subgroup of children with epilepsy. Univariable regression analysis of all children showed lower IE values if the child had epilepsy, if the child had infantile spasms, when a larger number of AEDs was used, if vigabatrin was used as first or second drug, if corticosteroid treatment was used, and if a child was older when it could walk independently. IE values were higher when seizure onset was at a later age, and when treatment success was achieved by the first AED. No significant effect of mutation in TSC1 or TSC2 was found.

In a multivariable regression analysis including all variables listed in table 2, a later age at seizure onset was the only variable that had a significant effect on IE. A delay of seizure onset of 12 months would mean an increase in IE of 14.0 points.

To assess predictive effects in children with epilepsy only, analyses were repeated excluding all children without epilepsy. Analysis of 88 children with epilepsy showed

Table 1. Description of data from all children (n=102), and the subgroup of children with epilepsy (n=88).

Variable	All children (n=102), number (%) / median (IQR)	Children with epilepsy (n=88), number (%) / median (IQR)
Gender male	53 (52)	46 (52)
Age at cognitive testing (years)	8.2 (4.7-12.0)	8.0 (4.0-11.9)
Intelligence equivalent (IE)	59 (39-78)	55 (30-73)
Mutation:		
TSC1	27 (27)	21 (24)
TSC2	67 (66)	60 (68)
NMI	6 (6)	5 (6)
Epilepsy	88 (86)	88 (100)
Infantile spasms	37 (36)	37 (42)
Age at seizure onset (months)	8 (4-24)	6 (3-16)
Number of AEDs used	1 (0-3)	2 (1-4)
Success of first AED	75 (74)	61 (70)
Vigabatrin used as:		
First AED	33 (32)	19 (22)
Second AED or later	30 (29)	30 (34)
Never used	39 (38)	39 (44)
Corticosteroid treatment	5 (5)	5 (6)
Age of walking independently (months)	18 (14-22)	18 (14-23)

IQR inter quartile range. NMI no mutation identified. AED anti-epileptic drug.

lower IE values if the child had infantile spasms, when a larger number of AEDs was used, if vigabatrin was used as first or second drug, if corticosteroid treatment was used, and if a child was older when it could walk independently. IE values were higher when seizure onset was at a later age. No correlation was found between IE and mutation in TSC1 or TSC2. The multivariable analysis showed that only a later age at seizure onset was independently predictive of IE. A delay of seizure onset of 12 months would mean an IE increase of 14.2 points in these children.

The correlation of IE with the age at seizure onset is shown in Fig. 2. Figure a shows the correlation with IE for all children, in whom 28% of IE variation is explained by the age at seizure onset. Figure b shows the correlation of IE variation with the age at seizure onset in children who had their first seizure within the first 24 months of life. 32% of IE variation is explained by age at seizure onset in this subgroup.

Table 2. Analysis results of all children (n=102).

Variable	Univariable β	95% CI	p value	Multivariable β	95% CI	p value
Mutation						
TSC1	17.7	-2.0 to 37.4	0.078	5.7	-12.3 to 23.7	0.534
TSC2	-3.3	-21.6 to 15.1	0.728	-3.5	-20.3 to 13.2	0.679
Epilepsy	-28.4	-42.3 to -14.6	0.000	-8.8	-28.5 to 10.9	0.383
Infantile spasms	-18.3	-28.4 to -8.2	0.000	2.0	-10.0 to 13.9	0.746
Age at seizure onset (months)	1.7	1.3 to 2.2	0.000	1.2	0.4 to 2.0	0.005
Number of AEDs used	-6.3	-8.6 to -4.0	0.000	-0.9	-4.7 to 2.9	0.634
Success of first AED	15.7	4.0 to 27.4	0.008	-0.2	-12.3 to 11.8	0.972
Vigabatrin used						
First AED	-0.9	-12.9 to 11.1	0.881	-3.3	-17.7 to 11.1	0.654
Second AED	-14.6	-26.9 to -2.3	0.020	-4.5	-17.8 to 8.8	0.509
or later	x	x	x	x	x	x
Never used						
Corticosteroid treatment	-33.2	-56.1 to -10.3	0.005	-11.8	-34.9 to 11.3	0.318
Age of walking independently (months)	-2.1	-3.2 to -0.9	0.000	-0.3	-1.5 to 0.9	0.622

CI confidence interval. AED anti-epileptic drug.

Table 3. Analysis results of children with epilepsy (n=88).

Variable	Univariable β	95% CI	p value	Multivariable β	95% CI	p value
Mutation						
TSC1	16.6	-4.4 to 37.5	0.121	4.1	-16.2 to 24.4	0.691
TSC2	-4.2	-23.4 to 15.0	0.666	-6.1	-24.8 to 12.6	0.523
Infantile spasms	-13.4	-24.0 to -2.8	0.013	1.7	-10.9 to 14.2	0.796
Age at seizure onset (months)	1.6	1.1 to 2.2	0.000	1.2	0.3 to 2.0	0.007
Number of AEDs used	-5.3	-7.8 to -2.8	0.000	-0.9	-4.8 to 3.1	0.661
Succes first AED	11.1	-0.8 to 23.1	0.068	0.5	-12.2 to 13.2	0.937
Vigabatrin used						
First AED	-16.3	-29.9 to -2.6	0.019	-3.2	-18.3 to 11.9	0.675
Second AED or later	-14.6	-26.4 to -2.7	0.016	-4.9	-18.9 to 9.0	0.491
Never used	x	x	x	x	x	x
Corticosteroid treatment	-29.3	-51.9 to -6.8	0.011	-11.8	-36.0 to 12.4	0.340
Age of walking independently (months)	-2.0	-3.2 to -0.8	0.001	-0.2	-1.6 to 1.3	0.828

CI confidence interval. AED anti-epileptic drug.

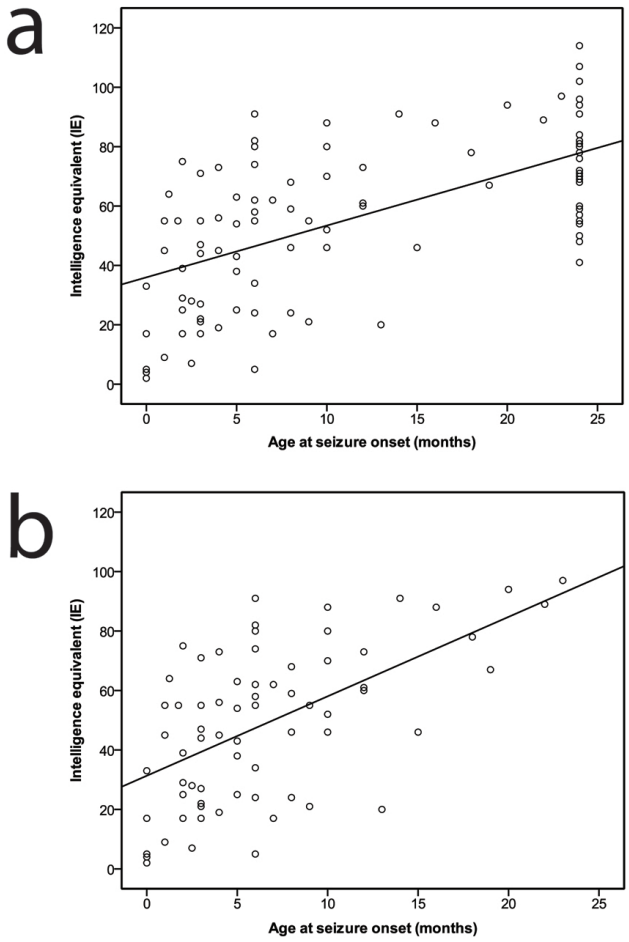

Figure 2. Correlation of Intelligence Equivalent (IE) with age at seizure onset.
(A) Correlation including all children (n=102). 37% of the variance in IE is explained by age at seizure onset. Children without epilepsy are set at 24, children in whom epilepsy started after 24 months are also set at 24. (B). Children in whom epilepsy started within 24 months of age (n=72). 32% of the variance in IE is explained by age at seizure onset.

DISCUSSION

In this study we used a multivariable regression model to confirm factors that were previously reported to contribute to IE, and to identify new factors contributing to IE. We specifically focused on the possible determinants in the first 24 months of life. We found that age at seizure onset could independently predict IE in our cohort of 102 TSC patients. Our analysis showed a 1.2 points increase of IE for every month the child does not develop epilepsy. This is an IE increase of 14 points for every year the child remains seizure free. This is a clinically relevant increase, as it could mean the difference between being dependent on other individuals throughout life, or going to school and leading an independent life. The variation in IE is explained for 28% by age at seizure onset in the total population, and for 32% in a subpopulation of children with epilepsy. Other factors, including details on the treatment of epilepsy, were found to be predictors of cognitive development in a univariable analysis, but had no significant effect after correcting for the age at seizure onset. Therefore, we could not make a prediction model for cognitive development in children with TSC.

Epilepsy is known to negatively influence cognitive functioning.[6] The age at seizure onset has also been previously correlated to cognition, and patients suffering from epilepsy starting in infancy are at high risk of reduced cognitive abilities. This has also been shown for patients with TSC.[7,9] Our study shows that, of all the factors included in our multivariable statistical model, only the age at seizure onset was an independent predictor of cognitive development.

Most studies correlating cognitive functioning to patient related factors have only used univariable analysis methods. Our study confirms that most factors related to epilepsy treatment, for example the number of AEDs and corticosteroid treatment, are correlated to cognitive function when analyzed separately. If they are corrected for the age at seizure onset however, this effect disappears. This underscores the importance of multivariable analyses, in which any association is corrected for possible confounding factors. Our model confirms previous findings from Jansen et al,[9] showing that factors related to cognitive development are highly intertwined, and we should be cautious about giving parents information on the development of their child based on studies using univariable analyses.

Even though studies suggest that early and effective treatment of epilepsy is beneficial for cognitive development,[8] the lack of direct significant correlations found in our analyses is not a new finding. Previous studies in patients with TSC have also struggled to show better cognitive development with successful epilepsy treatment, when trying to find variables independent of other factors.[10] This might indicate that the response to epilepsy treatment is dependent on intrinsic factors of the patient, including the age at seizure onset, and is therefore not an independent predictor. It is also possible that we

did not use the right epilepsy treatment variables. For our study, we explored the literature and found that most studies used response to AEDs and AED resistance as variables to correlate with IE. We attempted to make these variables more objective, by including the number of AEDs used, whether the child used corticosteroids or adrenocorticotropic hormone (ACTH), whether the first AED was successful (a seizure frequency decrease of at least 50%), and whether the child used vigabatrin as a first AED, later during treatment, or never. Furthermore, we explored the variable 'time between the first seizure and the first treatment', as it has been suggested that a treatment delay might cause a decrease of cognitive capabilities.[8] However, in our cohort, a treatment delay was mostly present in patients with a single seizure without an epileptic encephalopathy on EEG. Treatment delay was therefore not a marker of duration of uncontrolled epilepsy, and an AED was usually started when a second seizure occurred. Therefore, a long period between first and second seizure might in our cohort be a marker for a good prognosis. A delay in treatment of infantile spasms may still be an independent prognostic factor. However, due to the low number of children with infantile spasms in our study population, we could not analyze this group separately.

Besides factors related to epilepsy and epilepsy treatment, several other factors have also been correlated with cognitive functioning, including if the patient had a mutation in *TSC1* or *TSC2*.[5] A recent study made a new classification of mutations based on the length of the amino acid tail at the C-terminal in patients with a frameshift or nonsense mutation. A longer C-terminal was correlated with a decreased cognitive functioning in patients with a *TSC1* mutation, and increased cognitive function in patients with a *TSC2* mutation.[14] In contrast to these findings, in a univariable analysis of our cohort, *TSC1* mutations leaving a longer C-terminal tail seemed to correlate with better cognitive functioning. For *TSC2* mutations we could not find a relationship with the length of the C-terminal tail. However, the number of patients with a frameshift or nonsense mutation in our cohort was only 32. Larger cohorts may shed light on the relation between the C-terminal tail and cognitive functioning.

Another factor that has been previously correlated with cognitive function is lesion burden on brain MRI, including the number of cortical tubers, the proportion of brain occupied by tubers and the integrity of white matter.[11,12] We recognize that this lesion burden plays an important role in brain function, and is therefore likely important in the cognitive development of children with TSC. Unfortunately we did not have MRI data of all children in our study cohort, and could therefore not include data on lesion burden to our statistical analysis. Several ongoing studies are collecting data on epilepsy and MRI, including the EPISTOP study (clinicaltrials.gov NCT02098759). This study aims to find biomarkers for epileptogenesis, and will investigate the effect of treatment on epilepsy and cognitive functioning before seizures arise. Combining data from these studies allows the generation of a more detailed predictive model.

We have chosen to use data on possible determinants in the first 24 months of life, since we intended to search for factors in early life that could be correlated with IE. These could aid physicians in making a cautious prediction about the impact of epilepsy and the cognitive outcome of a child. Cognitive development in patients with TSC is very difficult to predict. It would be useful for parents to have an indication of the future abilities of their child, to find the appropriate support and schooling for their child. Finding predictors can also help select children at risk for cognitive problems for future intervention trials.

Our study had several limitations. The children in our study were all treated at a university hospital, which may have introduced a bias towards children who were more severely affected by TSC. Furthermore, some children were treated before the current treatment guidelines for TSC related epilepsy were introduced,[15,16] which might have led to treatment regimens that were not up to the current treatment standard. In addition, our analyses were not able to distinguish between correlation and causality. The question remains whether an early age at seizure onset causes decreased cognitive functioning, or whether the two are caused by the same intrinsic mechanism.

In conclusion, our study shows that, in our study population of 102 children with TSC, age at seizure onset is the only factor that independently predicts cognitive functioning later in life. Finding appropriate markers of early development that can help to predict the cognitive outcome of children with TSC could aid parents and physicians in finding the appropriate support and schooling for these patients, to optimize their chances and outcome in life.

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APPENDIX

A-Table 1. Supplemented values for 14 children without epilepsy.

Variable	Value for children without epilepsy
Epilepsy	no
Infantile spasms	no
Age at seizure onset (months)	24
Number of AEDs used	0
Treatment success of first AED	Yes
Vigabatrin used	First AED
Corticosteroid use	No

CHAPTER 5

Epilepsy in children with Tuberous Sclerosis Complex

Chance of remission and response to antiepileptic drugs

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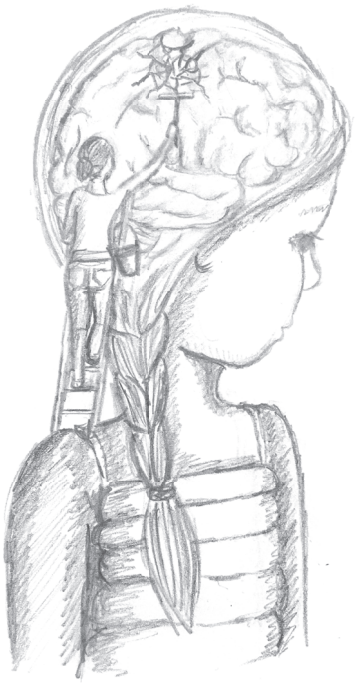

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ABSTRACT

Objective: To describe treatment and outcome of epilepsy in children with Tuberous Sclerosis Complex (TSC).

Methods: Seventy-one children with TSC and epilepsy treated at the ENCORE TSC expertise center between 1988 and 2014 were included. Patient characteristics and duration and effectiveness of antiepileptic treatments were extracted from our clinical database. Correlations were made between recurrence of seizures after response to treatment, and several patient characteristics.

Results: Median age at time of inclusion was 9.4 years (range 0.9-18.0). Seizure history showed that 55 children (77%) of 71 became seizure-free for longer than 1 month, and 21 (30%) of 71 for longer than 24 months. Remission of seizures was associated with higher IQ, and a trend was observed between seizure remission and age at onset of seizures. A total of 19 antiepileptic drugs (AEDs) were used. Valproic acid, vigabatrin, levetiracetam and carbamazepine were used most frequently. Nonpharmacological therapies (ketogenic diet, epilepsy surgery, and vagus nerve stimulation) were used 13 times. Epilepsy surgery was most effective, with four of five children becoming seizure-free. AEDs prescribed as first and second treatment were most effective. Valproic acid was prescribed most frequently as first and second treatment, followed by vigabatrin. Thirty-one children had infantile spasms, preceded by focal seizures in 18 children (58%). Vigabatrin was used by 29 children (94%), and was first treatment in 15 (48%). Vigabatrin was more effective than other AEDs when prescribed as first treatment.

Significance: We showed that, although 77% of children with epilepsy due to TSC reached seizure remission, usually after their first or second AED, this was only sustained for at least 24 months in only 38%. Almost half of those with 24 months remission later had relapse of seizures. Our results support vigabatrin as first choice drug, and show the need for better treatment options for these children.

INTRODUCTION

Tuberous Sclerosis Complex (TSC) is a genetic neurocognitive disorder, with an incidence of 1 in 6.000-10.000 live births.[1] Epilepsy is reported in up to 90% of TSC patients, and often starts in infancy.[2] Seizure semiology varies between patients and can change throughout a patient's lifetime. About 50% of children with epilepsy due to TSC present with infantile spasms, which are associated with a worse cognitive outcome.[3-5] Treatment of seizures due to TSC is notoriously difficult. Recently, a large study showed that more than half of all studied patients have refractory epilepsy.[6]

Clinical symptoms of patients with TSC are highly variable. During brain development, neural cells can develop aberrant morphology and migration, leading to typical features of cortical tubers and radial migration lines. These pathological changes in brain tissue have been implicated in causing epilepsy in TSC patients.[7] Possibly, mammalian target of rapamycin (mTOR) dysregulation in neurons can also independently contribute to epileptogenesis.[8] Developmental delay and autism spectrum disorders are also frequent consequences of TSC, with 40% of patients suffering from intellectual disability (IQ<70).[9]

A large number of antiepileptic drugs (AEDs) are available for seizure treatment, as well as non-pharmacological approaches such as surgery and the ketogenic diet. Recent clinical recommendations made by the International TSC Consensus Conference state that "anticonvulsant therapy in TSC should generally follow that of other epilepsies,"[10] and in 2012 the TSC expert panel specifically recommended vigabatrin as first line intervention for infants and AEDs with γ-aminobutyric acid (GABA)ergic mechanisms for older children[11]. In general, first line treatment for partial onset seizures in children is based on level C evidence (clinical trials with <20 participants) and expert experience.[12]

Etiology of epileptic seizures is known to be an important factor in the chance of intractability, and the International League Against Epilepsy (ILAE) recommends considering the underlying disease when choosing an AED. Vigabatrin (VGB) is most effective in the treatment of infantile spasms in TSC, and is the treatment of first choice.[11,13,14] For other seizure types, there is evidence for efficacy of lamotrigine (LTG),[15] levetiracetam (LEV),[16] and clobazam (CLB)[17] from small patient studies. For other AEDs specific effectiveness in TSC is unclear. Frequently used AEDs are valproic acid (VPA), LEV, topiramate (TPM), oxcarbazepine (OXC), and carbamazepine (CBZ).[18] Nonpharmacological epilepsy treatments shown to be effective in patients with TSC are the ketogenic or low glycemic index diet and epilepsy surgery.[19,20]

Recently, mTOR protein inhibiting drugs have been U.S. Food and Drug Asministration (FDA)/European Medicines Agency (EMA) approved as treatment for TSC-related subependymal giant cell astrocytomas (SEGAs) and renal angiomyolipomas (AMLs).[21,22] These drugs are not yet registered for the treatment of epilepsy in TSC, but animal studies and an open label study of 20 patients suggested a good anti-epileptic effect (60% good

response).[23] In the light of this potential new treatment option, acquiring more insight into the prognosis of seizure remission in TSC and into the benefit of conventional anti-epileptic treatments in TSC would be useful to help select patients that may benefit from new treatments. In this study we describe the use of anti-epileptic treatments in a large cohort of children with TSC and explore the response to antiepileptic treatments and the chance of remission after the first and following AED regimes.

METHODS

Patients

All children (0-18 years) with a clinically definite diagnosis of TSC according to TSC diagnostic criteria[24] who were treated at the ENCORE TSC expertise center at the Erasmus MC-Sophia Children's Hospital between 1988 and 2014 were potentially eligible for inclusion (n = 106). Ninety-four children ever had epileptic seizures requiring treatment. Of those, 23 of 94 could not be included because of lack of data completeness on epileptic seizures and the types of treatments that were used, despite our best efforts in obtaining all data, including interviewing the parents.

Data collection

All children treated at the TSC clinic are entered in a clinical database at their first visit. From then on, all data are collected prospectively. Data on the clinical history before their first visit is obtained from hospitals and other care-providers the patient has visited. Of 71 children, all data were included until the end of follow-up in February 2014, or until they reached the age of 18 years. Data extracted from our clinical database were demographics, TSC diagnostic criteria, results of genetic testing, seizure history of the child, and order of use, start and stop date of all seizure treatments, including anti-epileptic drugs (AEDs) , ketogenic diet, vagus nerve stimulation (VNS) and epilepsy surgery. If the exact start or stop date of a treatment was unknown, the best estimate was made as the middle date of the time period that was certain. Such an estimate was made for 219 start and stop dates (32%), and this period was longer than 30 days in 66 instances (10%).

The response to treatment was classified into five categories: seizure-free (defined as no seizures for at least 1 month), temporarily seizure-free (defined as the need for a new treatment strategy after a seizure-free period of at least 1 month), seizure reduction of >50%, no beneficial effect, or increase of seizure frequency. Treatment success was defined as seizure freedom or >50% seizure frequency reduction.

