A CLINICAL EVALUATION AND A GENETIC STUDY OF TUBEROUS SCLEROSIS COMPLEX IN A COHORT OF SRI LANKAN CHILDREN

BY

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Human Genetics Unit
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Declaration

I declare that the contents of this thesis are my own work, except for that detailed below, for which I would like to thank the following person:

- Ms. Ane- Marte Oye for sequencing and analysis of TSC1 and TSC2 genes.
CERTIFICATION

I certify that the contents of this dissertation are my own work and that I have acknowledged the sources where relevant.

Dr. L.D. Samudita Senaratne

This is to certify that the contents of this dissertation were supervised by the following supervisors:

Dr. Kaja Kristine Selmer
Dr. Jithangi Wanigasinghe

Dr. Dulika Sumathipala
Prof. V.H.W. Dissanayake
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Abstract

**Introduction:** Tuberous Sclerosis Complex is a heterogeneous genetic disorder with autosomal dominant inheritance that affects almost all organ systems. The disorder is characterized by development of hamartomas in almost all organ systems of the body. Other characteristic features of Tuberous Sclerosis Complex are epilepsy, intellectual disabilities, autism spectrum disorders and organ failure. Tuberous Sclerosis Complex is no more considered a rare disorder, yet no cure has been found up to date. The knowledge has expanded to understand the tumor suppressor effects of Tuberous Sclerosis Complex genes on the mTOR pathway, seeking for new treatment options for the disease. This study is the first attempt to look into the Sri Lankan genetic composition with regard to this disease.

**Objectives:** The aims of this study were to describe the clinical phenotype of the children with Tuberous Sclerosis Complex in Sri Lanka. The TSC1 and TSC2 mutations were analyzed and a genotype – phenotype correlation of infantile spasms and polycystic kidney disease has been assessed.

**Methods:** In this study, we describe 44 children including all major ethnicities in Sri Lanka. Clinical history, physical examination findings and investigation findings such as brain MRI, CT and 2D echocardiogram were documented. Sanger sequencing was done for all the exons of TSC1 and TSC2 genes as the first line of detection of point mutations and small indels. In failing to detect the above, Multiplex Ligation dependent Probe Amplification was done to look for large deletions or insertions. These two methods provide a sensitive strategy in molecular diagnosis.

**Results:** The mutation detection rate was 85.7% in this study population. There were nine patients (21.5%) with mutations detected in TSC1 gene and 27 patients (64.2%) with mutations in TSC2 gene.
**Conclusion:** Though the clinical characteristics of Tuberous Sclerosis Complex in Sri Lankan children were similar to the previously described phenotype, there were 22 novel mutations in *TSC1* and *TSC2* genes reported in this study population. Among them, fourteen mutations had a high potential of being pathogenic. The presence of renal cysts (Grade 3, 4) was seen in five patients and mutations were solely found in the Gap domain of *TSC2* gene. A total of 19 patients had a history of infantile spasms. Their mutations were documented to be predominantly in the *TSC2* gene (16/19) in the peripheral gene region (13/16). There were six patients (14.3%) with Tuberous Sclerosis Complex phenotype, but where no mutations were found in coding regions of either gene, suggesting the need for further investigation.
1. Introduction and Background

1.1. History of Tuberous Sclerosis Complex

Tuberous Sclerosis Complex (TSC [MIM 191092 and MIM 191100]) is a heterogeneous genetic disorder with autosomal dominant inheritance that affects almost all organ systems [1]. The history of TSC started 150 years ago when the German pathologist Friedrich Daniel von Ricklinghausen first described the disease in 1862 with a case report of a new born who presented with multiple heart and brain tumors and died after a short while [2]. In 1880, Magloire Bourneville, a French neurologist first characterized and coined this disease as Tuberous Sclerosis with autopsy findings in a patient with intractable seizures, mental retardation and facial angiofibromas [3]. The term “Tuberous Sclerosis” referred specifically to multiple ‘potato like’ sclerotic lesions that were found in the cerebrum. Twenty eight years later, in 1908, the German neurologist Heinrich Vogt established three major clinical criteria to diagnose TSC which is known as the “Vogt’s Triad”. This triad consists of epilepsy, mental retardation and adenoma sebaceum (facial angiofibromas). These were the first recorded crude diagnostic criteria for TSC [4]. Further exploration of the disease symptoms and signs, investigation into the cellular mechanisms that give rise to the disease and a quest for treatment has been a never ending process since then. Bourneville disease, Bourneville-Pringle disease, Tuberous Sclerosis (TS) and Tuberous Sclerosis Complex (TSC) are the different names used for the same disease [5]. At present this genetic disorder is commonly and more accurately termed Tuberous Sclerosis Complex in scientific literature in order to distinguish from Tourett’s syndrome, an unrelated neurological disorder, as well as giving the idea of the complexity of the disease, its phenotypes and its variable expressivity. Although TSC was recognized to be a genetic disease 100 years ago, the underlying molecular etiology was revealed in early 1990s with the discovery of the two causative genes, \textit{TSCI} and \textit{TSC2} [6, 7].
1.2. Genetics of Tuberous Sclerosis Complex