The treatment response was extracted from reports of the treating (pediatric) neurologist. The exact duration of seizure freedom was not always clear from the patient

records and parents' memory. Therefore, duration of seizure freedom was defined as the interval between the start of the AED causing seizure freedom, and the start of a subsequent treatment for seizure recurrence. Patients were considered to have sustained seizure freedom when they were seizure free for an interval of 24 months. Reasons for discontinuing AEDs were recorded.

Results of intellectual development testing were available for 57 children. Because of a broad range of developmental and cognitive functioning, abilities were assessed using the Wechsler intelligence scales (Wechsler Preschool and Primary Scale of Intelligence [WPPSI], Wechsler Intelligence Scale for Children [WISC], Wechsler Nonverbal Scale of Ability [WNV]) (n = 32, 56%) and the Bayley Scales of Infant Development (BSID) (n = 21, 37%). A minority of children were tested with the Snijders-Oomen Non-verbal Intelligence Scale (SON-R) (n = 3, 5%) and the Vineland Adaptive Behavior Scales (n = 1, 2%).

Data analysis

Continuous variables were not normally distributed. Chi-square tests were used for categorical data regarding the efficacy of the various order numbers of AEDs, and the analysis of genotype and epilepsy severity. Spearman's Rho was used for correlations of age at onset of epilepsy, infantile spasms, mutation type, and IQ with duration of seizure freedom. Comparisons were considered statistically significant if the two-sided alpha level was below 0.05. Survival graphs were made by Kaplan-Meier analyses. Data were analyzed by using IBM SPSS statistics 21.

RESULTS

Patient characteristics

Seventy-one children with TSC were included. Median age at end of follow-up was 9.4 years (range 0.9-18.0), and the median follow-up duration was 109 months (range 11-216). Genetic analysis showed a mutation of the *TSC1* gene in 20 (28%) of 71 patients, and a mutation in *TSC2* in 46 (65%) of 71. No mutation was identified in three patients, and two patients were not tested. Baseline demographics and epilepsy characteristics are shown in Table 1. Information about intellectual development was available for 57 children. Children with a mutation in the *TSC1* gene had a higher IQ, with a median of 65, compared to children with a *TSC2* mutation (median 47). Children who had suffered from infantile spasms had lower cognitive abilities (median IQ 43) than children who did not have infantile spasms (median IQ 60). Children with a *TSC2* mutation were more likely to have had infantile spasms ($p = 0.027$).

Table 1. Patient characteristics (n=71)

Age in months at time of data collection, median (25-75 percentile)	114 (72-185)
Gender male (%)	37 (52)
Mutation TSC1/TSC2/NMI/not tested (%)	20/46/3/2 (28/65/4/3)
Follow-up duration in months, median (25-75 percentile)	109 (68-177)
Age at first seizure in months, median (25-75 percentile)	7 (4-19)
Infantile spasms (%)	31 (44)
Ever had status epilepticus (%)	21 (30)
Intellectual quotient (n=58), median (range)	54.5 (5-97)

NMI, no mutation identified

Seizure freedom and relapse

Seizure freedom was reached in 55 (77%) of 71 patients for one or more periods of at least 1 month. Figure 1 shows the recurrence of seizures after the first seizure-free period. Of these 55 children, 13 (24%) had a relapse of seizures after 1-3 months. Thirty-two children (58%) had a recurrence of seizures during the first year after becoming seizure-free. Sustained remission (24 months of seizure freedom) was reached in 21 children (38%), of whom 11 discontinued their AED treatment after a mean period of 56 months (range 7-166). Nine of the 21 long-term seizure-free children (43%) had a recurrence after being seizure free for more than 24 months. This recurrence occurred after a period of up to 14 years. Figure 1a shows seizure relapse divided in age groups of seizure onset. Of the 21 children that reached sustained remission, almost half was older than 12 months at onset of epileptic seizures. Six children (28%) were aged 0-6 months when the first seizure was observed, five (24%) were aged between 7-12 months and 10 children (48%) were aged 13 months or older. Children with onset of epilepsy later in life seemed to have longer periods of seizure freedom (ns). Figure 1b,c show that there is no statistical difference in seizure relapse of children with or without infantile spasms, and of children with a *TSC1* or *TSC2* mutation. Intellectual development was assessed in 49 patients with sustained seizure freedom. Fifteen children (27%) had an IQ of 71 or higher, 34 (62%) had an IQ of 70 or lower (intellectual disability). Intellectually disabled children had a shorter duration of seizure freedom ($p = 0.006$).

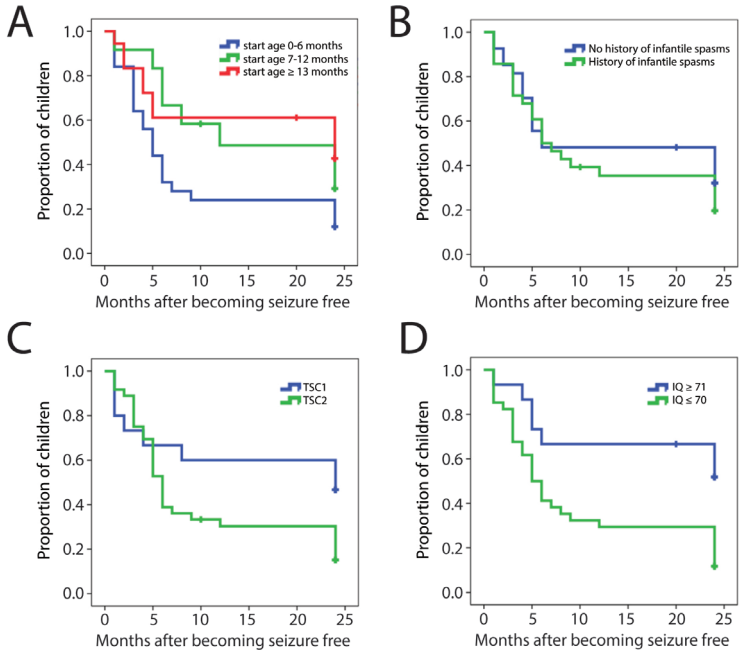

Figure 1. Proportion of children with sustained seizure remission (n=55).
(A) Seizure recurrence shown by age of onset of epilepsy; 0-6 months n=25 (45%), 7-12 months n=12 (22%), 13 months and older n=18 (33%). (B) Seizure recurrence shown by history of infantile spasms; no history of IS n=27 (49%) and history of IS n=28 (51%). (C) Seizure recurrence shown by mutation type; TSC1 n=15 (27%) and TSC2 n=36 (65%). (D) Seizure recurrence shown by intellectual ability; IQ ≥71 n=15 (27%) and IQ ≤70 n=34 (62%) (p=0.006).

Various AEDs used

The patients in our cohort used a total of 19 different AEDs. Table 2 shows the total number of AEDs and the number of treatments that were used simultaneously per child (polytherapy). The use of specific AEDs is presented in Figure 2. Most frequently used AEDs were VPA (85%), VGB (61%), LEV (46%), CBZ (41%), and CLB (41%). Other treatments used in our study population were epilepsy surgery (8%), ketogenic diet (8%) and vagus nerve stimulation (1%).

Response to AEDs per order number of treatment

We examined the order in which AEDs were used for which patients became seizure-free for the *first* time, and the total number of times the order in which AEDs were used caused seizure freedom, without taking the type of AED into account. Most children who became seizure-free experienced their first period of seizure freedom after the first or second AED. In total, 37 (52%) of 71 children reached seizure freedom after the first AED, and an additional 11 (16%) had a decrease in seizure frequency of >50%. Fourteen

Table 2. Seizure treatment characteristics

Total number of treatments used per child*, median (range)	4 (1-19)
Maximum number of treatments used simultaneously, median (range)	2 (1-5)
Patients who used ketogenic diet (%)	6 (8)
Patients who underwent epilepsy surgery (%)	6 (8)
Patients who used vagus nerve stimulator (%)	1 (1)

* Subsequently and simultaneously, treatments include total number of AEDs and all non-pharmacological treatments

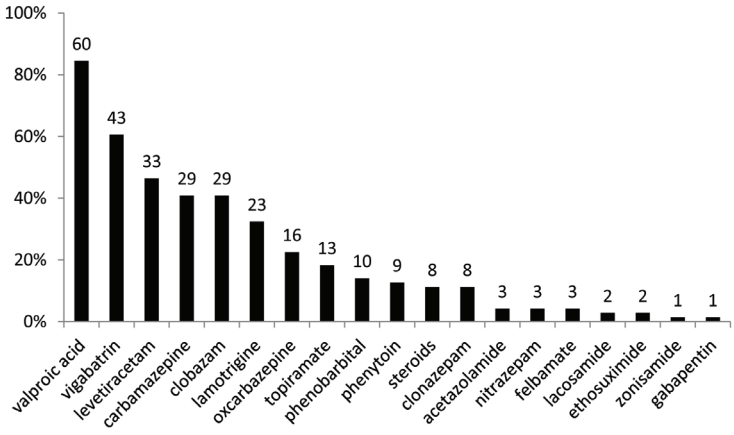

Figure 2. Percentage of children who used a specific AED during follow-up.
The Y-axis shows the percentage of the total study population. Numbers in the graph depict number of children who used that AED.

children (20%) became seizure free after the second AED, and four children (6%) after the third AED. One child (1%) had his first seizure remission after the fifth AED. No patient became seizure free for the first time on an AED later than the fifth order number. Figure 3 shows the order number of AEDs used during follow-up and their effectiveness in causing seizure reduction. The first and second prescribed AEDs were significantly more successful in attaining both seizure freedom and treatment success than AEDs used as fourth or later treatment (first AED $p < 0.001$ and $p = 0.002$, second AED $p = 0.002$ and $p = 0.002$). As is shown in Figure 3, seizure freedom did still occur with later treatment options, up to the tenth order number of AEDs. These are children who already experienced a seizure-free period earlier during their treatment, which was followed by seizure relapse. Although treatment benefit could still be achieved at a later order number of AEDs, it decreased rapidly with subsequent AEDs.

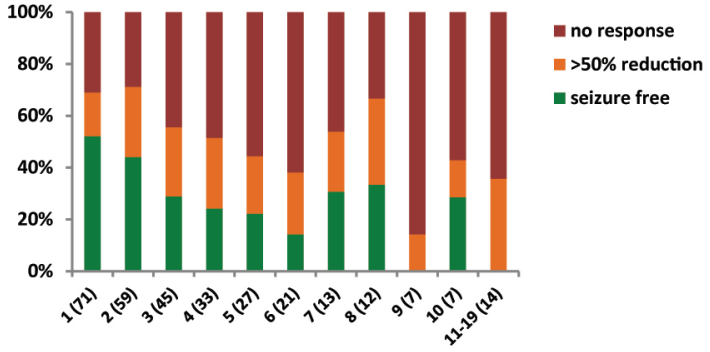

Figure 3. Response to AED use (n=322, 16 missing due to unknown response), according to their order number.
Between parentheses are depicted the number of times an AED was used as that order number. Due to small numbers, AEDs 11-19 are summarized in one bar.

Sequence of AEDs during treatment

Nine different drugs were prescribed as first treatment. VPA was prescribed most often (45%), followed by VGB (25%), phenobarbital (PB) (11%), CBZ (8%), and OXC (4%). LEV and phenytoin were both prescribed as first drug to two children, and LTG and nitrazepam to one child. Seizure freedom or treatment success was reached with VPA in 70% children, with VGB in 78%, with PB in 50%, with CBZ in 67%, and with OXC in 67%. All first used drugs were used as monotherapy.

Response to repeated use of the same AED

Fourteen children used 11 different AEDs on multiple occasions, with a maximum of three times per child. If the first trial of a specific AED caused seizure freedom, in 60% (6/10) the second trial also caused seizure freedom. If the first trial did not result in seizure freedom, only 14% (2/13) of second trials did. If the first treatment resulted in a 50% seizure reduction, in 11 out of 16 (69%) times the second time was also successful. However, when the treatment did not cause 50% seizure reduction the first time, none of the patients had a beneficial effect the second time (0/7).

Side effects

In 38 (13%) accounts of use, discontinuation of AED treatment was reported to be due to adverse effects. The mean percentage of discontinuation due to adverse effects per AED was 18% of all accounts, with a median of two times per AED (range 1-8). VGB was stopped least frequently due to side effects (4% of total VGB use). A total of seven different AEDs were reportedly associated with increased seizure frequency, including OXC, VPA, and CBZ.

Non-pharmacological epilepsy therapies

Seizure freedom was achieved in four out of the six children who underwent epilepsy surgery. Two children remained seizure-free, while the other two had a later recurrence of seizures. The fifth child had a reduction of seizure frequency of >50%. The ketogenic diet did not result in seizure freedom in any of the six patients on the diet; however, in two patients seizure frequency was reduced by more than 50%. A vagus nerve stimulator was used in one patient, but did not cause seizure reduction.

Treatment of infantile spasms

Thirty-one children in our cohort suffered from infantile spasms. Of these, 29 children had a seizure onset before the age of 1 year. Focal seizures preceded infantile spasms in 10 children (32%). Twenty-seven (87%) had at least one seizure free period. Eight children (26%) remained seizure free for more than 24 months. The number of treatments used in the first 24 months of life ranged from 1 to 10 (median 3). VGB was used most often (94% of patients), and was used as the first treatment in 15 children (48%), and second in 10 children (32%). The second most common treatment was VPA (74% of children). All other treatments were used by five children or less. In children receiving VPA as their first treatment, VGB was prescribed second (7/9), or third (1/9). One child was seizure free with VPA alone. Reasons for starting VPA before VGB included treatment in the time period before the benefit of VGB was evident (4/9), focal seizures at seizure onset (3/9), nontypical electroencephalography (EEG; 1/9) and other AED choice by a medical professional (1/9).

A second AED was used by 25 children (81%) and a third by 17 children (55%). No significant differences were found when treatment success of all accounts of all different types of AEDs was analyzed ($p = 0.160$). However, when the first AED was analyzed, VGB resulted in more seizure freedom and treatment success than other AEDs ($p = 0.05$ and $p = 0.04$, respectively).

Order number analysis showed that first, second, and third treatments were more effective in causing seizure freedom, when compared to effectiveness of later prescribed AEDs ($p = 0.005$, $p = 0.02$, $p = 0.04$, respectively). Steroids were used in five children, with one child receiving two courses of steroids. The order number of steroids during treatment ranged from 2 to 6, with a median of 3.5. Four of these children had been treated with VGB before steroids. On four accounts of steroid use the child became seizure-free. One account of steroids caused >50% seizure frequency reduction, and one child did not respond.

DISCUSSION

In this study, we confirm that epilepsy in TSC is difficult to treat and often relapses after a seizure-free period. In our cohort only 21 (30%) of 71 patients attained a remission of longer than 24 months with only 11 (15%) being able to taper their AEDs. VGB was the most successful AED in infantile spasms, but overall the first and second AED prescribed were most effective, independent of which specific AED was used.

AEDs used as a third or further option were still able to cause seizure reduction; however, chances of remission became small. Decreasing efficacy of AEDs used as later treatment options is also observed in the general epilepsy population.[25] Because effectiveness decreases rapidly after the first two or three failed AEDs, it might be advisable to start evaluating children for epilepsy surgery or other nonpharmacological options early during treatment. Nondrug therapies used in our cohort were epilepsy surgery, ketogenic diet, and vagus nerve stimulator. Epilepsy surgery was very effective in all five children, achieving seizure freedom in four children.

In our population, a better intellectual development is related to a more successful treatment outcome and longer periods of seizure remission. Children with a longer period of seizure freedom had an onset of seizures at a later age and had a higher IQ. In previous studies, the age of onset of epileptic seizures has been linked to the ability to reach seizure freedom.[26,27] These studies showed that intractability is associated with a higher rate of intellectual disability. It is not certain whether this is cause or effect, or the result of a common underlying brain pathology, but a good treatment response may be taken as a positive marker for development.

The patients in our cohort used several different AEDs, with VPA being the most frequently prescribed first treatment. This was also the most frequent drug in infants presenting with focal seizures, as was the practice at that time. New recommendations (2012) advise the use of VGB in all seizure types in infants with TSC. We hope that this will improve the outlook for newly presenting children. As randomized trials will not be feasible, comparing new cohorts with old cohorts such as the one in this study will give an idea of the additional benefit. In our cohort VGB was more effective than other AEDs when used as first treatment for infantile spasms,[13,14] but we could not show superiority of VGB for partial onset seizures or for infantile spasms when prescribed as a later treatment.

A limitations of our study is lack of data on the exact time periods of different seizure types per patient, so these could not be evaluated. Inherent to the retrospective nature of the study, data may not have been entirely complete, although we did have access to all patient records and correspondence regarding all hospital visits elsewhere. Missing or uncertain data were checked with parents of the patients. Because many children started their treatment many years ago, older guidelines were followed, leading to use of

AEDs that may be different from current practice. A strong point of our study is the long follow-up time of the children in our cohort, spanning nearly their entire childhood. Our follow-up was done at a TSC clinic in a university hospital, which may have introduced a bias toward more severely affected patients. However, we also had less severely affected children in our population, as eight children had only used one AED that caused them to be seizure free for long time periods.

New methods to improve the outcome of epilepsy in TSC are needed. Recent insights suggest that close EEG monitoring of children newly diagnosed with TSC can pick up epileptic discharges before the onset of clinical seizures.[28] Treatment of children with electrographic epilepsy with AEDs before the onset of clinical seizures may result in better seizure control and better cognitive development.[29] Another new promising treatment option is mTOR inhibitors. Case reports and an uncontrolled study suggested a good response in children with TSC and intractable epilepsy.[23] The efficacy of mTOR inhibitors in TSC is currently under extensive investigation in two large clinical trials (www.trialregister.nl NTR3178, www.clinicaltrials.gov NCT01713946). mTOR inhibitors may not only suppress seizures, but may also be able to reduce epileptogenesis in TSC.

Our findings confirm previous research that epilepsy in TSC is difficult to treat, and that better treatments are needed. We show that a period of sustained seizure freedom is achieved in 30% of children, but that almost half of them relapse even after 2 years of remission. After failure of the first two AEDs, chances of success with subsequent AEDs are lower. Epilepsy surgery is a good option for those that are eligible, and the adherence to new treatment guidelines may improve outcome in future cohorts. For others, mTOR inhibitors may be a promising option.

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CHAPTER 6

Sirolimus for epilepsy in children with tuberous sclerosis complex (RATE)

A randomized controlled trial

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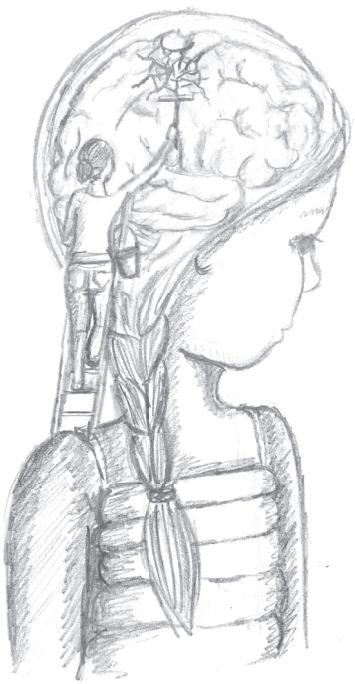

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ABSTRACT

Objective: To investigate whether mammalian target of rapamycin complex 1 (mTORC1) inhibitors could reduce seizure frequency in children with tuberous sclerosis complex (TSC).

Methods: Due to slow inclusion rate, target inclusion of 30 children was not reached. Twenty-three children with TSC and intractable epilepsy (age 1.8–10.9 years) were randomly assigned (1:1) to open-label, add-on sirolimus treatment immediately or after 6 months. Sirolimus was titrated to trough levels of 5–10 ng/mL. Primary endpoint was seizure frequency change during the sixth month of sirolimus treatment.

Results: Intention-to-treat analysis showed sirolimus treatment resulted in 41% seizure frequency decrease (95% confidence interval [CI] -69% to +14%; $p = 0.11$) compared to the standard-care period. Per protocol analysis of 14 children who reached sirolimus target trough levels in the sixth sirolimus month showed a seizure frequency decrease of 61% (95% CI -86% to +6%; $p = 0.06$). Cognitive development did not change. All children had adverse events. Five children discontinued sirolimus prematurely.

Conclusions: We describe a randomized controlled trial for a non–antiepileptic drug that directly targets a presumed causal mechanism of epileptogenesis in a genetic disorder. Although seizure frequency decreased, especially in children reaching target trough levels, we could not show a significant benefit. Larger trials or meta-analyses are needed to investigate if patients with TSC with seizures benefit from mTORC1 inhibition. This trial was registered at trialregister.nl (NTR3178) and supported by the Dutch Epilepsy Foundation.

Classification of evidence: This study provides Class III evidence that sirolimus does not significantly reduce seizure frequency in children with TSC and intractable epilepsy. The study lacked the precision to exclude a benefit from sirolimus.

INTRODUCTION

Tuberous Sclerosis Complex (TSC) causes epilepsy in 80-90% of patients, often starting in infancy.[1] Current treatment options include anti-epileptic drugs (AEDs), epilepsy surgery, ketogenic diet and vagus nerve stimulation.[2-4] Approximately two-thirds of patients do not reach adequate seizure control.[1] Cognitive development is negatively affected by epilepsy.[5,6]

TSC is caused by mutations in *TSC1* or *TSC2*,[7,8] resulting in upregulated activity of mammalian target of rapamycin complex 1 (mTORC1).[9] This upregulation disrupts neuronal migration and differentiation, causing regions of dyslaminated cortex, called cortical tubers, and dysplastic neurons throughout the brain.[10] Although the neurologic phenotype in TSC can be partly explained by the structural brain abnormalities, animal studies show that increased mTORC1 activity in the absence of anatomical abnormalities is sufficient to induce epilepsy.[11] Treatment with mTORC1 inhibitors can fully rescue this phenotype,[12] suggesting that mTORC1 inhibitors might be useful for targeted treatment of epileptogenesis in TSC.

Clinically used mTORC1 inhibitors include sirolimus and its derivative everolimus. Everolimus decreased subependymal giant cell astrocytoma (SEGA) and angiomyolipoma (AML) volume in patients with TSC.[13,14] Both sirolimus and everolimus may decrease seizure frequency (summarized in appendix 1). A prospective study showed clinically relevant seizure frequency reduction in 12/20 patients with TSC treated with everolimus.[15] However, because seizure frequency can fluctuate spontaneously, lack of a control group hampers interpretation of this study.[16,17]

We performed a randomized controlled crossover trial assessing the therapeutic benefit of sirolimus on seizure frequency in children with TSC and intractable epilepsy.

METHODS

Participants

Children between 3 months and 12 years with definite clinical diagnosis of TSC[18] were eligible for inclusion if they had at least 1 epileptic seizure per week and were resistant to at least two AEDs. Children with severe renal dysfunction, infection, or surgery less than 6 weeks before randomization were excluded. All data were collected at the ENCORE-TSC Expertise Centre, Erasmus MC-Sophia Children's Hospital in Rotterdam, The Netherlands.

Standard protocol approvals, registrations, and patient consents

The national and local institutional ethics review boards approved the trial protocol (registration number MEC-2010-362). The trial was performed in agreement with the Declaration of Helsinki (2008) and Good Clinical Practice guidelines. Oral and written informed consent was obtained from parents before randomization. This trial is registered at the Dutch Trial Register, reference number NTR3178. For the study protocol see http://www.erasmusmc.nl/encore/Poliklinieken/tsc1/wetenschondtsc/klinondtsc/Onderzoeksprotocol_RATE_studie_versie_3_20-01-2012.pdf/

Study design and treatment allocation

Patients participated for 12 months and were randomly assigned in a 1:1 fashion to receive add-on sirolimus treatment during the first or second period of 6 months, in a crossover design. Sample size was calculated based on an estimated baseline seizure frequency of 20 seizures per month, with an SD of 22, based on historical data. A power of 90% with an α level of 0.05 could show a minimal treatment effect of 0.75 SD on the primary outcome with 26 participants. Assuming a 10% dropout rate, target inclusion number was 30.

Patients received 1 mg/mL sirolimus oral solution (Rapamune; Pfizer, New York, NY), monitored and released through the Erasmus MC pharmacy. Sirolimus was titrated to blood trough levels of 5-10 ng/ml. Starting dose was based on body weight. Target trough levels were reached as quickly as possible, by adjusting the dose based on trough levels 1 week, 2 weeks and 1 month after starting sirolimus (appendix 2).

Administration was once a day, at a set time. In case of adverse events of grade two or higher, sirolimus was stopped until the adverse event resolved or reached grade one (appendix 2). AEDs taken at baseline were continued throughout the trial. Dose adjustments were made for cotreatment with CYP3A4-inducing AEDs. Parents and the patients' treating physicians were discouraged from changing AED regimes unless this would cause significant morbidity.

Randomization and masking

A random allocation sequence, computer generated with permuted block design (block size 4) and stratified by age (3-12 months, 1-4 years, 5-11 years) was provided by the Erasmus MC Department of Biostatistics. The neuropsychologist and neurophysiologist were masked to treatment.

Study procedures and outcomes

Primary outcome was seizure frequency, assessed by a daily seizure diary filled out by the parents, starting 1 month before randomization to determine baseline seizure frequency.

Secondary outcomes for epilepsy included proportion of responders to sirolimus and seizure severity. A responder was classified as having ≥50% reduction of seizure frequency in the last month of either study period relative to baseline. Children with secondarily generalized seizures were compared to measure change in seizure severity. The number of status epilepticus episodes in either period was also compared.

Another secondary outcome included analysis of electroencephalograms (EEGs) made at baseline, 6 months and 12 months. Thirty-minute EEG registrations were performed on a BrainRT system (OSG bvba, Rumst, Belgium) using 19 silver-silver chloride cup electrodes placed on the scalp and referenced to Fz electrode according to the 10-20 International System and analyzed by an experienced clinical neurophysiologist. Sampling frequency was 500 Hz, band pass filter was 0.16-70 Hz. Impedances were kept <5 kΩ. Amplitudes and frequencies of waveforms were measured manually using a longitudinal bipolar montage and by EEG spectral analysis after fast Fourier Transformation. Epileptiform activity was measured by spike index, presence of electrodecrements, and percentage of generalized epileptiform abnormalities. Spike index was assessed by the average percentage of 1-second bins showing spikes in 10-second epochs. Amplitude of delta activity was measured to determine encephalopathy (amplitude ≥200 μV). Other measurements included presence and frequency of occipital rhythm and presence of hypsarrhythmia.[19] EEG data were complete for 21 participants.

Secondary outcomes also included cognitive development and behavior. Neuropsychological assessments and questionnaires were performed at baseline and 6 and 12 months, and were selected to assess specific problematic behaviors in TSC.[20,21] Assessments included cognitive development (Bayley Scales of Infant and Toddler Development (BSID-III) or Wechsler Preschool and Primary Scale of Intelligence), adaptive behavior (Vineland Screener, 2008), sensory processing (Short Sensory Profile-NL, 2006), autistic features (Social Responsiveness Scale, 2007), and emotional and behavioral problems (Child Behavior Checklist, 2000). All participants were assessed by the same licensed neuropsychologist.

Blood samples were taken at baseline and 6 and 12 months for all participants and every visit during sirolimus treatment. Sirolimus trough levels were measured by high-performance liquid chromatography-mass spectrometry/mass spectrometry chromatography at the Erasmus MC pharmacy. Laboratory control values were measured by the Erasmus MC Department of Clinical Chemistry, including renal function (urea and creatinine), liver enzymes (aspartate transaminase, alanine transaminase, γ-glutamyltransferase), blood cell counts, total cholesterol, and triglycerides.

All data from all study visits and assessments were checked by at least two investigators.

The data safety monitoring board (DSMB) consisted of a pediatrician, pediatric neurologist, and statistician. The DSMB was provided with biannual progress reports and

was notified in case of a serious adverse event, and could stop or adapt the trial in case of safety concerns.

Adverse events were monitored throughout the trial, and were classified according to the WHO adverse reaction terminology and graded according to the National Cancer Institute common terminology criteria for adverse events.[22]

Classification of evidence

Our primary research question was whether sirolimus treatment could reduce seizure frequency in children with TSC and intractable epilepsy. This interventional study provides class III evidence that 6 months of sirolimus treatment does not seem to reduce seizure frequency in children with TSC and intractable epilepsy (41% decrease, $p = 0.11$). As our sample size is small, a beneficial effect is not ruled out, especially in children who reach the target trough level.

Statistical analysis

Data from all randomized participants (intention-to-treat) were used to analyze primary outcome, neuropsychological outcomes, spike index and delta amplitude. A linear mixed effects model was applied. For seizure frequency, data were log-transformed to obtain a normal distribution, and 0.5 was added to remove zero values. A multivariable model including the variables sirolimus treatment, month during the study (baseline, sixth month of first period, sixth month of second period), and randomization group was applied. The same model was used in the per protocol group of 14 children who reached the predefined effective trough levels in the sixth month of the sirolimus period.

A chi-square test was used to compare number of responders in the sirolimus and standard-care periods, to determine change in seizure severity, and to analyze changes in presence of electrodecrements, generalization of epileptiform discharges, occipital rhythm and location of spikes.

Cutoff level for significance was set at 0.05 (2-sided) for all tests. Interim analyses were not performed, and corrections for multiple testing were not made.

All data were analyzed using IBM (Armonk, NY) SPSS Statistics version 21 and the R 3.1.3 statistical package.

RESULTS

Study population

Between September 7, 2011, and December 4, 2013, 23 patients were randomly assigned to receive add-on sirolimus treatment immediately (n = 12) or after 6 months (n = 11). With consent of the local ethics committee, the DSMB, and the sponsor (Dutch

Epilepsy Foundation), we decided to stop inclusion at 23 patients without an interim analysis. This yielded 80% power to show a significant effect. One patient was lost to follow-up (figure 1). Baseline demographics and disease characteristics were well balanced between the randomized groups (table 1).

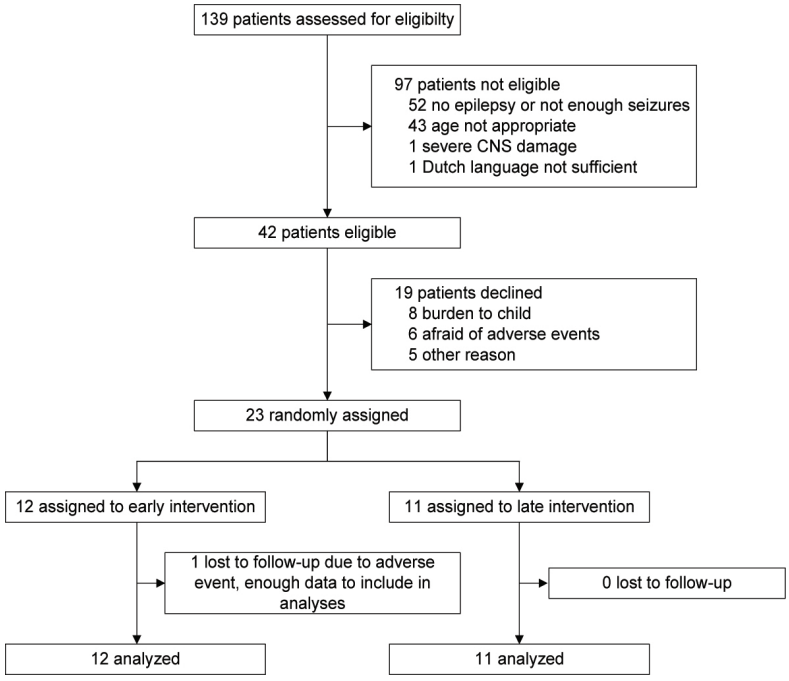

Figure 1. Flow diagram of study participants

Table 1. Baseline characteristics.

	Early sirolimus (n=12)	**Late sirolimus (n=11)**
Age at inclusion	5.5 (1.8-10.9)	5.1 (2.2-10.1)
Male gender	8 (67)	3 (27)
Mutation TSC1/TSC2/NMI	1/10/1 (8/83/8)	3/8/0 (27/73/0)
History of infantile spasms	8 (67)	6 (55)
Age (months) at first seizure	3 (0-14)	6 (0-83)
Seizure frequency in baseline month	48 (2-402)	35 (17-85)
Number of AEDs tried in total	4.5 (2-11)	5 (2-9)
Number of AEDs at baseline	2 (1-4)	3 (1-4)
BSID cognitive scale at baseline	9 (3-74)	18 (0.7-76)

AED = antiepileptic drug; BSID-III = Bayley Scales of Infant and Toddler Development, third edition, developmental level in months; NMI = no mutation identified. Data are median (range) or n (%).

Treatment

Mean daily sirolimus dose in the last month of sirolimus was 3.65 mg and ranged 0.9 to 8.0 mg. Eighteen children had interruptions of sirolimus treatment due to adverse events (median 2 events, median duration 7 days). Sirolimus was used on 82% of all days in the sirolimus period. AED treatment was adjusted during sirolimus in 3 children, and during standard-care in 8 children (chi-square 2.987 $p = 0.17$).

Primary outcome

Median seizure frequency at baseline was 35 seizures per month (interquartile range [IQR] 20-65), 25 (IQR 7-47) after the sirolimus period, and 32 (IQR 9-62) after the control period. In the intention-to-treat analysis, we found that during sirolimus treatment, patients had 41% less seizures than during standard-care (95% CI -69% to +14% $p = 0.11$). Seizure frequency change from baseline per patient is shown in figure 2, A and B, for sirolimus and standard-care respectively. Per protocol analysis, including the 14 patients who reached the target trough level of ≥5 ng/mL during the last month of the sirolimus period, showed a mean seizure frequency reduction of 61% (95% CI -86% to +6% p=0.06). Inspection of individual seizure frequency curves did not reveal evidence for a carryover effect.

Secondary outcomes

Nine children responded during the sirolimus period (≥50% seizure frequency reduction), of whom 3 became seizure free, while 6 children responded during the standard-care period, of whom 1 became seizure free (NS). Secondarily generalized seizures were present in 6 patients (26%), and their frequency was not affected by sirolimus treatment. No status epilepticus was recorded during the trial.

EEG analysis showed a median spike index of 50% at baseline, 40% after sirolimus treatment and 40% after standard-care ($p = 0.86$). No change was found between sirolimus and standard-care in presence of hypsarrhythmia or encephalopathy, occurrence of multifocal spikes, electrodecrements, generalization of epileptiform abnormalities, or the occurrence of an occipital rhythm.

No significant differences in cognitive or motor development, behavioral problems, adaptive behavior, or sensory processing were identified between sirolimus and standard-care (table 2). Data from the social responsiveness scale could not be analyzed, as many participants were too severely intellectually disabled.

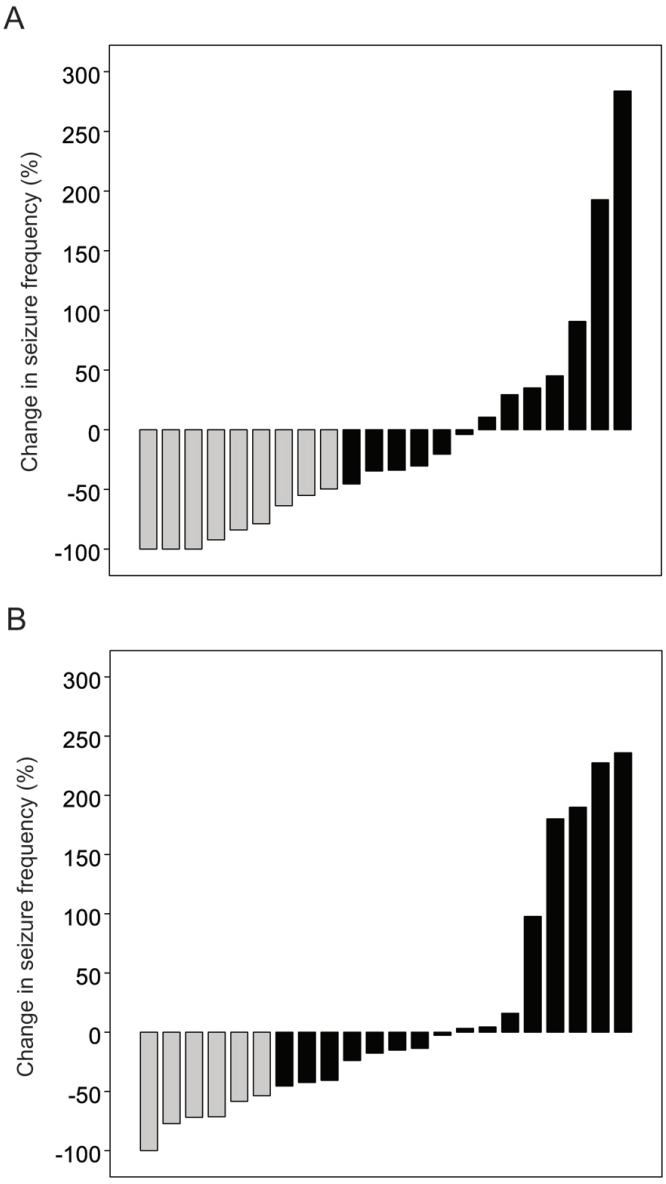

Figure 2. Seizure frequency change from baseline.
Decrease in seizure frequency is depicted by negative change, increase in seizure frequency is depicted by positive change. Every bar is 1 child. Grey bars represent children with a decrease of ≥50%. (A) Seizure frequency in the last month of the sirolimus period relative to baseline seizure frequency. (B) Seizure frequency in the last month of the standard-care period relative to baseline seizure frequency.

Table 2. Effect of sirolimus on secondary outcomes.

	Treatment effect (95% Confidence interval)	p value
Cognitive development (BSID-III n=21, WPPSI-III full-scale IQ n=2)	1.46 (-0.49 to 3.40)[a]	0.15
Fine motor development (BSID-III n=21)	1.18 (-0.52 to 2.88)[a]	0.18
Gross motor development (BSID-III n=21)	-0.42 (-1.73 to 0.90)[a]	0.54
Adaptive behavior (Vineland n=22)	0.44 (-1.21 to 2.09)[a]	0.60
Sensory processing (SP-NL n=21)	1.91 (-3.91 to 7.73)[a]	0.53
Total problem score (CBCL n=22)	-2.29 (-9.18 to 4.60)[b]	0.52
Internalizing problem score (CBCL n=22)	-0.77 (-3.58 to 2.05)[b]	0.60
Externalizing problem score (CBCL n=22)	-0.50 (-3.29 to 2.30)[b]	0.73

BSID-III = Bayley Scales of Infant Development; CBCL = Child Behavior Checklist; SSP = Short Sensory Profile; WPPSI-III-NL = Wechsler Preschool and Primary Scale of Intelligence Dutch version. BSID-III and Vineland depict developmental months, SP-NL and CBCL depict raw scores.
Data are missing for BSID-III fine and gross motor scale because the developmental level of 2 children was tested using WPPSI-III-NL. Data missing for other questionnaires due to incomplete answers to essential items on the checklist.
[a] Higher is better. [b] Lower is better.

Adverse events

Adverse events were consistent with the safety profile of sirolimus (table 3). All patients reported at least one adverse event, most commonly upper respiratory tract infections, gastrointestinal problems and acne-like skin lesions. Aphthous ulcers were observed only during the sirolimus period. Serious adverse events occurred in 5 patients. During sirolimus treatment, 3 individuals required hospitalization due to pneumonia, 1 following otitis media. Two patients were hospitalized during the standard-care period, 1 for a tonsillectomy and 1 for refusing food intake.

Sirolimus trough levels in the last month of the sirolimus period ranged from 2.3 to 14.2 ng/mL. Reduction of sirolimus dose due to adverse events was required in 12 children. Five children discontinued sirolimus due to adverse events. These included aphthous ulcers in 2 children, pneumonia requiring hospitalization, upper respiratory tract infection, and increased seizure frequency. Excluding the increased seizure frequency, all adverse events subsided after sirolimus was discontinued.

Clinically relevant laboratory results requiring follow-up occurred in 5 children (table 3). Three children had elevated cholesterol levels within the first month of starting sirolimus that normalized after dietary advice. None of the laboratory results led to discontinuation of sirolimus treatment.

Table 3. All adverse events during the sirolimus and standard-care period.

	Sirolimus period		Standard-care period	
	All grades	**Grade 3**	**All grades**	**Grade 3**
Upper respiratory tract infection	20 (87)	0	19 (83)	1 (4)
Gastro-intestinal	19 (83)	0	13 (57)	0
Acne-like skin lesions	17 (74)	0	8 (35)	0
Other infection	12 (52)	0	7 (30)	0
Aphthous ulcers	7 (30)	0	0	0
Fever	6 (26)	0	0	0
Injury due to accident	4 (17)	0	3 (13)	0
Fatigue	3 (13)	0	4 (17)	0
Behavioral change	3 (13)	0	0	0
Eczema	3 (13)	0	1 (4)	0
Pneumonia	3 (13)	3 (13)	0	0
Otitis media	2 (9)	1 (4)	2 (9)	0
Hemorrhagic disorders	2 (9)	0	2 (9)	0
Edema	2 (9)	0	0	0
Anorexia	1 (4)	0	1 (4)	1 (4)
Hair loss	1 (4)	0	0	0
Headache	1 (4)	0	0	0
Muscle pain	1 (4)	0	0	0
Polyuria	1 (4)	0	0	0
Red eye	1 (4)	0	0	0
Muscle weakness	0	0	1 (4)	0
Laboratory				
Cholesterol >6.5 mmol/l	3 (13)	0	0	0
Triglycerides >3.0 mmol/l	1 (4)	0	0	0
Alanine transaminase >100 units/l	2 (9)	0	0	0
Urea >8.0 mmol/l	0	0	1 (4)	0

Data are n (%) of children.

DISCUSSION

We report a randomized controlled trial of mTORC1 inhibitor sirolimus for treatment of intractable epilepsy in 23 children with TSC. We did not observe a significant therapeutic benefit of sirolimus on seizure frequency in the intention-to-treat analysis (41% decrease in seizures due to sirolimus; 95% CI -69% to +14%; $p = 0.11$). All children reported adverse events, for which sirolimus treatment was discontinued in 5. Due to these adverse events, not all children reached the predefined target trough level. Per protocol analysis of children who did reach the target trough level showed a seizure frequency decrease of 61% (95% CI -86% to +6%; $p = 0.06$).