Tuberous Sclerosis Complex is a neurocutaneous multisystem disorder which follows an autosomal dominant pattern of inheritance; therefore a pathogenic mutation in a single allele is sufficient to cause the disease [8]. It is also interesting to note that only 30% of the patients inherit the disease from either of their parents, while the remaining 70% is due to de novo mutations in TSC1 or TSC2 [9]. These de novo mutations are assumed to occur in the germ cells of either parent [10]. Theoretically, it can also occur as sporadic mutation in somatic cells of the early embryo. Germ line
mosaicism is also reported in families where parents without clinical manifestations have more than one child affected with TSC [8].

In autosomal dominant pattern of inheritance, the disease phenotype will be exhibited with a mutation in heterozygote state in an autosome (non sex chromosome). Therefore a parent with a mutant allele carries a risk of transmitting the disease allele to 50% of his or her offspring, irrespective of the gender. I.e. with each pregnancy there is a 50% risk of having an affected child. However, if a child is born of healthy parents who do not have detectable mutation in DNA from peripheral blood, the recurrence risk of having another child with TSC is considered to 1% to 2%. This small recurrence risk is due to germ line mosaicism. For the other relatives of the proband, depending on whether the parents carry the disease causing mutation and the degree of relationship, the risk varies [11]. Variable expressivity is the variable extent and intensity of phenotypic signs among people with a given genotype [12]. TSC is a very good example for variable expressivity. Variable expressivity results in great variation in clinical phenotype, ranging from single skin lesion in one member of the family to intractable seizures in another member [8]. Hence, expressivity is not determined by the specific gene mutation as different manifestations can be seen in members of the same family. Within the same family or among different families, there can be hundreds of phenotypic variants.

In some individuals disease manifestations are present at birth, but in others they might appear later in life. Age of onset does not seem to corelate with the severity of the disease [13]. The most common manifestations are the benign tumors in skin, brain, kidney, heart, eyes and lung. These may lead to organ dysfunction as the normal parenchyma is disrupted by tumor cells [2]. A range of neurobehavioral phenotypes with variable expression are associated with TSC and commonly seen are seizures, mental retardation, learning disabilities, attention deficit hyperactivity and autism [8]. TSC is typically a disease which
demonstrates pleiotropy, as this disease depicts a variety of apparently unrelated phenotypic features including skin hypopigmentation, multiple hamartomas in different organs, learning disability and multiple neurobehavioral phenotypes [14].

Penetrance is the frequency with which a genotype manifests itself in a given phenotype [12]. TSC is an autosomal dominant pleiotropic disorder with 100% or full penetrance [15]. Therefore, if the disease causing mutation is present in an individual, that will give rise to a disease phenotype even as a mild condition which can be clinically missed or undiagnosed. However, rare instances of non penetrance and reduced penetrance were reported in mid 1980s and early 1990s, but were later shown to be cases of germline mosaicism or occurrence of two disease causing mutations [16-18].

In some disease conditions, the age of onset becomes lower in successive generations while the disease severity increases. This phenomenon is called the anticipation [12]. This phenomenon is commonly seen with genetically transmitted diseases with triplet repeat expansions. Although the parents with a milder phenotype transmitting the diseased allele to a more severely affected offspring is commonly noted in TSC, anticipation is not reported [11].

Great improvement in the understanding of the underlying pathomechanism was made with the discovery of the two disease causative genes TSC2 in 1993 and TSC1 in 1997 respectively. TSC1 consists of a 3.4-kb coding region with 21 coding exons out of the total of 23 exons on 9q34 and TSC2 consists of 5.4-kb coding region with 41 exons on 16p13.3 [19]. At present there are more than 1000 pathological mutations reported in TSC1 and TSC2 [8]. TSC1 encodes the protein Hamartin and TSC2 encodes the protein Tuberin. Hamartin and Tuberin interact directly to form a cytosolic complex and both TSC1 and TSC2 genes act as tumor suppressor genes [9]. According to previous studies, mutation detection rate in TSC1
and TSC2 is approximately 85% to 89%. In the remaining 11% to 15% of the patients, no mutation is identified in these two genes, even if the clinical phenotype depicts TSC [2, 20]. Among all the mutations, TSC1 mutations are found in 20% of the patients while the remaining 80% is accounted by TSC2 mutations [21]. TSC1 mutations as well as the familial TSC2 mutations are less severe when compared to de novo TSC2 mutations. Patients with no mutations in either genes are in some studies reported to have less severe phenotype than the patients with either TSC1 or TSC2 mutations [22]. Table 1 summarises the characteristics of the TSC1 and TSC2 genes [8, 11].