The rationale of our study is based on the knowledge that mTORC1 inhibition directly targets the molecular mechanism underlying the pathophysiology in TSC-related epilepsy.[12] This would make mTORC1 inhibitors fundamentally different from current insufficient treatment with traditional AEDs aimed at seizure suppression. Mouse models have convincingly shown that mTORC1 inhibition can prevent and reverse epileptogenesis in TSC and epilepsies.[11,12,23,24] Several case series and an uncontrolled study have suggested a clinically relevant benefit in groups of patients with TSC treated with sirolimus or everolimus. These are summarized in A-Table 1 in appendix 1. Notably, when we specifically look at effects obtained during the treatment period, these published findings are very similar to our own findings, both with respect to seizure reduction as well as the number of responders. However, this effect is not statistically significant compared to a control period. Three children in our study became seizure-free on sirolimus treatment, which might indicate that sirolimus could be beneficial for some children. We did not find a different response rate when comparing children with a *TSC1* or *TSC2* mutation, children with or without infantile spasms, or a correlation with the number of months the child had seizures before trial start.

The decrease in seizures during standard-care may reflect spontaneous fluctuations of seizure frequency, and the tendency of individuals to participate in a trial when seizure frequency is high and regression to the mean is likely. Uncontrolled studies may overestimate the treatment effect, leading to an underestimation of the number of participants needed in a trial to achieve sufficient power.

Moreover, despite our efforts to keep patients on the same AED regimen during the entire study, several changes to AEDs were necessary, particularly during the 6-month standard-care period. This may have further contributed to the observed decrease in seizure frequency in the standard-care period, possibly resulting in underestimation of the effect of sirolimus.

We did not observe a beneficial effect of sirolimus treatment on cognition and behavior. Most children in our study were severely intellectually disabled, which may

complicate detection of an effect of sirolimus on cognitive and motor development. Improved seizure control is likely to be beneficial for cognition and behavior, but preclinical studies have also shown a therapeutic benefit of mTORC1 inhibitors on neuronal plasticity and cognitive and behavioral outcomes in the absence of seizures.[5,25] Future trials could address the value of mTORC1 inhibitors in treating TSC-associated cognitive and behavioral problems.

We were able to include 23 of the intended target of 30 participants, mainly because parents were reluctant to give their child an experimental drug and to minimize changes in treatment during the standard-care period. The decision to stop inclusion at 23 participants was made after consulting with the DSMB. We considered extending the study period further; however, the prospect of recruiting another 7 children within a reasonable time window was unrealistic. Although a larger study could have statistical power to show a significant effect, these results give an indication of the potential effect size and may help physicians in guiding parents and patients. Combined with other and future studies, possibly in a meta-analysis, our data could help position the role of mTORC1 inhibition in TSC-related epilepsy.

The choice of a crossover design was based on its large power to show an effect in a relatively small number of patients with a rare disease, as all participants function as their own control. Our trial did not include placebo treatment, and was not masked. We chose this trial design, together with the TSC parents association and parents of young children with TSC-related epilepsy, as having the least burden.

The sirolimus treatment period was 6 months, which is longer than most clinical trials for AEDs in intractable epilepsy.[26] A treatment period of 6 months is sufficient to investigate a clinically relevant effect of sirolimus on seizure frequency, especially in patients with frequent seizures. Continuing a therapy aimed at seizure reduction for more than 6 months without evidence of efficacy does not seem advisable. A longer treatment period would also necessitate an undesirably long control period.

We only selected patients with intractable epilepsy and high seizure frequency, which may limit the applicability of our results to the general TSC population. We aimed to keep children on blood trough levels of 5-10 ng/mL. Due to adverse events, only 14 children reached this target trough level in the last month of sirolimus treatment. A possible benefit of sirolimus might be present in children who are kept on the target trough level of sirolimus or even higher levels, as the per protocol analysis of this group showed a larger decrease in seizure frequency. However, all children in our study had adverse events, for which 5 children discontinued sirolimus treatment. These adverse events may limit clinical use of sirolimus. Our study is unsuitable for detecting rare adverse events, although the adverse events profile of sirolimus is well known from other indications.

We were unable to show a significant effect of sirolimus on seizure reduction in children with TSC and intractable epilepsy. A beneficial effect is not ruled out, however, and

further studies are needed to assess the value of mTORC1 inhibitors in the treatment of TSC-related epilepsy.

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A-Table 1 Summary of literature on mTORC1 inhibitor treatment for epileptic seizures in patients with Tuberous Sclerosis Complex.

Study	Study type	Number of patients	Age of patients	Drug	Dose	Duration of treatment	Control group?	Outcome
Muncy et al. 2009[27]	Case report	1	9 years	Sirolimus	0.15 mg/kg/day	10 months	No	Seizure clusters stopped, 1-5 seizures per day remained
Krueger et al. 2010[28]	Prospective open-label trial into SEGA treatment, with epilepsy as secondary outcome	16	Median 11 years (range 3-34) in total study population of 28 patients	Everolimus	Median 5.6 mg/m^2/day (range 1.5-10.5) in total study population of 28 patients	6 months	No	Frequency decrease in 9, no change in 6, increase in 1
Krueger et al. 2013[29]	Long-term follow up of patients in prospective open-label study	26 at baseline,23 at end of follow-up	Same as above	Everolimus	Median 5.3 mg/m^2/day (range 2.1-12.3) in total study population of 25 patients	Median 34.2 months (range 4.7-47.1) in total study population of 25	No	Percentage of patients who reported no seizures since their last visit increased from 38.5% to 65.2% at 24 months. Patients reporting at least one seizure per day decreased from 26.9% to 13.0% at 24 months
Perek-Polnik et al. 2012[30]	Case report	1	10 years	Everolimus	4.5 mg/m^2/day	12 months	No	Seizures ceased completely within first six weeks of treatment

A-Table 1 Summary of literature on mTORC1 inhibitor treatment for epileptic seizures in patients with Tuberous Sclerosis Complex. (continued)

Study	Study type	Number of patients	Age of patients	Drug	Dose	Duration of treatment	Control group?	Outcome
Franz et al. 2013[14]	Randomized controlled trial into SEGA treatment, with epilepsy as secondary outcome	117	Median 9.5 years (range 1.0-23.9) in 78 patients receiving everolimus	Everolimus	4.9 mg/m^2/day (range 2.3-11.8)	24 weeks	Yes, placebo	Change from baseline in seizure frequency was 0 in the everolimus and the placebo groups
Kotulska et al. 2013[31]	Long-term follow up of a selection of patients from randomized controlled trial	8	Median 24.5 months (range 12-35)	Everolimus	Median 4.5 mg/m^2/day (range 2.53-8.0)	Median 35 months (range 33-38)	No	Three patients seizure free at baseline, 1 complete and permanent cessation after two months treatment Two patients more than 50% reduction in the first six months One patient decreased markedly in the first year, then increased requiring a new AED One patient no impact on seizure frequency
Moavero et al. 2013[32]	Case report	1	20 years	Everolimus	Not reported	7 months	No	Seizure frequency decreased, generalized seizures stopped, patient continued to have focal-onset seizures

A-Table 1 Summary of literature on mTORC1 inhibitor treatment for epileptic seizures in patients with Tuberous Sclerosis Complex. (continued)

Study	Study type	Number of patients	Age of patients	Drug	Dose	Duration of treatment	Control group?	Outcome
Krueger et al. 2013[15]	Prospective open-label trial	20	Median 8 years (range 2-21)	Everolimus	8.4 mg/m^2/day (range 3.4-13.7)	12 weeks (4 weeks titration, 4 weeks early maintenance, 4 weeks final maintenance)	No	12 patients >50% reduction of seizure frequency Three patients 25-50% reduction Five patient <25% reduction Significant improvement only in the final maintenance period
Wiegand et al. 2013[33]	Case series	7	Median 5 years (range 2-12)	Everolimus	Median 3.5 mg/day (range 2.9-7.0)	36 weeks	No	Reduction of seizures in 4 patients, no alteration of seizures in 2 patients
Canpolat et al. 2014[34]	Case series	7	Median 13 (range 4-16)	Sirolimus	Median 2 mg/day (range 1.25-3)	12 months	No	All patient were seizure free after 0-9 months
Wiemer-Kruel et al. 2014[35]	Case report	1	13.5 years	Everolimus	5 mg	37 days	No	Clusters of severe seizures requiring hospitalization
Cardamone et al. 2014[36]	Case series	7	Median 6 years (range 3-17)	Sirolimus (6 patients) and Everolimus (1 patient)	Sirolimus: median 2.5 mg daily (range 1-5) Everolimus: 5 mg	Median 18 months (range 6-36 months)	No	One patient >90% reduction of frequency Four patients 50-90% reduction Two patients <50% reduction

AED anti-epileptic drug

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A-METHODS

Adverse event management

Adverse events were monitored throughout the trial, at every visit during sirolimus treatment, and through monthly telephone calls in the standard-care period. Parents were instructed to contact the investigator if their child became ill, or when they thought their child might have developed an adverse event.

Adverse events were classified according to the WHO adverse reaction terminology and graded according to the National Cancer Institute common terminology criteria for adverse events. For most types of adverse events, sirolimus was stopped in case of a grade two adverse event, and after the event was resolved or reached grade one, was restarted on the same dose. In case of a grade three adverse event, sirolimus was stopped until the event resolved or reached grade one, and was restarted at a lower dose. In case of a grade 4 adverse event, sirolimus was discontinued. For mouth ulcers, the sirolimus treatment schedule was more strict, as these can be very debilitating for young children. In case of grade one mouth ulcers, sirolimus was discontinued if necessary until the ulcer subsided, the dose was not changed when sirolimus was restarted, and parents were instructed to use non-alcoholic mouth-wash. In case of grade two or three mouth ulcers, sirolimus was discontinued until the ulcers were resolved or reached grade one, a lower dose was restarted, and non-alcoholic mouth wash and lidocaine oral gel was advised. In case of grade four mouth ulcers, sirolimus was discontinued.

If surgery was needed during the trial, sirolimus was stopped one week in advance should the surgeon wish, or earlier if needed. Sirolimus would then be started again at two weeks post-surgery or after wound healing, at the last dose that did not cause adverse events.

Sirolimus dosing

Patients received a 1 mg/ml oral solution of sirolimus (Rapamune, Pfizer), monitored and released through the Erasmus MC pharmacy. Starting dose was based on the normal starting dose of 2 mg daily for an adult of >50 kg or a body surface area of 1.73 m^2. This corresponds with a dose of 0.033 mg/kg/day, which was used as starting dose for participants. Participants using CYP3A4-inducing AEDs (carbamazepine, fenobarbitone, phenytoin) were started on a 25% higher dose. No loading dose was used. Sirolimus levels were originally titrated to blood trough levels of 10-15 ng/ml, but the target trough was lowered to 5-10 ng/ml after two serious adverse events, after deliberation with the DSMB. To ensure that participants reached this trough level as quickly as possible, trough levels and consequent dose adjustments were done after one week, two weeks and four weeks of starting sirolimus treatment. If dose increases were needed after the appointment at four weeks, blood was taken an additional time two weeks after

the dose increase. Administration of sirolimus was once a day, at a set time, always at the same time relative to a meal (for example always before breakfast). Parents were instructed to administer sirolimus by letting the child drink a solution of the sirolimus dose with at least 100 ml of liquid (water or juice), and rinse the glass with another 40 ml of liquid to ensure the entire dose was administered. During the sirolimus treatment period, the use of strong inhibitors or inducers of the CYP3A4 enzyme was to be avoided. These included ketoconazole, voriconazole, itraconazole, telithromycin, clarithromycin, rifampin, rifabutin, nicardipine, clotrimazole, fluconazole, troleandomycin, bromocriptine, cimetidine, danazol, protease inhibitors. Intake of grapefruit juice was also to be avoided during the trial. Parents and physicians of the participant were instructed to contact the investigators if the participant was to be prescribed one of these drugs, so an alternative could be found.

CHAPTER 7

Treatment of Cognitive Deficits in Genetic Disorders

A Systematic Review of Clinical Trials of Diet and Drug Treatments

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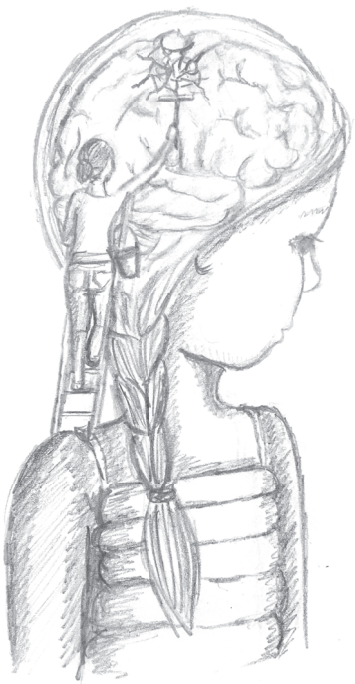

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ABSTRACT

Importance: Knowing the underlying etiology of intellectual disability in genetic disorders holds great promise for developing targeted treatments. Although successful preclinical studies and many positive clinical studies have been reported, it is unclear how many purported therapies have become established treatments. The quality of the clinical trials may be an important determinant for achieving clinical impact.

Objective: To evaluate clinical impact, strengths, and weaknesses of clinical trials of diet or drug treatments to improve cognitive function in patients with a genetic disorder.

Evidence review: MEDLINE, EMBASE, PsycINFO, and Cochrane databases were searched from inception date to January 26, 2014, for clinical trials with cognitive outcomes in patients with genetic disorders. Outcome measures of randomized clinical trials (RCTs) were compared between trial registries and reports, and trials were evaluated for the quality of design using the Jadad score and Consolidated Standards of Reporting Trials (CONSORT) criteria.

Findings: We identified 169 trial reports of 80 treatments for 32 genetic disorders. Seventy-five trials (44.4%) reported potential efficacy, of which only 2 therapies are now established treatments, namely, dietary restriction for phenylketonuria and miglustat for Niemann-Pick disease type C. The median sample size for RCTs was 25 (range, 2-537). Only 30 of 107 RCTs (28.0%) had acceptable Jadad scores exceeding 3. Reporting of key CONSORT items was poor. Reported outcome measures matched preregistered outcome measures in trial registries in only 5 of 107 RCTs (4.7%).

Conclusions and relevance: The number of trials in the field of cognitive genetic disorders is rapidly growing, but clinical impact has been limited because few drugs have become established treatments and the benefit of most drugs remains unclear. Most trials have small sample sizes and low quality of design. Predefinition of outcome measures, improved trial reporting and design, and international collaboration to increase recruitment are needed to unequivocally determine efficacy of drugs identified in preclinical research.

INTRODUCTION

Intellectual disability is a major societal and health care problem, with 1% to 3% of the population affected,[1] and many individuals are in need of lifelong care and support. Up to 40% of the cases have an identifiable genetic cause.[2] This proportion is likely to increase in the near future because whole-genome genetic diagnostics are linking more genes to intellectual disability.[3] Knowledge of genes and underlying mechanisms of genetic disorders has boosted expectations for novel, mechanism-based drugs, even for neurological consequences of genetic disorders. The availability of animal models has further accelerated optimism. For example, for fragile X syndrome, neurofibromatosis type 1, and tuberous sclerosis complex, genetically modified mouse models have resulted in promising candidate drugs.[4,5] However, it is unclear how often these have resulted in established treatments. Meta-analyses suggest that results from animal research often do not translate to humans.[6,7] High risks of biases and small sample sizes in the animal literature May explain part of this translational failure.[8,9] However, these meta-analyses included mostly animal models of common neurological disorders for which the models might be insufficiently analogous such as stroke, Parkinson disease, and Alzheimer disease.[10] Whether this poor translational value is also true for monogenetic disorders is unclear. Monogenetic disorders are unique because the exact etiological factor is known and faithful genetic models can be made. Therefore, the translational success rate is expected to be higher for such disorders.

The primary aim of this review was to investigate how often clinical trials reporting positive findings resulted in clinically accepted diet or drug treatments of cognitive deficits in genetic disorders. Because we found that this was surprisingly low, we subsequently investigated the quality of these clinical trials.

METHODS

We searched MEDLINE, EMBASE, PsycINFO, and Cochrane databases from inception date to January 26, 2014, for reports on clinical trials using cognitive outcome measurements in well-defined genetic disorders (eg, caused by single-gene mutations, aneuploidy, etc) (the Methods section in the Appendix describes the full search strategy and selection procedure). Studies using only behavioral outcomes or questionnaire-based outcomes were excluded (Figure 1). First, clinical impact of the treatments was evaluated by retrieving US Food and Drug Administration or European Medicines Agency registration status and other trials or meta-analyses. Strengths and weaknesses of the randomized clinical trials (RCTs) were assessed using Consolidated Standards of Reporting Trials (CONSORT) criteria checklists[11] for the quality of reporting and Jadad scores[12] as a measure of the

quality of design. The Jadad score summarizes the quality of randomization (up to 2 points), blinding (up to 2 points), and a description of patients who withdrew during the study (1 point). A score of 4 or 5 is considered higher quality.

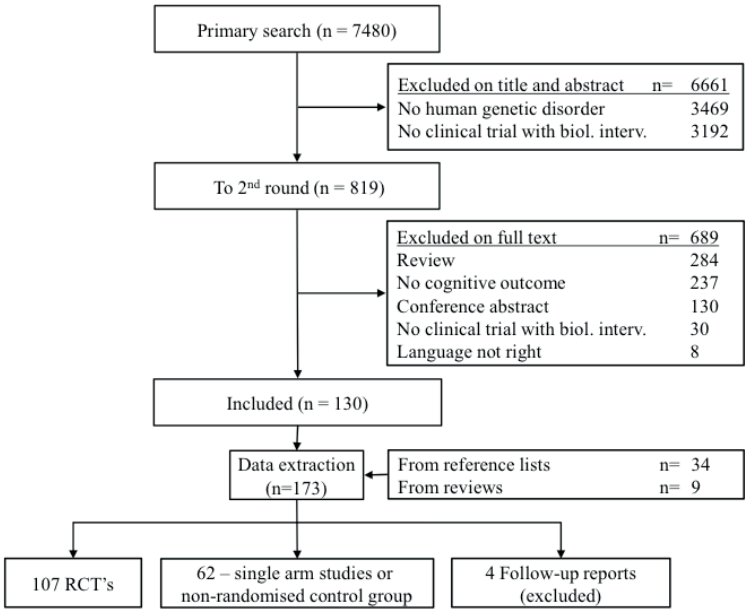

Figure 1. Search strategy and results.
In the first round, titles and abstracts were screened and excluded when articles were about nongenetic disorders, involved animal models, or did not describe a clinical trial with a biological intervention. In the second round, full texts were analyzed, and reviews, conference abstracts, and studies using only behavioral outcome measures or questionnaire-based outcomes were excluded. Articles written in English were selected. Four articles were follow-up reports, and these were excluded. RCTs indicates randomized clinical trials.

RESULTS

We identified 107 reports on RCTs and 62 reports on clinical trials without a control group (n = 34) or with a nonrandomized control group (eg, historic control subjects) (n = 28). The articles covered 32 genetic disorders, with 80 different combinations of disorders with treatments (Table 1). For 53 of 80 combinations (66.3%), we found only one report. Huntington disease, Down syndrome, phenylketonuria, and fragile X syndrome constituted 120 of 169 trial reports (71.0%).

Table 1. Clinical trials of therapies for cognitive symptoms of genetic disorders and their clinical impact.

	Total	Rationale	RCT's (n)	*Positive abstract*	Trials without randomised control group (n)	*Positive abstract*	Clinical impact plus trial identifiers of ongoing research
All included articles	**169**		**107**	***47***	**62**	***29***	
Huntington disease	**41**		**30**	***4***	**11**	***3***	
Unsaturated fatty acids	4	AM; HR; CD	4	*1*	-	-	Unclear
Food supplements & Vitamins	5	AM; HR; CD	3	*0*	2	*0*	Ongoing trials: NCT00592995; NCT00920699; NCT00608881
Acetylcholinesterase inhibitor	5	AM; HR; CD	2	*1*	3	*1*	Ongoing trials: NCT00652457; NCT01458470
Dopaminergic agents	5	AM; HR	3	*1*	2	*0*	Ongoing trials: NCT00724048
Psychostimulants and nootropics	3	AM; CD	3	*0*	-	-	Unclear
Sodiumchannel antagonist	3	AM; HR	2	*0*	1	*1*	Unclear
Anti-histaminergic drugs	2	HR	2	*1*	-	-	Ongoing trial; NCT01085266
NMDA-antagonist	2	AM; HR	2	*0*	-	-	Unclear
SSRI's	2	AM;HR	2	*0*	-	-	Unclear
GABA-A agonist	2	AM; HR	1	*0*	1	*0*	Unclear
Other therapies	8	AM; HR; CD	6	*0*	2	*1*	Ongoing trials: EUCTR2010-019444-39-FR (aminoacids); NCT01834911 (tetrabenazine)
Down syndrome	**37**		**22**	***7***	**15**	***6***	
Food supplements & Vitamins	11	AM; HR; CD	7	*0*	4	*1*	Ongoing trial: NCT00056329
Acetylcholinesterase inhibitor	9	AM; HR; CD	6	*3*	3	*3*	Ongoing trials: NCT01084135; NCT01112683
Folic acid and folic acid like drugs	2	HR	2	*1*	-	-	Ongoing trial: NCT01576705
Hormones, antidiuretic	2	AM; HR	2	*2*	-	-	Unclear
Hormones, growth	2	HR	-	-	2	*0*	Unclear

Table 1. Clinical trials of therapies for cognitive symptoms of genetic disorders and their clinical impact. (continued)

	Total	Rationale	RCT's (n)	*Positive abstract*	Trials without randomised control group (n)	*Positive abstract*	Clinical impact plus trial identifiers of ongoing research
Amino acids and derivates	4	HR; CD	2	*0*	2	*1*	Unclear
Hormones, thyroid	2	HR	1	*0*	1	*0*	Ongoing trial: NCT01576705
Other therapies	5	HR; CD	2	*1*	3	*1*	Unclear
Phenylketonuria	**25**		**15**	***12***	**10**	***2***	
Amino acids and derivates	7	AM; HR	5	*3*	2	*1*	Unclear
Phenylalanine-low diets	16	HR	9	*9*	7	*1*	Standard care
Other therapies	2	AM; CD	1	*0*	1	*0*	Unclear
Fragile X mental retardation syndrome	**17**		**11**	***6***	**6**	***3***	
Folic acid and folic acid like drugs	8	5	6	*3*	2	*0*	Unclear
Food supplements & Vitamins	2	5	2	*2*	-	-	Ongoing trial: NCT01855971
Other therapies	7	AM; HR	3	*1*	4	*3*	Ongoing trials: NCT01555333 (GABA-agonist); EUCTR2011-004349-42-GB (mGluR5-agonist); NCT01120626 (acetylcholinesterase inhibitor)
Turner syndrome	**6**		**6**	***5***	**-**	**-**	
Hormones, sex	3	HR	3	*3*	-	-	Unclear
Hormones, steroids	2	HR	2	*2*	-	-	Unclear
Hormones, growth	1	HR	1	*0*	-	-	Unclear
Prader-Willi syndrome							
Hormones, growth	5		5	*4*	-	-	Unclear
Neurofibromatosis type 1	**5**		**3**	***1***	**2**	***2***	

Table 1. Clinical trials of therapies for cognitive symptoms of genetic disorders and their clinical impact. (continued)

	Total	Rationale	RCT's (n)	*Positive abstract*	Trials without randomised control group (n)	*Positive abstract*	Clinical impact plus trial identifiers of ongoing research
HMG CoA reductase inhibitors	4	AM	3	*1*	1	*1*	Ongoing trials: EUCTR2009-010965-22-NL; NCT00352599; NCT00853580
Psychostimulants and nootropics	1	CD	-	-	1	*1*	Ongoing trials: ACTRN12611000765921; NCT00169611
Velocardiofacial syndrome	**3**		**2**	***1***	**1**	***1***	
Psychostimulants and nootropics	2	CD	1	*1*	1	*1*	Unclear
Amino acids and derivates	1	HR	1	*0*	-	-	Unclear
Mucopolysaccharidosis type III	**2**		**1**	***0***	**1**	***1***	
Substrate reduction therapy	1	CD	1	*0*	-	-	Ongoing trial: EUCTR2006-004661-34-FR
Topoisomerase inhibition	1	AM	-	-	1	*1*	Ongoing trial: NTR2402
Rett syndrome							
Other therapies	2	HR; none	2	*1*	-	-	Unclear
Angelman syndrome							
Other therapies	2	AM; HR	1	*1*	1	*0*	Unclear
Gaucher disease, type III							
Other therapies	2	CD	1	*0*	1	*1*	Unclear
Niemann-Pick disease, type C							
Substrate reduction therapy	2	AM	1	*1*	1	*1*	EMA approved, FDA pending
Ceroid Lipofuscinosis, neuronal 3	2						
Food supplements & Vitamins	1	HR	-	-	1	*0*	Unclear

Table 1. Clinical trials of therapies for cognitive symptoms of genetic disorders and their clinical impact. (continued)

	Total	Rationale	RCT's (n)	*Positive abstract*	Trials without randomised control group (n)	*Positive abstract*	Clinical impact plus trial identifiers of ongoing research
Unsaturated fatty acids	1	HR	-	-	1	*1*	Unclear
Single disorder/treatment combinations	**18**	**AM; HR; CD**	**7**	***4***	**11**	***8***	**Ongoing trials: NTR3178; NCT01730209; ISRCTN09739757; NCT01070316 (Tuberous Sclerosis Complex); NCT01681940 (Alpha-mannidosis); NCT01801709 (MPS-I)**

AM, animal model; CD, treatment efficacious for comparable disorder (eg, acetylcholinesterase inhibitors are effective for Alzheimer disease; therefore, they might be effective for Down syndrome); EMA, European Medicines Agency; FDA, US Food and Drug Administration; HR, human research (eg, results from blood testing indicate involvement of hormone); NA, not applicable; RCTs, randomized clinical trials.

[a] Trials are grouped by disorder or treatment category and are sorted by disorder, starting from the disorder with the most trials. For the ratings of all trials, see A-Table 2. Ongoing international clinical trials were retrieved through theWorld Health Organization search portal (http://www.who.int/trialsearch). NCT-numbered records are available at the US National Institutes of Health clinical trials website (http://www.clinicaltrials.gov), EUCTR-numbered records are available at the European Union Clinical Trials Register (http://www.clinicaltrialsregister.eu), NTR-numbered records are available at the Nederlands Trial Register (http://www.trialregister.nl), and ACTRN-numbered records are available at the Australian New Zealand Clinical Trials Registry (http://www.anzctr.org.au).

Increasing contribution of animal models as the rationale for cognitive trials

Among 169 trial reports, the trial was predominantly based on data from animal models in 48 articles (28.4%), and the rationale was based on previous findings in human participants in 76 articles (45.0%). In 35 reports (20.7%), the rationale was derived from a comparable disorder. For example, because children with neurofibromatosis type 1 display signs of attention-deficit/hyperactivity disorder (ADHD), methylphenidate (a drug approved for ADHD) was evaluated in children with neurofibromatosis type 1.[13] In the remaining 10 reports (5.9%), the rationale was unclear. The steep increase in clinical trial reports over time is shown in Figure 2, with a growing proportion based on animal research.

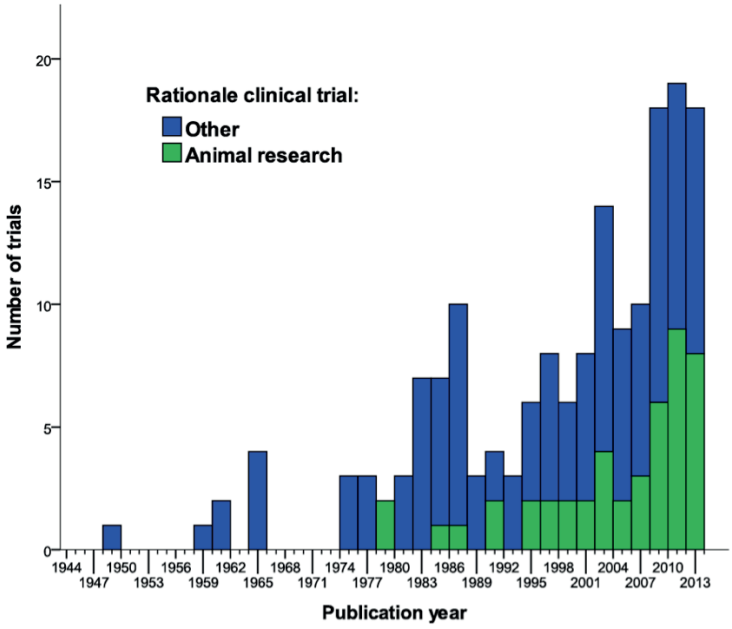

Figure 2. Clinical trials reporting cognitive outcomes in genetic disorders over time and their rationale. Studies based on animal models are increasingly published. Other rationales include empirical clinical data from human studies (eg, biomarkers and functional magnetic resonance imaging) or efficacy in a related disorder.

Association between trial conclusions and drug approval

Efficacy or possible efficacy of the treatment on cognitive functioning was implied in the abstract of 75 of 169 articles (44.4%).This percentage varied substantially among disorders (Table 1) but did not differ between RCTs and other types of design such as single-arm studies. In 77 of 169 articles (45.6%), the authors concluded that the drug had no significant effects. The remainder of studies concluded that the intervention

worsened cognitive function (8 studies) or that the data did not allow any conclusions on efficacy (9 studies). We next investigated the status of all studied combinations of disorder and treatment. Clinical impact is listed in the far right column of Table 1. The only approved drug (by the European Medicines Agency [US Food and Drug Administration approval is pending]) for treatment of cognitive dysfunction in a genetic disorder is miglustat, for neurological symptoms of Niemann-Pick disease type C.[14] This disease is a rare autosomal recessive lysosomal storage disease that leads to ataxia, eye movement disturbance, progressive dementia, and dysarthria and is generally fatal between 5 and 10 years after diagnosis. Miglustat decreases glycolipid biosynthesis by inhibiting glucosylceramide synthase, which catalyzes glycosphingolipid synthesis and leads to amelioration of the ataxia phenotype and increased longevity in the mouse model of Niemann-Pick disease type C.[15] In an RCT, miglustat improved horizontal saccadic eye movements.[14] A secondary cognitive outcome measure, the Mini-Mental State Examination, improved in the treated group after 12 months and deteriorated in the untreated controls.

Results of Cochrane systematic reviews

To identify effective treatments without US Food and Drug Administration or European Medicines Agency approval, we retrieved systematic reviews of the interventions listed in Table 1 that had been published in the Cochrane database in the last 5 years. In A-Table 2 in the Appendix, the findings and recommendations of these systematic reviews are summarized. For example, treatment of children having phenylketonuria with a phenylalanine-low diet to prevent severe cognitive deficits is supported by sufficient evidence from all-or-none case series (level 1c). Attempts to alter the phenylalanine-low diet (eg, stopping the diet at a certain age or adding extra tyrosine) have not resulted in evidence-based changes.[16] In fragile X syndrome, folic acid has been investigated as a potential treatment. Six RCTs included cognitive outcome measures, and 3 studies presented a positive conclusion in the abstract (Table 1). However, none met the quality criteria for a Cochrane review.[17] Down syndrome is associated with an increased incidence of Alzheimer dementia, and studies have assessed the effect of acetylcholinesterase inhibitors, a class of drugs used in dementia, on cognitive functioning in patients with the syndrome. A series of systematic reviews identified only one eligible study[18] in patients with Down syndrome, which was small and found no significant effects of donepezil hydrochloride on cognitive functioning. An additional study[19] published in 2012 showed no benefit of memantine hydrochloride (an *N*-methyl-D-aspartate receptor antagonist) over placebo in adults with a combination of Down syndrome and Alzheimer disease. For Huntington disease, no intervention reduced symptoms or slowed disease progression, but it was noted that further trials with greater methodological quality are necessary.[20,21] Since then, 2 reports have been published on pridopidine (a

dopaminergic stabilizer) that found no effect on cognitive end points, although one found a possible effect on motor end points.[22] The Cochrane reviews concluded that most trials did not meet basic requirements for meta-analysis.

Ongoing follow-up trials

We searched clinical trial registries to identify ongoing follow-up studies for the intervention-disorder combinations (Table 1). Follow-up clinical trials were found in registries for 22 of 80 intervention disorder combinations (27.5%). In total, 31 trials in progress are registered. Most ongoing trials are for food supplements and vitamins to treat Huntington disease, hydroxymethylglutaryl coenzyme A reductase inhibitors (statins) to treat neurofibromatosis type 1, and mammalian target of rapamycin–pathway inhibitors (rapamycin and related drugs) to treat tuberous sclerosis complex. For 58 intervention-disorder combinations, we did not identify a follow-up study (labeled as unclear).

Quality of design and reporting of cognitive RCTs for genetic disorders

We identified 75 of 169 clinical trials in which some efficacy was reported, but only 2 interventions for cognitive deficits have led to accepted treatments. Hence, we investigated reasons for the lack of clinical impact of the retrieved clinical trials. Data on the sample size, outcome measurements, and reporting were analyzed for the subgroup of 107RCTs. Nonrandomized and single-arm studies were excluded from this part of the review because they can be informative only when the expected benefit is dramatic (eg, the difference between severe intellectual disability and normal cognitive functioning). Randomized control groups are necessary to account for child development, natural disease course, training of patients by repeated cognitive testing, and placebo effects.

Sample size and justification

The median number of participants in the 107 retrieved RCTs was 25 (range, 2-537). Only 35 of 107 articles (32.7%) described how the authors determined the sample size. When the sample size calculation is reported, the sample size tends to be much larger (median, 63 participants) compared with the group of trials without a justification of the sample size (median, 19 participants) (Mann-Whitney test, $P < .001$). A systematic review of trials that were not selected for a specific field had a median sample size of 52, and this was considered alarmingly low by the authors.[23] The power of most of the studies in this review seems inadequate to detect clinically meaningful and scientifically reasonable effects. Because 31 of 52 disorder-intervention combinations (59.6%) in this review were tested in one RCT and because many of them were tested in small trials, we conclude that most interventions were not appropriately assessed.

Risk of bias in RCTs for cognitive genetic disorders

In total, 75 of 169 trial reports (44.4%) claimed some efficacy of treatment but led to few accepted treatments. Because a high risk of bias excludes trials from approval by medical authorities, systematic reviews, or meta-analyses, we investigated whether these studies were at elevated risk of reporting bias. Indications of elevated risk of bias are correlated with the quality of reporting of clinical trials.[24] Hence, we used CONSORT criteria to identify gaps in reporting.[25] Table 2 lists all 25 CONSORT items separately for articles before and after 2001, when the second version of the reporting guideline was published and received widespread attention.[11,26] Reporting has improved on most items since 2001. Older trials reported poorly on all aspects, except for description of trial design (with 62.0% [31 of 50] adequately reporting) and details on the intervention, including How and when they were administered (with 54.0% [27 of 50] adequately reporting).

Table 2. Reporting of essential trial properties in RCTs according to CONSORT criteria.

Nr.	CONSORT-item	All trials, n/n applicable (%)	≤ 2001 n/n applicable (%)	> 2002 n/n applicable (%)
1	Identification as a randomised trial in the title; structured summary of trial design, methods, results and conclusions	25/107 (23)	3/50 (6)	22/57 (39)
2	Scientific background, explanation of rationale, specific objectives or hypotheses	63/107 (59)	21/50 (42)	42/57 (74)
3	Trial design including allocation ratio, important changes to protocol	70/107 (65)	31/50 (62)	39/57 (68)
4	Eligibility criteria for participants, settings and locations were data were collected	44/107 (41)	13/50 (26)	31/57 (54)
5	Details of interventions, including how and when they were administered	64/106 (60)	27/50 (58)	37/56 (66)
6	Completely defined pre-specified primary and secondary outcomes, including how and when they were assessed	18/107 (17)	4/50 (8)	14/57 (25)
7	How sample size was determined	35/107 (33)	7/50 (14)	28/57 (49)
8	Method used to generate the random allocation sequence, type of randomisation, details on restriction	21/107 (20)	1/50 (2)	20/57 (35)
9	Mechanism used to implement the random allocation sequence, including steps to conceal the sequence until interventions were assigned	28/107 (26)	3/50 (6)	25/57 (44)
10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	29/107 (27)	6/50 (12)	23/57 (40)
11	Who was blinded after assignment to interventions and how, similarity of interventions	35/104 (33)	11/47 (19)	24/57 (42)
12	Statistical methods used for primary analysis and additional analyses (such as subgroups)	63/106 (59)	19/49 (39)	44/57 (77)
13	Participant flow through trial, including losses and exclusions, together with reasons	44/107 (41)	11/50 (22)	33/57 (58)

Table 2. Reporting of essential trial properties in RCTs according to CONSORT criteria. (continued)

Nr.	CONSORT-item	All trials, n/n applicable (%)	≤ 2001 n/n applicable (%)	> 2002 n/n applicable (%)
14	Dates defining periods of recruitment and follow-up, why the trial ended	17/107 (16)	4/50 (8)	13/57 (23)
15	Baseline characteristics for both groups	57/107 (53)	17/50 (34)	40/57 (70)
16	For each group, numbers of participants included in each analysis and whether analysis was by original assigned groups	59/107 (55)	23/50 (46)	36/57 (63)
17	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	34/107 (32)	6/50 (12)	28/57 (44)
18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	29/101 (27)	9/48 (19)	20/53 (38)
19	All important harms or unintended effects in each group	43/106 (40)	12/50 (24)	31/56 (55)
20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	42/107 (39)	11/50 (22)	31/57 (54)
21	Generalizability (external validity, applicability) of the trial findings	47/107 (44)	15/50 (30)	32/57 (56)
22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	52/106 (49)	19/50 (38)	33/56 (59)
23	Registration number and name of trial registry	21/107 (20)	0/50 (0)	21/57 (37)
24	Where the full trial protocol can be accessed, if available	3/107 (3)	0/50 (0)	3/57 (5)
25	Sources of funding and other support (such as supply of drugs), role of funders	28/107 (26)	6/50 (12)	22/57 (39)

CONSORT, Consolidated Standards of Reporting Trials; RCTs, randomized clinical trials.

[a] On most items, reporting improved after 2001, when CONSORT criteria were first implemented.

Reporting of randomization, blinding, and attrition

Randomization, blinding, and attrition are particularly linked to risk of bias.[12] Only 35.1% (20 of 57) of articles published after 2001 adequately described the method that was used to generate the randomization list, 43.9% (25 of 57) of recent articles described how the allocation sequence was concealed from investigators, and 40.4% (23 of 57) of reports described the roles of personnel in the implementation of the randomization. The method of blinding was described in 42.1% (24 of 57) of the recent trials. The number of participants who were lost to follow-up, including reasons, should be reported for each study arm to prevent attrition bias. Participant flow through the trial, including losses and exclusions, was adequately described in 57.9% (33 of 57) of the recent trials. A validated 5-point scoring scale for the quality of randomization, blinding, and attrition has been established by Jadad and colleagues.[12,27] Of 107RCTs, 26 (24.3%) received the maximal score of 2 points on randomization, 44 (41.1%) received the maximal score of 2 points for blinding, and 47 (43.9%) scored positive on description of withdrawals (1 point). On the Jadad scoring scale, 77 of 107 reports (72.0%) were considered poor, with a total score of 3 or less. Poor design was associated with a positive tone in the abstract. In total, 53.2% (41 of 77) of trials with a total Jadad score of 3 or less reported positive conclusions in the abstract while only 23.3% (7 of 30) of higher-quality trials (total Jadad score of 4 or 5) had a positive abstract ($P = .03$, Fisher exact test).

Reporting of outcome measurements and preregistration

The median number of cognitive outcome measures per RCT in this review was 7 (range, 1-77). Ten of 107 trials (9.3%) applied correction for multiple hypothesis testing. A distinction between primary and secondary outcome measures was made in 41 of 107 RCTs (38.3%). Sufficient details on outcome measures, including the notation that they were predefined, and specifics on how and when they were assessed were reported in 18 of 107 RCTs (16.8%) (CONSORT item 6 in Table 2). The extreme variability in the use of cognitive outcome measures, even within one disorder, precludes an in-depth analysis in this review. In 22 RCTs, we could assess changes observed in the placebo group. Of these, a significant improvement in the placebo group was observed in 16 RCTs (72.7%). The remaining 6 RCTs demonstrated a stable placebo control group. These results highlight the need for a placebo group in cognitive trials.

Of 107 RCTs in the review, only 22 trials were registered in a publicly accessible registry before outcome data were collected. This represents 22 of 45 trials (48.8%) published in 2006 or later, when preregistration of trials became mandatory for publication in major clinical journals.[28] Preregistration of outcome measures in a clinical trial registry is necessary to demonstrate that outcome measures were not changed or added during the trial or analysis and to ensure that there was no selective reporting.[29] Seven trials registered outcome measures only after final data collection was complete, which we counted as

nonregistered. Next, we compared trial registrations with trial reports and found that only 12 of 22 trials included sufficient details of the neuropsychological tests that were used. Other registrations used terminology such as *cognitive functioning*. Seventeen of 22 trials reported on extra outcome measures that were not in the registry. This might be appropriate when designated as exploratory, but this was not the case. Only 7 trials had all the reported outcomes adequately registered, and only 9 trials finally reported all predefined outcome measures. Of 22 timely trial registrations, 17 had 1 or more of the problematic issues described above. In 6 cases, the tone of the abstract was altered because of the added or changed outcome measure. For example, a secondary outcome measure in a trial of arbaclofen for fragile X syndrome was added and showed significant improvement, changing the implication in the abstract.[30] In a trial of simvastatin for cognitive deficits in neurofibromatosis type 1, a secondary outcome measure was the single significant improvement, but this outcome measure was a subtest of the registered test.[31] The authors commented that this result could be spurious, and the overall outcome was regarded as negative. Readers should be informed that such analyses were of an exploratory nature and were not predefined. Taken together, 51.1% (23 of 45) of the recent trials have not been registered. When registered, details on outcome measures were lacking or incomplete, indicating a high risk of selective outcome reporting bias.

DISCUSSION

To our knowledge, this is the first comprehensive overview of cognitive clinical trials for genetic disorders. Despite a growing number of trials and many reports presenting positive conclusions in the abstracts, only 2 treatments have established clinical impact, namely, dietary restriction for phenylketonuria and miglustat for Niemann-Pick disease type C. To identify an explanation for this translational failure, we analyzed the characteristics, strengths, and weaknesses of trials and found that most trials were of poor quality as determined by CONSORT criteria and Jadad scores or had small sample sizes.