**TABLE 1: Characteristics of the TSC1 and TSC2 genes[8, 11]**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>TSC1</th>
<th>TSC2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Localization</td>
<td>9q34</td>
<td>16p13.3</td>
</tr>
<tr>
<td>Structure</td>
<td>23 exons—8.6 kb transcript alternate splicing in the 5' UTR</td>
<td>41 exons—5.5 kb transcript exons 25, 26 and 31 alternatively spliced</td>
</tr>
<tr>
<td>Mutations</td>
<td>Small truncating mutations</td>
<td>Large deletions/rearrangements</td>
</tr>
<tr>
<td></td>
<td>Missense mutations indels</td>
<td>small truncating mutations</td>
</tr>
<tr>
<td></td>
<td></td>
<td>missense mutations</td>
</tr>
<tr>
<td>Occurrence</td>
<td>10%-15% of sporadic cases</td>
<td>70% of sporadic cases</td>
</tr>
<tr>
<td></td>
<td>30% of familial cases</td>
<td>50% of familial cases</td>
</tr>
<tr>
<td>LOH in hamartomas</td>
<td>Rare</td>
<td>Frequent</td>
</tr>
<tr>
<td>Product</td>
<td>Hamartin</td>
<td>Tuberin</td>
</tr>
<tr>
<td>Subcellular localization</td>
<td>Cytoplasmic,? cortical</td>
<td>Cytoplasmic,? Golgi associated</td>
</tr>
<tr>
<td>Animal models</td>
<td>Knockout mice under development</td>
<td>Eker Rat Knockout mice, Drosophila (gigas)</td>
</tr>
</tbody>
</table>


### 1.3. Basic Disease Mechanism

The mammalian target of rapamycin (mTOR) is an evolutionarily conserved serine-threonine kinase. This kinase is known to sense the environmental and cellular nutrition and energy status. Growth factors, nutrients and mitogens stimulate the activation of the two mTOR complexes, mTOR1 and mTOR2 to regulate diverse functions such as cell growth, development, proliferation, angiogenesis, memory, longevity, autophagy and innate as well as adaptive immune responses [23].
*TSC1* and *TSC2* genes encode the proteins Hamartin and Tuberin, respectively. These two proteins form a complex in the cytoplasm and modulate cell functions such as cell growth, differentiation, migration and proliferation via mammalian target of rapamycin (mTOR) signaling cascade [24]. Figure 3 demonstrate the role of *TSC1*-TSC2 complex in the mTOR signaling pathway [25].

Fig 3: Place of *TSC1*-TSC2 complex in mTOR signaling pathway (with kind courtesy of Malgorzata Urbariska, MSc, International Institute of Molecular and Cell Biology, Warsaw, Poland)

The hamartin-tuberin protein complex does not act directly on mTOR. This complex acts as a tumor suppressor by activating GTP- binding protein Ras homolog enriched in brain (Rheb). This small G protein Rheb inhibits the mammalian target of rapamycin (mTOR) [26]. Co-factor of mTORC1 is the regulatory associated protein of mTOR (Raptor). Raptor activates the protein kinase domain of mTOR and thereby increases the mRNA transcription and protein synthesis. Mutation in either the *TSC1* or *TSC2* gene will inactivate the tumor suppressor action of the hamartin-tuberin complex, and thereby will also the inhibitory action of Rheb be. This will result in abnormal cell proliferation, and thereby formation of
nonmalignant tumors, the hamartomas. Rapamycin insensitive component of mTOR (Rictor) is the co-factor of mTORC2 complex which regulate protein synthesis in a manner different to mTORC1 and is unaffected by Rheb [27].

The molecular mechanisms in TSC reveal that the hamartomas can develop in almost all body systems by constitutive activation of the protein kinase mTOR due to haplo-insufficiency of either of the TSC1 or TSC2 gene [27]. The most commonly affected organ is the skin followed by the brain, kidney, heart and lung. Less commonly affected are the eye, liver, gut, bone, mouth and teeth.