Although poor trial quality is an important issue in any area of medicine, this field of cognitive trials is particularly vulnerable to the consequences of weak study design. First, cognitive outcome measures are sensitive to placebo or retest effects and thus to bias. We identified 16 of 22 placebo-controlled trials (72.7%) in this review in which the placebo group significantly improved, highlighting that single-arm studies in this field are inappropriate to study efficacy of drugs. Spontaneous improvements can be caused by training of patients on outcome measures (owing to repeated assessments), natural development of children, regression to the mean, or strong contribution of a placebo effect. Therefore, rigorous randomization and blinding procedures are needed. Second, cognitive trials are susceptible to selective outcome reporting given the many variables

that can be reported. In this review, the median number of 7 reported cognitive outcome measures emphasizes the high risk of false-positive findings. Moreover, few trials had unambiguous predefined cognitive outcome measures. Hence, these weaknesses in design may lead to false-positive study results and the risk of inappropriate off-label prescription. It is our clinical experience at a reference center for rare disorders that many parents and patients are well aware of trials concerning their diseases and that off-label prescriptions are common, particularly when trial abstracts have a positive tone. In A-Table 2 in the Appendix, we provide a scoring table with the quality scores of individual trials, which indicates the strength of evidence for each drug.

Another complication in this field of rare disorders is the small sample sizes. Small samples have low power to detect differences and are prone to false-negative results (type II error).[32] Although multiple small trials with low risk of bias may be combined in a meta-analysis, this requires standardized outcome measurements and treatment modalities, as well as reporting of standardized effect sizes, which are not common practice in cognitive trials. Hence, it has been proposed that studies with small samples should be discouraged because they are likely to be inconclusive and may pose unnecessary risk and burden on participants.[33] Moreover, when studies with small samples are not repeated because of nonsignificant findings, such trials impede drug development. There are several recommendations to improve the current status of cognitive trials. First, because large sample sizes of patients with a rare disorder are difficult to achieve for any single center, multicenter international collaborations are needed to improve recruitment. This has been demonstrated by the large and high-quality trials for Huntington disease, a disorder with a prevalence of approximately 1 per 18 000 (A-Table 2 in the Appendix). The basis of this success is international collaboration promoted by patient advocacy groups.[34] In addition, researchers may join existing initiatives to increase recruitment (eg, the Rare Diseases Clinical Research Network of the National Center for Advancing Translational Sciences in the United States). The European Commission has recently adopted a directive on the recognition and funding of European reference networks for rare diseases, highlighting the need to concentrate care and research for rare disorders in specialized referral centers. Second, CONSORT guidelines should be followed for developing trial protocols and for writing reports. Third, and probably most challenging, is the recommendation to identify appropriate end points for cognitive trials. Infrastructure and design of clinical trials should be discussed early, even before testable drugs become available. In our experience, the development of a clinical trial protocol is time consuming and requires good natural history studies to identify end points and perform realistic power calculations. Assistance from patient advocacy groups for selection of relevant outcome measures should also be encouraged. In addition, because it is largely unknown how long a treatment should last to establish significant improvement in cognitive function, the inclusion of neurophysiological outcome measures in clinical

studies is recommended. Positive changes in functional magnetic resonance imaging, transcranial magnetic stimulation, or electroencephalography may inform future trials and drug development, even in the absence of significant changes in cognitive function.

CONCLUSIONS

In addition to enhancement of the quality of preclinical drug studies,[8-10,32] improvement in clinical studies is needed to allow the successful translation of early findings and the establishment of new treatments. Current practice may lead to undesired off-label prescription or result in premature abandonment of promising drugs.

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APPENDIX

SUPPLEMENTAL METHODS

SEARCH STRATEGY:

PubMed

(Cognition[mesh] OR Cognit*[tiab] OR neurocognit*[tiab] OR neuropsychol*[tiab] OR neuro psychol*[tiab] OR Learning[mesh] OR Learn*[tiab] OR memor*[tiab] OR Thinking[mesh] OR Thinking[tiab] OR Thought[tiab] OR problem solv*[tiab])
AND
(Genetic Diseases, Inborn[mesh] OR Genetic Disease*[tiab] OR Genetic Disorder*[tiab] OR Genetic syndrom*[tiab] OR Genetic condition*[tiab] OR Genetic defect*[tiab] OR Hereditary Disease*[tiab] OR Hereditary Disorder*[tiab] OR Hereditary syndrom*[tiab] OR hereditary condition*[tiab] OR Hereditary defect*[tiab])
AND
(randomized controlled trial [pt] OR controlled clinical trial [pt] OR randomized [tiab] OR placebo [tiab] OR randomly [tiab] OR trial [tiab] OR groups [tiab] OR Clinical Trial[pt]) NOT (animals [mh] NOT humans [mh])

Embase

(Cognition/exp OR Learning/exp OR Thinking/exp OR (Cognit* OR neurocognit* OR neuropsychol* OR (neuro NEXT/1 psychol*) OR Learn* OR memor* OR Thinking OR Thought OR (problem NEXT/1 solv*)):ab,ti) AND ((‘genetic disorder’/exp NOT ‘multi-factorial genetic disorder’/exp) OR ((Genetic* OR Hereditar*) NEAR/3 (Disease* OR Disorder* OR syndrom* OR defect*)):ab,ti) AND (random*:ab,ti OR factorial*:ab,ti OR crossover*:ab,ti OR (cross NEXT/1 over*):ab,ti OR placebo*:ab,ti OR ((doubl* OR singl*) NEAR/5 blind*):ab,ti OR assign*:ab,ti OR allocat*:ab,ti OR volunteer*:ab,ti OR ‘crossover procedure’/de OR ‘double-blind procedure’/de OR ‘randomized controlled trial’/de OR ‘single-blind procedure’/de OR ‘clinical trial’/exp OR trial:ab,ti) NOT ([animals]/lim NOT [humans]/lim)

Cochrane Library

(Cognit* OR neurocognit* OR neuropsychol* OR “neuro psychol*” OR Learn* OR memor* OR Thinking OR Thought OR “problem solv*”) AND (Genetic* OR Hereditar*) NEAR/3 (Disease* OR Disorder* OR syndrom* OR defect*)

PsycINFO

((Cognit* OR neurocognit* OR neuropsychol* OR "neuro psychol*" OR Learn* OR memor* OR Thinking OR Thought OR "problem solv*") AND (Genetic* OR Hereditar*) ADJ3 (Disease* OR Disorder* OR syndrom* OR defect*)).tw. AND (randomized OR placebo OR randomly OR trial OR groups).tw.

During a first screen on title and abstract, references were discarded when they described non-Mendelian disorders (e.g. Alzheimer's disease, Parkinson's disease, except when they included only patients with Mendelian forms of these disorders) or described a non-biological intervention (e.g. behavioral therapy) or no intervention study. During screening on full-text, references were discarded when they described conference abstracts or reviews, clinical trials without cognitive outcome measure (e.g. outcome assessment only based on questionnaires) or were non-English. For additional records, we searched reference lists of retrieved records and from identified reviews (figure 1). T.V. has performed the selection of articles and I.E.O checked a random 10% sample for agreement. Within this 10% sample, agreement was 100%. Data extraction was performed with a pre-specified standardized form by T.V. and likewise checked by I.O. with a 30% random sample of articles. Only minor differences were found, not of influence for the review. Outcomes that were collected were samples sizes, characteristics, CONSORT-items, Jadad-items, placebo-effects in the placebo-groups and trial registration status.

A-Table 1. Clinical impact of clinical trials measured by Cochrane systematic reviews during the last five years.

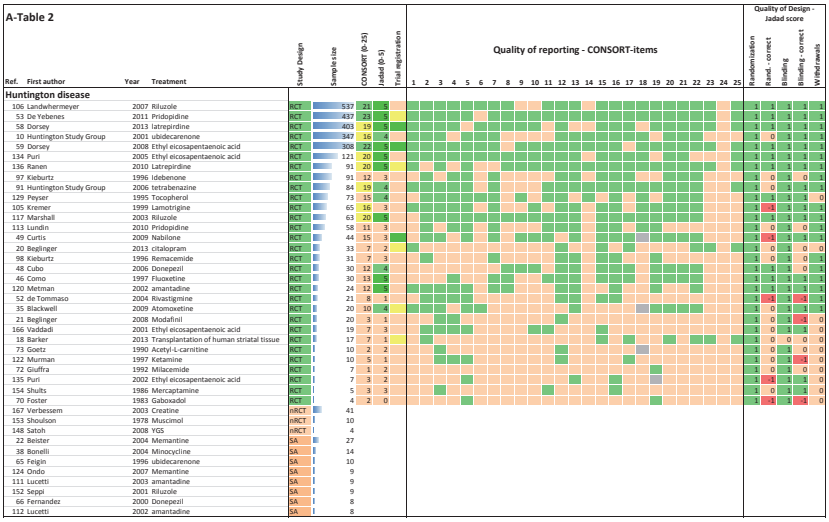
	Disorder	Intervention	Findings	Reviewer's conclusions
Rueda et al (2011)[35]	Fragile X syndrome	Folic acid	Studies were generally poorly reported and only one study was classified as low risk of bias.	The quality of available evidence is low and not suitable for drawing conclusions about the effect of folic acid on fragile X syndrome patients. It consists of few studies with small samples of patients, all of them male, with little statistical power to detect anything other than huge effects.
Webster & Wildgoose (2010)[36]	Phenylketonuria	Tyrosine	Three trials were included. Blood tyrosine concentrations were significantly higher in the participants receiving tyrosine supplements than in those receiving placebo. No significant differences were found on any of the other outcomes measured.	From the available evidence no recommendations can be made about whether tyrosine supplementation should be introduced into routine clinical practice. Further randomised controlled studies are required to provide more evidence.
Mohan et al (2009)[37-40]	Down syndrome and Alzheimer's disease	Rivastigmine, galantamine, memantine and donepezil	No clinical trials were eligible for rivastigmine, galantamine or memantine; One small-sized study of donepezil found no significant benefit[18]	Well-designed, adequately powered studies are required to investigate the effects of Alzheimer treatments on adults with Down syndrome.
Mestre et al (2009)[21]	Huntington's disease (Disease progression)	All available	No trials produced positive results for the selected efficacy outcome measures.	Further trials with greater methodological quality should be conducted using more sensitive biological markers. Pre-symptomatic mutation carriers should be included in future studies.

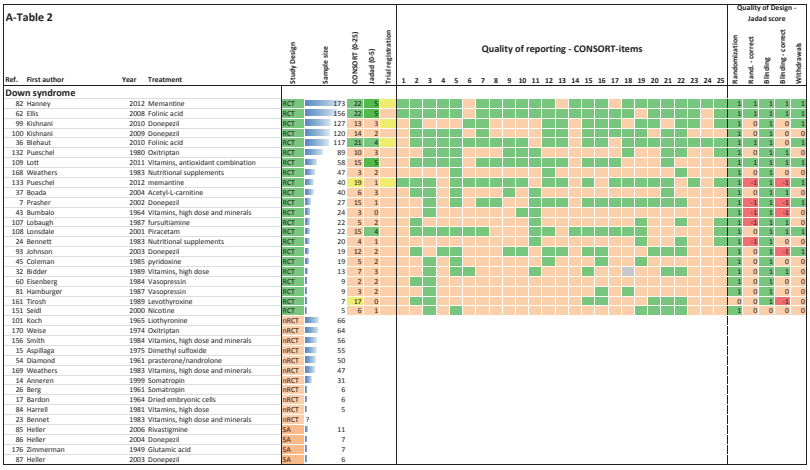
A-Table 1. Clinical impact of clinical trials measured by Cochrane systematic reviews during the last five years. (continued)

	Disorder	Intervention	Findings	Reviewer's conclusions
Mestre et al (2009)[20]	Huntington's disease (Symptom management)	All available	Only tetrabenazine showed a clear efficacy for the control of chorea, but no effect on cognitive functioning. The remaining pharmacological interventions revealed no clear effectiveness.	No intervention proved to have a consistent symptomatic control in HD. Tetrabenazine is the anti-choreic drug with the best quality data available. Other symptomatic areas should be explored by well-designed randomised placebo-controlled studies.

APPENDIX

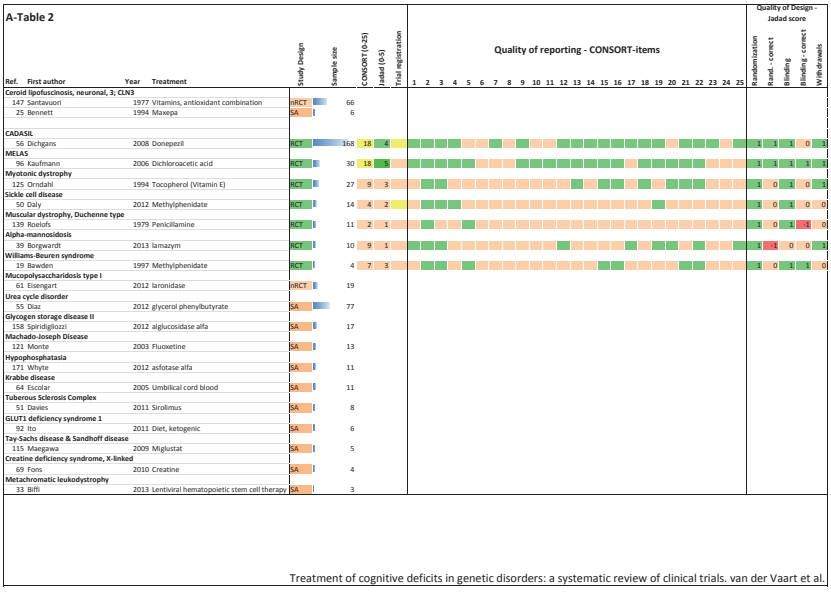
A-Table 2. Summary of all trials included in this review and their ratings.






A-Table 2
Ref. First author Year Treatment
Study Design
Sample size
CONSORT (0-25)
Jadad (0-5)
Trial registration
Quality of reporting - CONSORT-items
Quality of Design - Jadad score
Randomization
Rand. - correct
Blinding
Blinding - correct
Withdrawals
Phenylketonuria
172 Williamson 1981 Diet, phenylalanine low RCT 132 6 2
16 Azen 1996 Diet, phenylalanine low RCT 118 0 1
102 Koch 1982 Diet, phenylalanine low RCT 115 10 0
57 Dobson 1977 Diet, phenylalanine low RCT 111 3 1
174 Yi 2011 Docosahexaenoic acid RCT 33 16 5
130 Pietz 1995 Tyrosine RCT 24 6 3
157 Smith 1998 Tyrosine RCT 21 7 3
95 Kalkanoglu 2005 Diet, phenylalanine low RCT 19 0 1
150 Schindeler 2007 Amino acids, large neutral RCT 16 5 3
27 Berry 1990 Amino acids, valine, isoleucine, leucine RCT 16 4 1
77 Griffiths 1998 Diet, phenylalanine low RCT 16 4 3
110 Lou 1987 Tyrosine RCT 14 2 3
88 Holtzman 1975 Diet, phenylalanine low RCT 10 10 0
44 Clarke 1987 Diet, phenylalanine low RCT 9 3 2
160 ten Hoedt 2011 Diet, phenylalanine low RCT 9 10 4
68 Fisch 1984 Diet, phenylalanine low nRCT 85
90 Hsia 1958 Diet, phenylalanine low nRCT 24
137 Realmuto 1986 Diet, phenylalanine low nRCT 13
42 Bruhl 1964 Diet, phenylalanine low nRCT 10
104 Krause 1985 Diet, phenylalanine low nRCT 10
119 Mazzocco 1992 Tyrosine nRCT 9
164 Ullrich 1994 Levodopa nRCT 9
47 Crowley 1990 Diet, phenylalanine low SA 10
94 Jordan 1985 Amino acids, valine, isoleucine, leucine SA 5
13 Anderson 1976 Diet, phenylalanine low SA 4
Fragile X mental retardation syndrome
162 Torrioli 2008 Acetyl-L-carnitine RCT 63 4 3
28 Berry-Kravis 2012 arbaclofen RCT 62 16 5
29 Berry-Kravis 2006 CX-516 RCT 49 13 5
79 Hagerman 1986 Folic acid RCT 25 3 3
146 Sahu 2012 donepezil RCT 20 18 5
163 Torrioli 1999 Acetyl-L-carnitine RCT 17 0 2
159 Strom 1992 Folinic acid RCT 16 5 3
80 Hagerman 1988 Folic acid RCT 15 3 3
71 Froster-iskenius 1986 Folic acid RCT 10 1 1
40 Brown 1986 Folic acid RCT 5 3 1
41 Brown 1983 Folic acid RCT 2 3 1
30 Berry-Kravis 2009 Fenobarn nRCT 25
138 Roberts 2011 Methylphenidate/Dexamfetamine nRCT 12
114 Madison 1986 Folic acid nRCT 3
140 Rosenblatt 1985 Folic acid nRCT 2
31 Berry-Kravis 2008 Lithium SA 15
63 Erickson 2011 Riluzole SA 6


A-Table 2
Ref. First author Year Treatment
Study Design
Sample size
CONSORT (0-25)
Jadad (0-5)
Trial registration
Quality of reporting - CONSORT-items
Quality of Design - Jadad score
Randomization
Rand. - correct
Blinding
Blinding - correct
Withdrawals
Turner syndrome
141 Ross 2000 ethinylestradiol RCT 54 3 2
142 Ross 2003 Oxandrolone RCT 51 6 2
143 Ross 1998 ethinylestradiol RCT 47 1 2
144 Ross 2009 Oxandrolone RCT 44 10 3
145 Ross 1997 Somatropin RCT 40 3 2
177 Zuckerman-Levin 2009 methyltestosterone RCT 16 4 4
Prader-Willi syndrome
155 Siemensma 2012 Somatropin RCT 50 4 1
67 Festen 2008 Somatropin RCT 29 3 1
123 Myers 2007 Somatropin RCT 25 3 1
89 Hoybye 2005 Somatropin RCT 19 6 2
83 Haqq 2003 Somatropin RCT 14 2 2
Neurofibromatosis type 1
165 Vaart van der 2013 Simvastatin RCT 84 26 5
103 Krab 2008 Simvastatin RCT 61 24 5
116 Mainberger 2013 lovastatin RCT 11 4 1
11 Acosta 2011 Lovastatin SA 23
118 Mautner 2002 Methylphenidate SA 23
Velocardiofacial syndrome
75 Green 2011 Methylphenidate RCT 34 7 3
76 Green 2012 ademetionine RCT 12 4 2
74 Gothelf 2003 Methylphenidate SA 12
Mucopolysaccharidosis type III
78 Guffon 2011 Miglustat RCT 25 11 4
131 Piotrowska 2008 Genistein SA 10
Rett syndrome
127 Percy 1994 Naltrexone RCT 22 11 3
175 Zappella 1990 Bromocriptine RCT 10 1 0
Angelman syndrome
128 Peters 2010 Betaine, folic acid RCT 48 3 3
34 Bird 2011 Multivitamins, other combinations nRCT 90
Gaucher disease type III
149 Schiffmann 2008 Miglustat RCT 30 11 1
12 Altarescu 2001 imiglucerase SA 21
Niemann-Pick disease, type C
126 Patterson 2007 Miglustat RCT 41 21 3
173 Wraith 2010 Miglustat SA 29



Non-randomized and single-arm studies were not scored for quality. RCT: randomized controlled trial; nRCT: trial with a non-randomized control-group; SA: single-arm study, no control group; CONSORT: CONsolidated Standards Of Reporting Trials. Total number of items reported in the trial. 21-25 items reported: green/good. 16-20 items reported: yellow/questionable. 0-15 items reported: red/inadequate. Individual CONSORT-items: see main table in paper. Green means items reported, red means item not reported. Jadad-score: 0-5 points in total, 2 for randomization, 2 for blinding and 1 for the description of withdrawals and reasons for withdrawal. One point was deducted if the method of randomization/blinding was described, but the followed method was considered inadequate.

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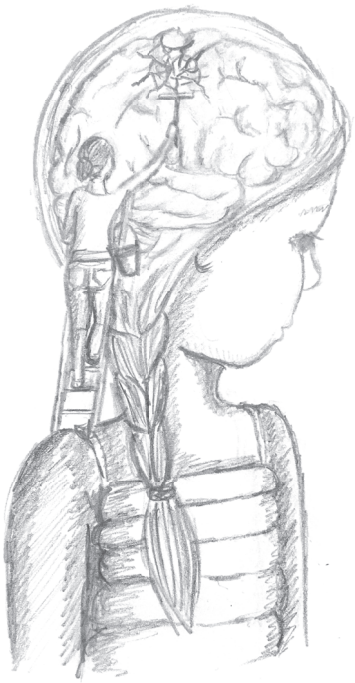
CHAPTER 8

GENERAL DISCUSSION



Tuberous Sclerosis Complex is a rare genetic disorder with a highly variable phenotype. Patients suffer from hamartomas in the kidneys, heart, lungs and eyes, and are often recognized by hypomelanotic macules and angiofibromas on the skin. Neurologic symptoms have the greatest impact on the functioning of the patient in daily life. Neurologic morbidity includes structural changes in the brain, for example cortical tubers and SEGA. Others features associated with TSC are epilepsy, cognitive impairment and autism. The studies described in this thesis contribute to more understanding on the neurologic and behavioral phenotypic variability of TSC, and more insight in targeted treatments for TSC- related epilepsy and cognitive impairment.