1.4. Affected organ systems

**Skin:** Skin is the most commonly affected organ in TSC and more than 96% of the patients are found to have skin manifestations [28]. Table 2 summaries the common skin manifestations in TSC [2, 3, 28].
<table>
<thead>
<tr>
<th>Skin manifestation</th>
<th>Prevalence</th>
<th>Age of presentation</th>
<th>Diagnostic importance</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypopigmented patches</td>
<td>&gt;90%</td>
<td>Birth, infancy, childhood</td>
<td>Major diagnostic criterion</td>
<td>Presenting sign in TSC. Number increases with age</td>
</tr>
<tr>
<td>Facial angiofibromas (FA)</td>
<td>75%</td>
<td>2-5 years</td>
<td>Major diagnostic criterion, when =/&gt; 3</td>
<td>Presentation in adulthood taken as minor diagnostic criterion after confirmation with skin biopsy</td>
</tr>
<tr>
<td>Fibrous cephalic plaques</td>
<td>25%</td>
<td>2-5 years</td>
<td>Major diagnostic criterion, when =/&gt; 3</td>
<td>Most specific skin lesion. Histologically similar to FAs. Paired with FA in diagnosis</td>
</tr>
<tr>
<td>Shagreen patches</td>
<td>50%</td>
<td>1st decade of life</td>
<td>Major diagnostic criterion</td>
<td>Large plaques on lower back with bumpy or orange peel appearance. Nearly pathognomonic to TSC</td>
</tr>
<tr>
<td>Ungual fibromas</td>
<td>&lt; 20% (overall) 80% (old population)</td>
<td>2nd decade of life</td>
<td>Major diagnostic criterion, when =/&gt; 2.</td>
<td>&gt;80% noted in older adults</td>
</tr>
<tr>
<td>Confetti skin lesions</td>
<td>3% (paediatric population) 58% (overall)</td>
<td>1st decade of life</td>
<td>Minor diagnostic criterion</td>
<td>1-3mm hypopigmented maculae. Commonly scattered over arms and legs</td>
</tr>
<tr>
<td>Café au lait patches</td>
<td>30%</td>
<td>Birth, infancy, childhood</td>
<td>Not a diagnostic criterion</td>
<td>Hyperpigmented maculae.</td>
</tr>
</tbody>
</table>

=/>: equal or more
Brain: The greatest morbidity and mortality in TSC is associated with brain manifestations. TSC associated structural abnormalities of the brain, epilepsy and neuropsychiatric disorders are the three major phenotypes associated with the central nervous system [2].

Cortical tubers, a form of focal cortical dysplasias, are the hallmark of TSC. Histologically they consist of giant neurons and astrocytes [27]. Cortical dysplasia are congenital abnormalities where a group of neurons fail to migrate to the expected area of the brain during embryonic development [2]. Cortical tubers are found in 90% of the patients with TSC by neuro imaging such as Magnetic Resonance Image (MRI). Other forms of cortical dysplasia such as cerebral white matter radial migration lines can be found together with cortical tubers in TSC and when there are two or more areas of cortical dysplasia, it is considered as a major diagnostic criterion. Cortical dysplasias are commonly associated with intractable epilepsy and learning difficulties in TSC patients [2].
Other structural manifestations of the brain are the subependymal nodules (SEN) and the subependymal giant cell astrocytomas (SEGA). SEN can be detected in 80% of the patients with TSC prenatally or at birth. They are non-malignant tumors with slow progression which develop along the wall of the ependymal lining of lateral and third ventricles [2].

SEGAs are histologically similar to SEN and incidence in TSC is 5-15%. They can be present prenatally or at birth, but are much more likely to arise during childhood or adolescence. They seldom occur after 20 years. It is accepted that SEGA arises from SEN near the foramen of Monro. Although they are slow growing they can lead to critical situations where urgent surgical resection is necessary as they might obstruct foramen of Monro, leading to high intracranial pressure. Both SEN and SEGA may progressively calcify and stop growing [2, 27].

According to recent studies, 90% of the patients with TSC have epilepsy [29]. Common types of seizures are the infantile spasms (IS), which occur in 30 – 40% of the patients, and partial seizures. Partial seizures can progress in to generalized tonic clonic seizures due to rapid bilateral synchronization [27]. Several studies show early onset of infantile spasms is associated with poorer prognosis with developmental delay, autism and intractable partial epilepsies later in life [27]. In TSC most of the patients with IS do not depict the typical electro encephalogram (EEG) pattern with hypsarrythmia and therefore the diagnosis of IS in TSC patients can be masked [29]. Hence it is advisable to treat IS in TSC purely on clinical grounds independent of EEG. For patients with medically intractable partial epilepsies, a remedy is soughed with methods such as the ketogenic diet, vagal nerve stimulation and respective epilepsy surgery [27].

Among the patients with TSC, 50-60% has neuropsychiatric disorders such as autism, mental retardation, attention deficit hyperactivity, learning disabilities and other behavioural problems.
Kidney: Renal complications are the second most common cause of morbidity and mortality [30] and renal angiomyolipomas are present in 75% - 80% of the patients with TSC [3]. Angiomyolipomas are benign tumors composed of thickened blood vessels, adipose tissue and smooth muscle and they are relatively specific to TSC. Two or more angiomyolipomas in any organ is considered as a major diagnostic criterion in TSC and the most common organ affected is the kidney [2]. The renal manifestations in TSC begin in infancy and progress with age. The rapid growth of angiomyolipomas are noted during childhood and adolescence and slow down towards adulthood [27]. Females are more commonly affected, with a female to male ratio of 4:1 [3]. The smaller angiomyolipomas are said to be asymptomatic, but when the lesions exceed 4cm in size, they can give rise to life threatening complications such as haemorrhage, acute renal failure or shock [3]. Lesions greater than 4cm are usually found in post pubertal patients [27]. The second most common lesions found in the kidney are the renal cysts which are present in 45% of the patients with TSC [27]. The combination of renal cysts and angiomyolipomas is characteristic of TSC [31]. Renal micro-cysts can be present in patients with both TSC1 and TSC2 mutations and appear earlier in life than the angiomyolipomas [3]. Severe polycystic kidneys appears only in
patients with TSC2 gene deletions that gives rise to contagious gene syndrome involving the PKD1 gene deletions [32]. PKD1 gene is transcribed in the opposite direction to TSC2 gene and is only 48 base pairs away from TSC2 gene on chromosome 16.3 [2]. This contagious deletion is reported in 2% of the patients with TSC [27] and gives rise to TSC phenotype combined with a more aggressive autosomal dominant polycystic kidney disease (ADPKD) presenting during infancy and childhood [2].

![Renal MRI scan with bilateral renal cysts and Renal Ultrasound Scan right kidney with angiomyolipomas](image)

Fig 6: Renal involvements in TSC (with permission from Herscot Center for TSC at Massachusetts General Hospital, Boston, Massachusetts, USA)

**Lung:** Lymphangiomyomatosis (LAM) is a late presentation in TSC and appear in 2\textsuperscript{nd} or 3\textsuperscript{rd} decade of life nearly exclusively in females with TSC. However, some male TSC cases have been reported to have LAM findings in biopsy material [27]. LAM is the most common lung manifestation in TSC and is observed in 30-40\% of the female patients in child bearing age, though only 1\% of them are found to be symptomatic [2, 3]. Histologically, LAM is diffuse infiltration of lung tissue with atypical smooth muscle cells. Gradual replacement with cysts in pulmonary parenchyma occur at a later stage [27]. As LAM affects almost exclusively females, there is strong evidence of a hormonal component being involved in the pathomechanism [32]. LAM is the third most common cause of mortality in TSC and is a
major diagnostic criterion in TSC. Presence of LAM without TSC is classified as sporadic LAM. Angiomyolipoma is a finding in one third of the patients with sporadic LAM. Therefore when both LAM and angiomyolipomas are present in a patient with TSC, it is considered as one major diagnostic criterion rather than two [2].

Fig 7: MRI scans of lungs affected with LAM. (The images are from the National Heart, Lung, and Blood Institute (NHLBI) at the National Institutes of Health (NIH) in Bethesda, MD, USA.)

Heart: Cardiac rhabdomyomas (CR) are present in approximately 50% of all patients with TSC and are usually congenital as they develop between 22 to 26 weeks of gestation [3]. CRs are mostly asymptomatic and can be a finding in routine checkups. It might also present as a cardiac murmur, a rhythm disturbance, the Wolff-Parkinson-White syndrome or as cyanosis. In general CRs are multiple, well circumscribed hyperechogenic masses frequently located in interventricular septum or the free wall of the heart [33]. These solid tumors regress spontaneously and disappear completely or partially within infancy to early childhood in more than 50% of all cases [34]. The ones that cause serious cardiovascular complications such as outflow tract obstruction, reduced cardiac function or rhythm disturbances may need surgical or medical intervention [35].
Eye: Astrocytic hamartoma (AH) is a characteristic retinal finding and is also considered as a major criterion of diagnosing TSC. They commonly present as multiple lesions, and are histologically similar to cortical tubers in the brain [2]. As a AH usually does not cause visual impairment, it rarely require intervention and is a good marker of TSC. Eye manifestations are found in approximately 35% of the patients with TSC. Retinal Achromic Patches(AP) might also be observed but, are less common and are considered as a minor criterion [36].