PHENOTYPIC VARIABILITY IN TSC

In chapters 2, 3 and 4 we investigated the neurologic and behavioral phenotypic variability of TSC, and examined which factors might be influencing this variability. When examining the type of genetic mutation and TSC-related brain pathology , we found that patients with a *TSC2* mutation had on average more cortical tubers (chapter 2). Surprisingly, mutations fully inactivating the TSC2 protein did not cause a more severe phenotype than mutations leaving the TSC2 protein intact. We also found that cortical tubers were strongly related to autism spectrum disorder severity (chapter 3). Other associations between TSC-related brain pathology and ASD were also examined, however most were mediated by IQ. When examining the variability of intellectual functioning in a large group of TSC patients, we found that an older age at the start of seizures was the only independent predictor of intellectual functioning in our model (chapter 4).

How does phenotypic variability originate?

The spectrum of symptoms associated with TSC was based on clinical observations in patients. This led to the clinical criteria with major and minor TSC features described in table 1 in the general introduction. Upon discovery of the genetic cause of TSC, the full phenotypic spectrum of TSC was confirmed to be due to a mutation in *TSC1* or *TSC2*. Many studies compared the phenotype of patients with a *TSC1* and *TSC2* mutation, and found that, overall, a *TSC2* mutation caused a more severe phenotype. This makes sense on a genetic level, as the TSC2 protein holds the GAP domain that inhibits Rheb and therefore decreases mTORC1 activity. However, since there is a lot of phenotypic overlap between patients with a mutation in *TSC1* or *TSC2*, a prediction for the individual patient cannot be made. Recent studies have therefore focused more on the type of mutation in patients. Different ways to divide the types of mutations have been published, and all result in different associations with symptoms. A study on epilepsy severity classified mutations based on their location on the gene, and assumed mutations in exon 1-22

would influence the interaction between the TSC1 and TSC2 proteins, and mutations in exons 34-41 would likely affect the GAP domain.[1] Mutation in exons lying between those regions were found to associate with a reduced incidence of infantile spasms, and therefore a milder TSC phenotype. A study investigating variability of IQ found that frameshift mutations leading to longer C-terminal tails led to lower IQ in *TSC1* mutations, and higher IQ in TSC2 patients.[2] Unfortunately, the authors proposed no mechanism for this finding, leaving the biochemical consequences of such C-terminal tails unclear. In chapter 2, we divided *TSC2* mutations in two groups: those that leave the TSC2 protein functional, and those that cause nonsense mediated decay and therefore leave no TSC2 protein. We found that such a distinction does not explain the variability in TSC associated brain lesions.

Most of these studies, including ours, include a limited number of patients. This is usually due to the rare nature of TSC itself. Additionally, since so many different types and locations of mutations are possible in the TSC1 or TSC2 gene, even a population of 200 patients would still mean that for many mutations only one or two patients can be examined. Ideally, research should include such a large group of patients that every mutation would be found in at least five patients, so that a phenotypic correlation between genotype and phenotype can be determined with more certainty.

Such a study would follow the assumption that the answer to the phenotypic variability question indeed lies in the type of mutation. Many researchers however are starting to believe that the type of mutation is not what is causing the variability at all. From cancer research, we know that loss of heterozygosity (LOH) can also aggravate a phenotype. The term LOH is used when a germ line mutation is heterozygous, but the second allele is also mutated, leaving a cell within a functioning copy of that gene. This is most often the result of chance. Mutations happen in every cell, and the chance that it affects the second allele of an already mutated first allele is equal to any other mutations. If such an LOH mutation occurs early in embryologic development, an entire body part may develop out of cells that have lost the function of the affected gene. This has been proposed as the mechanism for cortical tubers.[3] The LOH hypothesis could explain the familial phenotypic variability in TSC. Members of one family carry the same mutation, and the previously mentioned location of a genetic mutation could therefore not be the (full) explanation for why some children in the same family suffer from severe morbidity due to TSC, while others might not even know they have TSC. Variability within families and even within twins has been repeatedly shown.[4-6] A new study performed at the Erasmus MC will examine the variability of IQ in twin pairs with TSC (clinicaltrials.gov NCT02436746). As they share the exact same DNA, any variability in phenotype is likely to be from a stochastic process like LOH.

Another option includes the entire genome, with all genetic material, genetic modifiers and epigenetic changes. As we have learned over the years, even though DNA has

a certain contents of genes, every individual has numerous mutation throughout the entire genome. All these alterations together decide the appearance and character of a person, and with the current state of science, it is not yet possible to determine the interplay between all these genes and mutations. All these variations in our genome will also likely influence the TSC phenotype.

As all the above mentioned genetic mechanisms take place in all TSC patients, it is highly likely that the genetic cause of the variability in phenotype in patients with TSC is a combination of all these mechanisms.

What is cause and what is consequence?

Even though much focus has been on finding a genetic explanation for phenotypic variability, not all TSC-related features might entirely be due to genetics. Studies examining clinical TSC features have posed the question whether for example intellectual functioning in TSC is due to genetic changes, or whether the severity of TSC-related brain pathology might also play a large part in determining a patients intellectual abilities. This question was also raised by us in chapter 3, when we observed that even though the number of cortical tubers is associated with the severity of ASD, many of the other associations between TSC-related brain pathology and ASD are mediated through IQ. Moreover, as many patients with TSC also have epilepsy, is it epilepsy that decreases intellectual functioning, or is it the TSC-related brain pathology that causes epilepsy and therefore decreases intellectual functioning?

For this reason, we examined the variability of intellectual functioning in chapter 4. We added several epilepsy variables and the genetic mutation to our statistical model, and found that only an older age at the onset of epilepsy could independently cause a positive influence on intellectual abilities. A genetic mutation in *TSC1* or *TSC2* did not have a correlation with intellectual abilities. This raised the question whether it might indeed be the epilepsy that has the most influence on intellectual functioning.

Unfortunately, we did not have enough data on TSC-related brain pathology to add for example the number of cortical tubers to our model. Previous studies have found that cortical tubers are indeed associated with intellectual functioning.[7,8] As our model included only data on the first two years of life, many other factors could influence intellectual functioning in these children. TSC-related factors such as co-morbidity, the frequency of hospital visits and possible interaction problems with parents also suffering from TSC could play a role, but non-TSC-related factors such as social economic status are important for any child's development, regardless of having TSC. This complicates the search for a model that can predict the intellectual outcome of a child with TSC.

Can we determine the precise interplay between TSC associated factors using clinical studies?

Ideally, every parent of a child with TSC would like to know the development and future complications of their child. Not only which intellectual abilities their child will face, but also from which other physical TSC consequences they will suffer, and at what age these consequences will emerge. Science will quite possibly never have an answer to such an elaborate question, which is why most research focusses only on quantifiable problems such as the number of cortical tubers or IQ. As mentioned above, the problem with focusing on one feature is that the influence of features that are not measured will not become apparent, and associations that are measured are actually mediated by another factor. The best way to solve this problem would be to meet halfway. Prediction models that are based on a very large study population can include more clinical variables, and can therefore better determine which factors are of independent importance to the TSC phenotype. Such models can also be made to change the outcome, and to determine whether TSC-related brain pathology influences epilepsy, and in what way epilepsy in turn influences IQ, or whether brain pathology and epilepsy can independently influence intellectual abilities. TSC-related as well as non-TSC-related factors should be collected prospectively and longitudinally, and should be entered in a standardized database. A team of medical professionals with knowledge on the development of children should decide which variables might be of importance to an individual's development and functioning. A team of TSC experts should decide which TSC-related factors could be important to the TSC phenotype. Combining all those factors in a prospective database, ideally including more than 1000 TSC patients followed from a young age, will enable researchers to build statistical models that can narrow down which factors influence the TSC phenotype.

How can basic studies help to determine cause and consequence?

Besides studying clinical features and their interaction, another approach is to manipulate one aspect of a disease in animal models. Numerous TSC mouse models have been developed that recapitulate parts of the TSC phenotype. Using these models, a direct consequence of changing one aspect of the TSC phenotype can become apparent, in a population of animals that is otherwise exactly the same. Genes can be manipulated in a specific location of the gene, or in a specific cell population in a part of the body. Changes to genes can also be made postnatally, so that mice develop normally and changes in protein activity only become active when they are adult. One such example is the study of Abs et al.[9] In this study, the *TSC1* gene was inactivated in adult mice. While these mice had no brain pathology, they started having seizures within nine days of the onset of gene deletion. This shows that the deletion of the *TSC1* gene alone, and therefore the increase of mTORC1 activity, can cause epilepsy, independent of any TSC-

related brain pathology. In another study, *TSC1* mutant mice without brain pathology or seizures were shown to have impaired hippocampal learning and social behavior, indicating that intellectual abilities and social skills might not entirely due to TSC-related brain pathology.[10]

These animal models represent parts of the TSC phenotype, and some researchers therefore argue that they are not good models to study TSC, as they do not recapitulate all TSC features. However, as we are currently searching for the cause of certain TSC features, these animal studies provide valuable information. As long as we do not know the exact interplay between all TSC features, it is important to keep performing basic studies into the genetic and biological mechanisms of TSC, so that hopefully one day we can fully understand how all TSC features interact.

TREATMENTS FOR TSC

In chapter 5, we showed that the symptomatic treatment of TSC-related epilepsy with regular AEDs is insufficient in many patients, as only 77% of children reached seizure remission, and only 38% of those stayed seizure free for over 24 months. Surgical removal of SEGA caused an increase of seizures and vision disorders in a group of 47 TSC patients,[11] and removing one spot of renal AML does not prevent new spots of AML from forming.[12] This has led to the search for a treatment that targets the entire spectrum of TSC features, by inhibiting the mTORC1 protein thought to underlie all TSC symptoms. The identification of the mTORC1 inhibitors sirolimus and everolimus has subsequently changed the treatment of TSC patients substantially.

Could mTORC1 inhibition treatment be the answer for treating TSC-related epilepsy?

After confirming that SEGA and AML decrease in volume upon treatment with mTORC1 inhibitors,[13,14] the focus shifted toward one of the TSC-related symptoms thought to cause most morbidity: epilepsy. Sirolimus was shown repeatedly to reduce or stop seizures in several TSC mouse models.[15-17] Results for human TSC patients however have not been that obvious. Several human case series showed that seizures were reduced in many patients using mTORC1 inhibitors, although these case series never included a control group, and numbers of patients were small. In chapter 6, we present the results of the first controlled trial investigating mTORC1 inhibitors in intractable epilepsy in TSC. Our data shows no significant effect of sirolimus on seizure frequency. However, in patients with sirolimus trough levels above 5 ng/ml, we observed a greater reduction of seizure frequency, even though this was also not significant.This suggests that mTORC1

inhibitors might indeed be effective in reducing seizure frequency in TSC-related intractable epilepsy if it is dosed high enough.

A recently published large international trial included 366 individuals with TSC between 1-65 years old.[18] Patients were treated with a low dose of everolimus (titrated to 3-7 ng/ml), a high dose of everolimus (titrated to 8-13 ng/ml) or placebo. Preliminary results indicated that patients taking a higher dose more often had a decrease in the number of seizures, and taking either a high or low dose of everolimus was more effective than placebo. However, even in the group taking a high dose of everolimus, only 3% of the patients was completely seizure free.

These results are somewhat disappointing, especially since animal models have shown such a strong anti-epileptic effect of mTORC1 inhibitors. One obvious difference between animal and human treatment is that the studies in patients have included patients with intractable epilepsy, while animals are treated with an mTORC1 inhibitor as a first drug. In addition, animals usually only have seizures for a short period of time before mTORC1 inhibitor treatment is initiated, while patients may have already had interictal discharges or even seizures for a long period before any treatment is initiated. Furthermore, the dose of mTORC1 inhibitors in animals is high compared to the human dose. Our study suggested that patients taking a higher dose could be more likely to have a reduction of seizure frequency, suggesting the dose should be increased for an optimal effect. This might be a problem as mTORC1 inhibitors also cause side effects, which can be especially invalidating in young children. Side effects in our study mostly included upper respiratory tract infections and aphthous ulcers in the mouth. These side effects are extra problematic in young, intellectually disabled children, as they are not able to tell their parents they aren't feeling well, and for example stop eating and drinking and therefore get even more ill.

How can we interpret the varying individual response to mTORC1 inhibitors?

When looking through the results of our trial in chapter 6, it is apparent that the included children had very different responses to mTORC1 inhibitor treatment. Some children became seizure free, while others did not have any change in seizure frequency, or even had an increase of seizures. We tried to create a homogeneous patient group by restricting the included children's age, a minimum number of AEDs tried, and a minimum number of seizures per week. The different outcome per child may be because these children were not all the exact same age, and they had not suffered epilepsy for the same amount of time. Also, their seizure type varied, and they took different types of AEDs. To determine the exact effect of mTORC1 inhibitor treatment on all these factors requires a very large group of treated TSC patients. Due to the rare nature of TSC, such a large trial is not feasible. Even though the largest international trial included 366 patients, the age

range of these patients was large, varying from 1-65 years. The included patients also had varying durations and types of epilepsy.

These variations between patients might change the outcome of the effectiveness of mTORC1 inhibitors, but at least they can be registered. There are several other factors that may change the effectiveness of mTORC1 inhibitors that we cannot determine clinically. For example, the degree of LOH might influence the hyperactivation of mTORC1. If more cells have LOH, mTORC1 inhibition by the TSC1-TSC2 complex is lost in more cells, and therefore more mTORC1 is hyperactive. This might require a larger dose of mTORC1 inibitors, or might even cause mTORC1 inhibitors not to be effective. Mutations in other genes associated with the mTOR pathway might also be influencing the phenotype, and might possibly require additional medication.

An obvious solution to this problem would be combining treatments. For epilepsy, this might include combining mTORC1 inhibitors with other epilepsy treatments such as AEDs or the ketogenic diet. Since testing all these combinations of treatments in a randomized controlled trial will never be possible in a rare disorder such as TSC, this is something that should be tried in every patient individually.

Another approach is to investigate drugs that modulate the mTORC1 pathway but do not act on mTORC1 itself. mTORC1 is only one protein in an elaborate protein pathway, and several other targets for therapy have been described. For example, the protein 5' adenosine monophosphate-activated protein kinase (AMPK) directly inhibits components of the mTORC1 complex, and could therefore be stimulated to decrease mTORC1 activity.[19] Phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) has also been described as part of the mTOR pathway, and dual inhibition of these proteins can inhibit mTORC1.[20]Other proteins in the mTOR pathway that could be targeted are the additional proteins within mTORC1, such as DEP-domain containing mTOR-interacting protein (DEPTOR). Overexpression of DEPTOR downregulates the activity of mTORC1,[21] and could therefore be effective as an mTORC1 inhibitor.

Although all the above mentioned treatments might reduce TSC symptoms, the only option that could cure TSC entirely is gene therapy. Replacing a mutated gene with a healthy one has been attempted for numerous disorders, such as immune deficiencies[22]and sickle cell disease.[23] Though many advances have been made using gene therapy in the last decade, treating TSC with such a therapy causes many challenges that do not have a solution to date, the most important one being that cells containing the TSC mutation are present throughout the whole body, and a therapy would probably need to be effective in the majority of cells to ameliorate the TSC phenotype. Furthermore, such a treatment should be initiated at a very young age, preferably during embryologic development, as some TSC features, like cortical tubers, cannot be cured even if a mutated gene is replaced with a healthy one.

When should mTORC1 treatment be initiated?

As more drug therapies are developed for TSC, the question arises at what time during a patient's life such treatments should be initiated to have an optimal effect. As mentioned earlier, the results of clinical trials investigating epilepsy might be disappointing compared to the promising results of animal studies, because patients are treated when they have had seizures for many years, and have tried several other epilepsy therapies before trying an mTORC1 inhibitor. mTORC1 inhibitors might be more useful if they are the first treatment after seizures start, of maybe they should be started before the first seizure. Or, considering the broad spectrum of symptoms associated with TSC, maybe such a treatment should be started directly at birth, or even before that. The discussion on when to start these drugs would be a lot simpler if they did not cause any side effects. mTORC1 inhibitors have been shown to have many different side effects, and the long term effects of treating children for a long time have never been examined. Moreover, as the phenotype of TSC is so variable, treating patients preventatively would mean treating patients for symptoms they might never have. Therefore, determining when to start a treatment requires more knowledge on which patient will develop which symptoms. For epilepsy, such a study is now ongoing (clinicaltrials.gov NCT02098759), in which treatment with the AED vigabatrin is started before the onset of clinical seizures. Children with a TSC diagnosis before the age of three months are intensively monitored by EEG, to determine possible epileptic activity. When this activity becomes apparent, children are randomized to either be treated immediately with vigabatrin, or after clinical seizures arise. This study will give insight in which EEG parameters might predict the development of clinical seizures, and whether preventatively treating children will reduce the severity and frequency of seizures, and possibly even improve intellectual development. Such benefits from preventative treatment have been suggested before.[24-26] Besides the effect from epilepsy, intellectual abilities themselves are also thought to benefit from early treatment. Trials in TSC patients examining changes in intellectual ability with an mTORC1 inhibitor are ongoing (clinicaltrials.gov NCT01289912 and NCT01730209), and hopefully they will tell us whether, if intellectual abilities can be improved with mTORC1 inhibitors, the effect on intellectual abilities is larger when the treatment is started at a younger age.

Another problem is the duration of mTORC1 inhibitor treatment. Although mTORC1 inhibitors reduce the volume of SEGA, the volume increases after subsequent stopping of mTORC1 inhibitor treatment.[27] This might indicate that patients with TSC should be treated with mTORC1 inhibitors their entire life. Even though many side effects have become apparent from the use of these drugs in the past decades, the effects of lifetime treatment are not yet known, especially not if this treatment is started in children. For example, as mTORC1 inhibitors reduce cell proliferation, children could develop growth retardation. Long-term use effects of these drugs should be strictly monitored.

IMPROVING CLINICAL TRIALS IN RARE DISEASES

How can we optimize the evaluation of a new drug?

TSC is not the only genetic condition in which patients suffer from intellectual disability. Disorders like Neurofibromatosis type I and Fragile X syndrome are also associated with intellectual disability, and, similar to TSC, no effective treatment has yet been identified for this debilitating feature of these conditions. This absence of effective treatment has not been for lack of trying, as shown by the numerous reports on animal and patient studies published on treatments possibly influencing cognitive development in these disorders. In chapter 7, we wondered why so little treatments get registered as effective therapy, while so many treatments seem to be examined. We found that small sample size and low quality of study design are probably the cause for failure of treatment trials in patients. International collaboration is need to increase the number of patients in the clinical trials. In addition, trial design should be improved. Clinical trials should include a control group where possible, and should randomize participants. Also, the reporting and design of trials should be improved and outcome measures should be predefined.

How can recruitment issues be improved?

Randomized clinical trials are considered the highest standard of evidence for determining whether a new treatment is effective. Such trials are the most valuable for patients, as they involve the actual patients who will be eligible to undergo the treatment once efficacy has been established. However, the inclusion of patients in such trials is often the largest hurdle. Many clinical trials suffer from low inclusion rates. The reasons patients offer for not joining a clinical trial are usually related to time investment, travel distance, and fear for side effects. Even though participating in research has become a lot more common in the past decade, and people are much more informed about research into their own condition, these recruitment issues still remain.

One way to reduce this problem is to increase the number of patients who are eligible for trial participation. Investigators could contact other hospitals and treatment facilities within their own country, or even more preferably collaborate with treatment centers in other countries. Establishing such international trial collaboration will enable patients from different countries to join a clinical trial, and will increase the number of included patients and therefore improve trial quality.

Another way to get more patients interested in a trial is to verify whether the outcomes of a trial are of actual importance to patients. Doctors and researchers might observe that a certain feature of a disorder is present in many patients and want to examine whether a drug is useful to reduce this symptom, however patients may find this only a minor symptom and be much more interested in the treatment of another feature of their disorder. Discussing the possible outcomes of a clinical trial with patients is best

done through a patient organization, as they know many patients and family members and are also interested in scientific research. Together, a clinical trial can be designed that is of interest to researchers as well as patients.

How can we improve the quality of trials and their reporting?

Besides the number of included children, chapter 7 also shows that the quality and reporting of trials might cause treatments to fail. The outcome measures or trial design are often not suitable for the research question, and not enough information is included in a publication for readers to thoroughly understand how the trial was performed. Most investigator initiated trials are designed by doctors who were never thoroughly educated on how to perform a clinical trial. Therefore, university hospitals and research facilities should offer guidance on how to design and perform such expensive and time consuming research.

Research journals publishing clinical trials should also take large responsibility in this matter. High impact journals already require trials to be registered in a trial database, and adherence to the CONSORT statement for quality reporting of trials. However, low impact journals often do not require any of these, and will publish any research submitted to them. If these journals would enforce a stricter publication policy on the quality of clinical trials, researcher would know that a poorly designed and analyzed clinical trial will not have any chances of being published, and will be forced to adhere to the strict guidelines. And even more important, patients would not be exposed to treatment and assessments that do not lead to any useful conclusions.

CONCLUSIONS

Our studies show that the phenotype in patients with TSC is highly variable, and that much additional research is needed to identify the cause and complicated interplay of this variability. This requires large study populations with prospective follow-up. Increasing the number of patients in studies will also improve clinical trials investigating the new treatments that are eagerly awaited for by patients with a rare disorder. For TSC, much hope is fixed on mTORC1 inhibitors. With many clinical trials ongoing, we might soon have an answer whether mTORC1 inhibitors are indeed the solution to treating TSC, or whether new therapeutic strategies are needed to tackle this complicated genetic disease.