Fig 8: 2D Echocardiogram of Multiple CRs in a patient with TSC (With kind complement of Dr. Duminda Samarasinghe, Consultant Paediatric Cardiologist- Lady Ridgeway Hospital for Children, Colombo, Sri Lanka)

Fig 9: Image of a retinal hamartoma in a patient affected with TSC (with permission from Herscot Center for TSC at Massachusetts General Hospital, Boston, Massachusetts, USA)
Mouth and Teeth: Intra oral fibromas (when there are two or more) and dental enamel pits (when there are three or more) are considered as minor diagnostic criteria in TSC [2]. Intra oral fibromas are said to be present in 20 – 50% of the patients with TSC, but with a higher frequency in adulthood [28, 37]. Dental enamel pits are significantly more common among adult TSC patients when compared to the normal healthy adults [38]. Prevalence among the paediatric population is not known.

![Dental enamel pit](image1)

![Intra oral fibroma](image2)

Fig 10: Minor diagnostic criteria associated with mouth & teeth. (These photos are courtesy of Greg Mlynarczyk, DDS, a member of the TS Alliance Professional Advisory Board.)

Other clinical, but less frequent characteristics of TSC include lesions in bone, in gastrointestinal system and in endocrine organs [2]. Bone cysts are less frequently seen in TSC and are not specific to the disease. They are rarely identified when the other features of TSC are absent, and are therefore no more considered as a diagnostic criterion. The same is true for the hamartomatous rectal polyps. Liver angiomiolipomas are acknowledged as nonrenal angiomylipomas [2].
1.5. Clinical Aspects of TSC

TSC is a neurocutaneous disorder that affects almost all body systems. There are many neurocognitive and neurobehavioral phenotypes also associated with TSC. Therefore a multi disciplinary approach is required in treating patients with TSC. This multi disciplinary effort includes the neurologist, dermatologist, nephrologists, ophthalmologist, cardiologist, pulmonologist, psychiatrist, radiologist, dentist and surgeons of various disciplines. Geneticists and genetic counselors also play a major role with the discovery of genetic mutations and alterations in molecular mechanisms related to disease causation. At the 2012 International Tuberous Sclerosis Consensus Conference, the diagnostic criteria for TSC were updated. Clinical features of TSC continues to be the principal means of diagnosis while changes compared to 1998 criteria are the inclusion of genetic test results and reducing diagnostic classes from three (possible, probable, definite) to two (possible and definite) [2].

Table 3 shows the updated diagnostic criteria for TSC in 2012 at the International Tuberous Sclerosis Consensus Conference. It mainly consists of 2 parts, genetic diagnostic criteria and clinical diagnostic criteria. Clinical criteria are again subdivided into major and minor criteria. It emphasizes that diagnosing a pathological mutation in either TSC1 or TSC2 genes is sufficient to diagnose TSC [2].

Testing for mutations in TSC1 and TSC2 genes is not only important in diagnosis of TSC, but it is also important in family screening, risk assessment and counseling according to the genetic makeup of each and every family member.
TABLE 3: Updated diagnostic criteria for tuberous sclerosis complex 2012 [2].

A. Genetic diagnostic criteria
The identification of either a TSC1 or TSC2 pathogenic mutation in DNA from normal tissue is sufficient to make a definite diagnosis of tuberous sclerosis complex (TSC).
A pathogenic mutation is defined as a mutation that clearly inactivates the function of the TSC1 or TSC2 proteins (e.g., out-of-frame indel or nonsense mutation), prevents protein synthesis (e.g., large genomic deletion), or is a missense mutation whose effect on protein function has been established by functional assessment (www.lovd.nl/TSC1, www.lovd/TSC2, and Hoogeveen-Westerveld et al., 2012 and 2013).
Other TSC1 or TSC2 variants whose effect on function is less certain do not meet these criteria, and are not sufficient to make a definite diagnosis of TSC. Note that 10% to 25% of TSC patients have no mutation identified by conventional genetic testing, and a normal result does not exclude TSC, or have any effect on the use of clinical diagnostic criteria to diagnose TSC.

B. Clinical diagnostic criteria

Major features
1. Hypomelanotic macules (≥3, at least 5-mm diameter)
2. Angiofibromas (≥3) or fibrous cephalic plaque
3. Ungual fibromas (≥2)
4. Shagreen patch
5. Multiple retinal hamartomas
6. Cortical dysplasias*
7. Subependymal nodules
8. Subependymal giant cell astrocytoma
9. Cardiac rhabdomyoma
10. Lymphangioleiomyomatosis (LAM) y
11. Angiomyolipomas (≥2)y

Minor features
1. “Confetti” skin lesions
2. Dental enamel pits (>3)
3. Intraoral fibromas (>2)
4. Retinal achromic patch
5. Multiple renal cysts
6. Nonrenal hamartomas

Definite diagnosis: Two major features or one major feature with ≥2 minor features
Possible diagnosis: Either one major feature or ≥2 minor features
* Includes tubers and cerebral white matter radial migration lines.
y A combination of the two major clinical features (LAM and angiomyolipomas) without other features does not meet criteria for a definite diagnosis

Once the diagnosis of TSC has been established according to the above mentioned guidelines, it is important to manage and follow up the patients according to the standard protocols. Due to the diverse and varied presentation and progression of disease manifestations management plan will be exclusive to each and every patient. Depending on the availability of resources and the other practicalities management practices are highly variable among region and
Table 4 & 5 shows the standardized approach for clinical management of patients with TSC which was reviewed and recommended at the 2012 International Tuberous Sclerosis Consensus Conference. These recommendations are applicable for the newly diagnosed patients as well as the patients with established diagnosis [1].

**TABLE 4: Management recommendations for the newly diagnosed and suspected TSC [1].**

<table>
<thead>
<tr>
<th>Organ System or Specialty Area</th>
<th>Recommendation</th>
</tr>
</thead>
</table>
| Genetics                      | - Obtain three-generation family history to assess for additional family members at risk of TSC  
- Offer genetic testing for family counseling or when TSC diagnosis is in question but cannot be clinically confirmed |
| Brain                         | - Perform magnetic resonance imaging (MRI) of the brain to assess for the presence of tubers, subependymal nodules (SEN), migrational defects, and subependymal giant cell astrocytoma (SEGA)  
- Evaluate for TSC-associated neuropsychiatric disorder (TAND)  
- During infancy, educate parents to recognize infantile spasms, even if none have occurred at time of first diagnosis  
- Obtain baseline routine electroencephalogram (EEG). If abnormal, especially if features of TAND are also present, follow-up with a 24-hr video EEG to assess for subclinical seizure activity |
| Kidney                        | - Obtain MRI of the abdomen to assess for the presence of angiomyolipoma and renal cysts  
- Screen for hypertension by obtaining an accurate blood pressure  
- Evaluate renal function by determination of glomerular filtration rate (GFR) |
| Lung                          | - Perform baseline pulmonary function testing (pulmonary function testing and 6-minute walk test) and high-resolution chest computed tomography (HRCT), even if asymptomatic, in patients at risk of developing lymphangioleiomyomatosis (LAM), typically females 18 years or older. Adult males, if symptomatic, should also undergo testing  
- Provide counsel on smoking risks and estrogen use in adolescent and adult females |
| Skin                          | - Perform a detailed clinical dermatologic inspection/exam |
| Teeth                         | - Perform a detailed clinical dental inspection/exam |
| Heart                         | - Consider fetal echocardiography to detect individuals with high risk of heart failure after delivery when rhabdomyomas are identified via prenatal ultrasound  
- Obtain an echocardiogram in pediatric patients, especially if younger than 3 yr of age  
- Obtain an electrocardiogram (ECG) in all ages to assess for underlying conduction defects |
| Eye                           | - Perform a complete ophthalmologic evaluation, including dilated funduscopy, to assess for retinal lesions and visual field deficits |
TABLE 5: Management recommendations for patients already diagnosed with tuberous sclerosis complex (TSC) [1].