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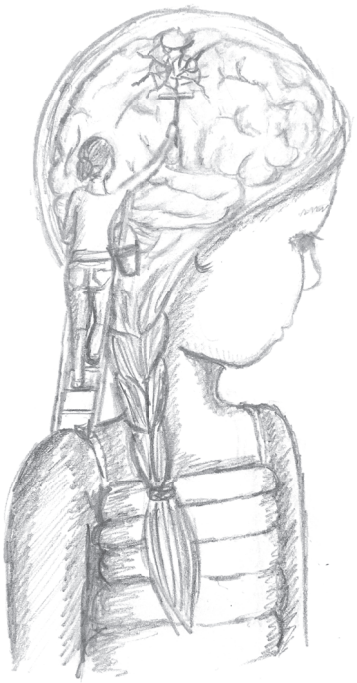
APPENDICES



I ENGLISH SUMMARY

Tuberous Sclerosis Complex (TSC) is a rare genetic disorder with an incidence of 1 in 6000 births. The genetic cause is a mutation in either the *TSC1* or *TSC2* gene, leading to a hyperactivation of the mTORC1 pathway. Clinical features of TSC range from hamartomas in kidneys, heart and eyes, to malformations of brain development called cortical tubers. Patients can also suffer from epilepsy, intellectual disability and psychiatric morbidity such as autism. This wide spectrum of TSC features is not present in all patients, and the severity of symptoms varies among patients. Recently, research has focused on finding a cure for TSC based on the molecular pathology in TSC.

As large variability in symptoms exists among TSC patients, we have performed several studies to uncover the origin of this variation, and the correlation between various disease characteristics. For many years, it has been known that patients with a *TSC2* mutation have on average a more severe phenotype. In chapter 2, we investigated different types of mutations in *TSC2* in a group of 64 children aged 1.4-17.9 years with TSC, to determine whether certain mutations cause a more severe phenotype than others. We subdivided *TSC2* mutations into mutations that leave functioning protein (*TSC2*p), and those that leave no functioning protein (*TSC2*x). We found that patients with a *TSC2* mutation indeed had more and larger cortical tubers, more radial migration lines and more subependymal nodules. However, no large differences were found in TSC-related brain pathology when patients with a *TSC2*p and *TSC2*x mutation were compared.

To further investigate the TSC phenotype, we examined whether TSC related brain pathology is related to the severity of autism spectrum disorder (ASD) features. In chapter 3, we examined 52 children with TSC, and found that cortical tubers and radial migration lines were indeed positively related to the severity of ASD. However, when the results were corrected for IQ, only the total number of cortical tubers and the number of tubers in the frontal lobe was correlated with ASD severity. This suggests that IQ mediates the relation between TSC-related brain pathology and autism spectrum disorder.

In chapter 4, we studied which factors influence intellectual functioning in TSC. We collected data on the first 24 months of life of 102 patients with TSC. Our outcome measure was intellectual equivalent, including both intellectual and developmental quotient. In univariable analyses, many factors were associated with the intelligence equivalent. Predictors of lower intellectual equivalent were the presence of infantile spasms, a larger number of antiepileptic drugs used, vigabatrin not used as the first drug, use of corticosteroid treatment, and a later age at which the child could walk independently. Higher intellectual equivalent was associated with an older age at seizure onset. However, when we applied a multivariable analysis, only the age at seizure onset was significantly correlated to intellectual equivalent, contributing to 28% of the variation in intellectual

equivalent. This suggests that results from multivariable analysis approaches are most valuable for our understanding of phenotypic variability in TSC.

Epilepsy is an important feature in TSC, and treatment strategies have not been optimal so far. In chapter 5 and 6, we examined the treatment of epilepsy in patients with TSC. In chapter 5, we assessed all epilepsy treatments in a group of 71 children with TSC, to determine which treatments patients use most often, and which treatments give the best chance of seizure frequency reduction. We found that 55 children (77%) became seizure-free for longer than 1 month, and 21 (30%) for longer than 24 months. Antiepileptic drugs prescribed as first and second treatment were most effective. Non-pharmacological therapies like the ketogenic diet, epilepsy surgery, and vagus nerve stimulation were used 13 times, and epilepsy surgery was most effective, with four of five children becoming seizure-free. In children with infantile spasms, vigabatrin was more effective than other AEDs when prescribed as first treatment. We concluded that, as only 30% of children become seizure free for longer than 24 months, the treatment with regular antiepileptic drugs is not sufficient for TSC-related epilepsy. Therefore, we examined treatment with mTORC1 inhibitors in TSC-related epilepsy in chapter 6. We treated 23 children with TSC and intractable epilepsy with sirolimus for six months in a cross-over design. In our primary outcome of seizure frequency reduction, our intention-to-treat analysis showed a non-significant reduction of 41% (95% confidence interval -69% to +14%; p=0.11). Our pre-specified analysis of 14 children who reached the target trough level showed a seizure frequency reduction of 61% (95% confidence interval -86% to +6%; p=0.06). No differences were found on cognitive development. All children had side effects. Unfortunately, our study was underpowered to show an effect of sirolimus on seizure frequency. We concluded that sirolimus might be a useful treatment for TSC-related epilepsy, especially if the sirolimus trough level is in the target range.

In chapter 7, we examined clinical trials that investigate treatment for intellectual disability in genetic disorders. To date, very few treatments are registered for this indication, even though a large number of pre-clinical and clinical trials are published each year. We identified 169 trial reports, of which 75 (44%) reported potential efficacy, of which only two are now established treatments for intellectual disability. Assessment of the trial reports using JADAD and CONSORT guidelines showed that many trial reports were of poor quality. Low sample size, low quality and design of trials, and failure to predefine outcome measures are common problems in clinical trial reporting. Improvements in these areas are essential to improve research for new treatments of intellectual disability in genetic disorders.

In chapter 8, we discuss our research and the future endeavors that are needed to understand TSC, and to optimize treatment. Large study populations with prospective follow-up will improve the understanding of TSC, and the relation between various TSC-

characteristics. Future trials with mTORC1 inhibitors or drugs targeting other proteins in the mTOR pathway might be the best way to treat symptoms related to TSC.

II NEDERLANDSE SAMENVATTING

tubereuze Sclerose Complex (TSC) is een zeldzame genetische aandoening met een incidentie van 1 op 6000 geboortes. De genetische oorzaak is een mutatie in het *TSC1* of het *TSC2* gen. Deze mutatie leidt tot hyperactivatie van de mTORC1 eiwit-cascade. Klinische kenmerken van TSC variëren van hamartomen in de nieren, het hart en de ogen, tot malformaties van de hersenontwikkeling die corticale tubers genoemd worden. Patiënten kunnen ook epilepsie, mentale retardatie en psychiatrische aandoeningen zoals autisme hebben. De symptomen van dit brede TSC spectrum zijn niet bij alle patiënten aanwezig, en de ernst van de symptomen varieert tussen patiënten. De meest recente onderzoeken zijn gericht op het vinden van een geneesmiddel voor TSC op basis van de moleculaire pathologie die TSC veroorzaakt.

Gezien de grote variatie in symptomen tussen TSC patiënten, hebben wij een aantal studies verricht om de oorzaak van deze variatie te achterhalen, en om de correlatie tussen verschillende TSC aspecten te ontdekken. Sinds langere tijd is het bekend dat patiënten met een mutatie in het *TSC2* gen gemiddeld een ernstiger fenotype hebben. In hoofdstuk 2 hebben we verschillende typen mutaties in *TSC2* onderzocht in 64 kinderen met TSC en een leeftijd tussen 1.4 en 17.9 jaar, om vast te stellen of bepaalde mutaties een ernstiger fenotype veroorzaken dan andere. We hebben *TSC2* mutaties onderverdeeld in mutaties die functionerend TSC2 eiwit mogelijk maken (*TSC2*p), en mutaties die geen functionerend TSC2 eiwit mogelijk maken (*TSC2*x). We vonden dat patiënten met een *TSC2* mutatie inderdaad meer en grotere corticale tubers, meer radiaire migratie lijnen en meer subependymale nodules hadden. Maar we vonden geen grote verschillen in TSC-gerelateerde hersenpathologie tussen patiënten met een *TSC2*p en *TSC2*x mutatie.

Om het fenotype nog verder te onderzoeken, hebben we onderzocht of TSC-gerelateerde hersenpathologie gerelateerd is aan autisme spectrum stoornis (ASS) kenmerken. In hoofdstuk 3 hebben we 52 kinderen met TSC onderzocht, en hebben we gevonden dat corticale tubers en radiaire migratie lijnen een positieve correlatie hebben met de ernst van ASS. Maar toen we deze resultaten corrigeerden voor het IQ van de patiënten, bleven alleen het totaal aantal corticale tubers en het aantal corticale tubers in de frontale kwab nog gerelateerd aan de ernst van ASS. Dit doet ons denken dat het IQ belangrijk is in de relatie tussen TSC-gerelateerde hersenpathologie en ASS.

In hoofdstuk 4 hebben we bestudeerd welke factoren van invloed zijn op het intellectuele functioneren van TSC patiënten. We hebben data verzameld over de eerste 24 levensmaanden van 102 patiënten met TSC. Onze uitkomstmaat was het intellectueel equivalent, waarbij zowel het intellectueel quotiënt als het ontwikkelingsquotiënt zijn meegenomen. In univariabele analyses waren veel factoren gerelateerd aan het intellectueel equivalent. Voorspellers van een lager intellectueel equivalent waren de

aanwezigheid van infantiele spasmen, een groter aantal anti-epileptica dat de patiënt gebruikt had, als vigabatrine niet gebruikt was als eerste medicijn, het gebruik van corticosteroïden en een oudere leeftijd waarop het kind voor het eerst los kon lopen. Een hoger intellectueel equivalent was geassocieerd met een oudere leeftijd waarop epilepsie aanvallen begonnen. Maar toen we een multivariabele analyse deden was alleen de leeftijd waarop epilepsie aanvallen begonnen nog significant gerelateerd aan het intellectueel equivalent. Dit verklaarde voor 28% de variatie in het intellectueel equivalent. Dit zou kunnen betekenen dat resultaten van multivariabele analyses het meest waardevol zijn voor onze kennis van het variabele fenotype van TSC.

Epilepsie is een belangrijk kenmerk van TSC, en behandelstrategieën hiervoor zijn momenteel niet optimaal. In hoofdstuk 5 en 6 onderzochten we de behandeling van epilepsie bij patiënten met TSC. In hoofdstuk 5 hebben we alle verschillende epilepsie behandelingen van een groep van 71 kinderen met TSC bestudeerd, om te bepalen welke behandelingen patiënten het meest gebruiken, en welke behandelingen de beste kans geven op aanvalsvermindering. We vonden dat 55 kinderen (77%) aanvalsvrij werden voor een periode van langer dan een maand, en dat 21 kinderen (30%) langer dan 24 maanden aanvalsvrij werden. Anti-epileptica die als eerste of tweede voorgeschreven werden waren het meest effectief. Niet-medicamenteuze behandelingen zoals het ketogeen dieet, epilepsiechirurgie en de nervus vagus stimulator werden 13 keer gebruikt. Epilepsiechirurgie was het meest effectief; vier van de vijf kinderen die dit ondergingen werden aanvalsvrij. Bij kinderen met infantiele spasmen was vigabatrine effectiever dan andere anti-epileptica als het als eerste behandeling werd voorgeschreven. We concludeerden dat, gezien maar 30% van de kinderen langer dan 24 maanden aanvalsvrij werden, de behandeling met reguliere anti-epileptica niet genoeg is voor de behandeling van TSC-gerelateerde epilepsie. Daarom hebben we in hoofdstuk 6 de behandeling van TSC-gerelateerde epilepsie met mTORC1 remmers onderzocht. We hebben 23 kinderen met TSC en moeilijk behandelbare epilepsie zes maanden lang behandeld met sirolimus in een cross-over onderzoek. Onze primaire uitkomst was de vermindering van het aantal epilepsie aanvallen. In onze intention-to-treat analyse vonden we een niet-significante reductie van epilepsie aanvallen van 41% (95% confidence interval -69% tot +14%; p=0.11). In onze van tevoren gespecificeerde analyse van de 14 kinderen die de doel-bloedspiegel hebben behaald, vonden we een aanvalsvermindering percentage van 61% (95% confidence interval -86% tot +6%; p=0.06). We vonden geen verschillen in het cognitieve ontwikkelingsniveau. Alle kinderen hadden bijwerkingen van sirolimus. Helaas had onze studie te weinig power om een effect van sirolimus op aanvalsfrequentie aan te tonen. We concludeerden dat sirolimus een bruikbaar medicijn kan zijn in de behandeling van TSC-gerelateerd epilepsie, vooral als de doel-bloedspiegel van sirolimus behaald kan worden.

In hoofdstuk 7 bekeken we klinische trials die behandelingen voor mentale retardatie door genetische syndromen onderzoeken. Tot op de dag van vandaag zijn er maar zeer weinig behandelingen geregistreerd voor deze indicatie, ook al wordt er elk jaar een groot aantal pre-klinische en klinische trials gepubliceerd. We identificeerden 169 trial beschrijvingen, waarvan 75 (44%) mogelijke effectiviteit van de behandeling beschreven. Daarvan zijn er twee momenteel geregistreerde behandelingen voor mentale retardatie. Het scoren van de trial beschrijvingen met gebruik van de JADAD en CONSORT richtlijnen liet ons zien dat veel trial beschrijvingen van slechte kwaliteit zijn. Een laag aantal deelnemers aan het onderzoek, slechte kwaliteit en ontwerp van studies en het niet van tevoren definiëren van de uitkomstmaat zijn veel voorkomende problemen in het rapporteren van klinische trials. Verbeteringen op deze gebieden zijn essentieel om onderzoek naar nieuwe behandelingen voor mentale retardatie door genetische aandoeningen te verbeteren.

In hoofdstuk 8 bediscussiëren we ons onderzoek en de toekomstige inspanningen die nodig zijn om TSC beter te begrijpen en om de behandeling te optimaliseren. Grotere onderzoekspopulaties met prospectieve follow-up zullen ons begrip van TSC en de relatie tussen verschillende TSC kenmerken verbeteren. Toekomstige trials met mTORC1 remmers of medicijnen die op een ander eiwit in de eiwit-cascade ingrijpen zouden wel eens de beste manier kunnen zijn om de symptomen die gerelateerd zijn aan TSC te kunnen behandelen.

III LIST OF ABBREVIATIONS

AED	Anti-Epileptic Drug
Akt	Protein Kinase B
AML	Angiomyolipoma
AMPK	5'adenine monophosphate-activated protein kinase
ASD	Autism Spectrum Disorder
CNS	Central Nervous System
DEPTOR	DEP-domain containing mTOR-interacting protein
DQ	Developmental Quotient
FKBP12	FK-506 binding domain
FRB	FKBP-rapamycin binding domain
GAP	GTP-ase Activating Protein
GDP	Guanine Di-Phosphate
GTP	Guanine Tri-Phosphate
IE	Intellectual equivalent
IS	Infantile Spasms
IQ	Intellectual Quotient
LAM	Lymphangioleiomyomatosis
LOH	Loss of Heterozygosity
MRI	Magnetic Resonance Image
mTORC1	Mammalian Target of Rapamycin Complex 1
NAWM	Normal Appearing White Matter
PI3K	Phosphoinositide 3-kinase
pS6	Phosphorylated Serine 6
Rheb	RAS-homolog Enriched in Brain
RML	Radial Migration Lines
SEGA	Subependymal Giant Cell Astrocytoma
SEN	Subependymal Nodule
TAND	Tuberous sclerosis complex Associated Neuropsychiatric Disorders
TSC	Tuberous Sclerosis Complex
TSC1	Tuberous Sclerosis Complex 1 (gene)
TSC2	Tuberous Sclerosis Complex 2 (gene)
TSC1-TSC2	Protein complex of *TSC1* and *TSC2*

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V LIST OF PUBLICATIONS

Mous S, Overwater IE, Vidal Gato R, Duvekot J, ten Hoopen LW, de Wit MCY, Dieleman GC. Brain pathology and quantitative autistic traits in children with Tuberous Sclerosis Complex – a clinical epidemiological study. *Manuscript in preparation*

Schreiber J, Grimbergen LA, Overwater IE, van der Vaart T, Mozaffari M, Kruit R, Stedehouder J, Kushner SA, Jaarsma D, Elgersma Y. Mechanisms underlying cognitive deficits in a mouse model for Costello syndrome are distinct from other RASopathy mouse models. *Manuscript in preparation*

Overwater IE, Verhaar BJH, Lingsma HF, Bindels-de Heus K, van den Ouweland AMW, Nellist MD, ten Hoopen LW, Moll HA, de Wit MCY. Interdependence of factors predicting cognition in children with Tuberous Sclerosis Complex. *Journal of Neurology (2017) Jan;264(1):161-167*

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VI PHD PORTFOLIO

Erasmus MC Department: Neurology
Research School: ONWAR/NIHES
PhD period: August 2011 – May 2017
Promotors: prof.dr. Y. Elgersma, prof.dr. H. A. Moll
Co-promotor: dr. M. C. Y. de Wit

1. PHD TRAINING

General academic skills	Year	Workload
BROK course: Basiscursus Regelgeving en Organisatie voor Klinisch onderzoekers	2011	1.0
CPO mini course	2011	0.5
PhD day Erasmus MC	2011	0.5
Scientific intergrity	2012	1.0
EEG reading	2013	2.0
Current issues in Clinical Neuroscience: Epilepsy	2014	2.0
Biomedical English writing	2014	4.0
BROK recertification	2015	0.5
Research skills		
Neuroscience department weekly research meeting	2011-2015	2.0
Neuroscience weekly meeting Elgersma lab	2011-2015	2.0
Neuroscience weekly journal club	2011-2015	2.0
Study design (NIHES curriculum)	2011	2.5
Biostatistical methods I: basic principles (NIHES curriculum)	2011	5.0
Repeated measurements (NIHES curriculum)	2015	1.0
Course on NONMEM: nonlinear mixed effects modeling	2015	0.5
National conferences		
ONWAR annual PhD meeting, Driebergen (oral and poster presentations)	2011-2015	2.0
Symposiums Dutch TSC patient federation, various locations (oral presentations)	2011-2015	3.0
mTOR symposium, Rotterdam (oral presentation)	2012	0.5
Symposium child- and adolescent psychiatry, Rotterdam (oral presentation)	2013	0.5
Dutch federation for pediatric neurology (NVKN), Alkmaar (oral presentation)	2014	0.5
Symposium of pediatricians specialized in genetic disorders, Eindhoven (oral presentation)	2015	0.5
Sophia research day, Rotterdam (oral presentation)	2015	0.5
International conferences		
TSC International Research Conference, Belfast	2011	1.0

Society for the Study of Behavioral Phenotypes, Leuven	2012	1.0
International TSC congress, Naples (poster presentation)	2012	1.0
International Research Conference TSC, Washington (oral presentation)	2013	1.5
European Pediatric Neurology Society, Brussels (oral presentation)	2013	1.5
International TSC Research Conference, Windsor (oral presentation)	2015	1.5
International Child Neurology Conference, Amsterdam (oral and poster presentation)	2016	1.5

2. TEACHING

Lecturing	Year	Workload
Workshops for Master of Neuroscience students	2011	0.5
Anatomy classes for medical students	2011-2015	1.0
Psychiatrists in training (lecture on synaptic plasticity)	2013-2014	0.5
Pediatric neurology minor (lecture on Tuberous Sclerosis Complex)	2013-2015	0.5
Neurologists (lecture on Tuberous Sclerosis Complex)	2015	0.5
Pediatricians (lecture on Tuberous Sclerosis Complex)	2015	0.5

Supervising theses

W. Dommisse, high school student	2013	1.0
M. Zandijk, high school student	2013	1.0
B. Verhaar, bachelor student medicine, University of Amsterdam	2014	3.0
E. van der Ende, master student medicine, Erasmus University	2014	3.0
K. Hanemaaijer, master student medicine, Erasmus University	2014	3.0

3. OTHER

Organizational committee of annual ONWAR PhD meeting	2013-2014	4.0

VII DANKWOORD

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VIII ABOUT THE AUTHOR

Iris Overwater was born on the 9th of September 1988 in Vlaardingen, The Netherlands, and raised in Maassluis. After graduating from Atheneum at Maascollege/Accent-college in Maassluis in 2006, she successfully completed the decentral selection procedure for medical school at the Erasmus Medical Center in Rotterdam. During her bachelor studies, which she finished in 2010, she also completed a master of neuroscience degree in the laboratory of professor Ype Elgersma. During this master, she investigated the effect of Hras mutations in several animal models, to discover the effect of the $Hras^{G12V}$ mutation in excitatory and inhibitory neurons. In 2011, she started a PhD project on mTORC1 inhibitor treatment of Tuberous Sclerosis Complex (TSC). This PhD project was a combined effort of the Departments of Neuroscience, Pediatrics and Neurology, and was led by prof. dr. Ype Elgersma, prof. dr. Henriëtte Moll and dr. Marie-Claire de Wit. During her PhD project, Iris led two clinical trials involving patients with Tuberous Sclerosis Complex. The first trial examined the effect of sirolimus on epilepsy in children with TSC, the second investigated the effect of everolimus on cognitive functioning in children with TSC. Besides these trials, Iris also started a database for children with TSC who visited the outpatient clinic of the ENCORE TSC expertise center.

While finishing her PhD, Iris started her internships to finish medical school. After medical school, Iris will work at the Department of Neurology of the Erasmus Medical Center in Rotterdam. She wants to become a pediatric neurologist.

Iris has a relationship with Floris. She lives in Rotterdam together with her bunnies Sammie and Mara.