<table>
<thead>
<tr>
<th>Organ System or Specialty Area</th>
<th>Recommendation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genetics</td>
<td>Offer genetic testing and family counseling, if not done previously, in individuals of reproductive age or newly considering having children</td>
</tr>
</tbody>
</table>
| Brain                          | Obtain magnetic resonance imaging (MRI) of the brain every 1-3 yr in asymptomatic TSC patients younger than age 25 yr to monitor for new occurrence of subependymal giant cell astrocytoma (SEGA). Patients with large or growing SEGA, or with SEGA causing ventricular enlargement but yet are still asymptomatic, should undergo MRI scans more frequently and the patients and their families should be educated regarding the potential of new symptoms. Patients with asymptomatic SEGA in childhood should continue to be imaged periodically as adults to ensure there is no growth.  
- Surgical resection should be performed for acutely symptomatic SEGA. Cerebral spinal fluid diversion (shunt) may also be necessary. Either surgical resection or medical treatment with mammalian target of rapamycin complex (mTOR) inhibitors may be used for growing but otherwise asymptomatic SEGA. In determining the best treatment option, discussion of the complication risks, adverse effects, cost, length of treatment, and potential impact on TSC-associated comorbidities should be included in the decision-making process.  
- Perform screening for TSC-associated neuropsychiatric disorders (TAND) features at least annually at each clinical visit. Perform comprehensive formal evaluation for TAND at key developmental time points: infancy (0-3 yr), preschool (3-6 yr), pre-middle school (6-9 yr), adolescence (12-16 yr), early adulthood (18-25 yr), and as needed thereafter. Management strategies should be based on the TAND profile of each patient and should be based on evidence-based good practice guidelines/practice parameters for individual disorders (e.g., autism spectrum disorder, attention deficit hyperactivity disorder, anxiety disorder). Always consider the need for an individual educational program (IEP). Sudden change in behavior should prompt medical/clinical evaluation to look at potential medical causes (e.g., SEGA, seizures, renal disease).  
- Obtain routine electroencephalograph (EEG) in individuals with known or suspected seizure activity. The frequency of routine EEG should be determined by clinical need rather than a specific defined interval. Prolonged video EEG, 24 hr or longer, is appropriate when seizure occurrence is unclear or when unexplained sleep, behavioral changes, or other alteration in cognitive or neurological function is present.  
- Vigabatrin is the recommended first-line therapy for infantile spasms. Adrenocorticotropic hormone (ACTH) can be used if treatment with vigabatrin is unsuccessful. Anticonvulsant therapy of other seizure types in TSC should generally follow that of other epilepsies. Epilepsy surgery should be considered for medically refractory TSC patients, but special consideration should be given to children at younger ages experiencing neurological regression and is best if performed at epilepsy centers with experience and expertise in TSC. |
| Kidney                         | Obtain MRI of the abdomen to assess for the progression of angiomyolipoma and renal cystic disease every 1-3 yr throughout the lifetime of the patient. |
- Assess renal function (including determination of glomerular filtration rate [GFR]) and blood pressure at least annually.
- Embolization followed by corticosteroids is first-line therapy for angiomyolipoma presenting with acute hemorrhage. Nephrectomy is to be avoided. For asymptomatic, growing angiomyolipoma measuring larger than 3 cm in diameter, treatment with an mTOR inhibitor is the recommended first-line therapy. Selective embolization or kidney-sparing resection are acceptable second-line therapy for asymptomatic angiomyolipoma.

### Lung
- Perform clinical screening for lymphangioleiomyomatosis (LAM) symptoms, including exertional dyspnea and shortness of breath, at each clinic visit. Counseling regarding smoking risk and estrogen use should be reviewed at each clinic visit for individuals at risk of LAM.
- Obtain high-resolution computed tomography (HRCT) every 5-10 yr in asymptomatic individuals at risk of LAM if there is no evidence of lung cysts on their baseline HRCT. Individuals with lung cysts detected on HRCT should have annual pulmonary function testing (pulmonary function testing and 6-min walk) and HRCT interval reduced to every 2-3 yr.
- mTOR inhibitors may be used to treat LAM patients with moderate to severe lung disease or rapid progression. TSC patients with LAM are candidates for lung transplantation but TSC comorbidities may impact transplant suitability.

### Skin
- Perform a detailed clinical dermatologic inspection/exam annually.
- Rapidly changing, disfiguring, or symptomatic TSC-associated skin lesions should be treated as appropriate for the lesion and clinical context, using approaches such as surgical excision, laser(s), or possibly topical mTOR inhibitor.

### Teeth
- Perform a detailed clinical dental inspection/exam at minimum every 6 months and panoramic radiographs by age 7 yr, if not performed previously.
- Symptomatic or deforming dental lesions, oral fibromas, and bony jaw lesions should be treated with surgical excision or curettage when present.

### Heart
- Obtain an echocardiogram every 1-3 yr in asymptomatic pediatric patients until regression of cardiac rhabdomyomas is documented. More frequent or advanced diagnostic assessment may be required for symptomatic patients.
- Obtain electrocardiogram (ECG) every 3-5 yr in asymptomatic patients of all ages to monitor for conduction defects. More frequent or advanced diagnostic assessment such as ambulatory and event monitoring may be required for symptomatic patients.

### Eye
- Perform annual opthalmologic evaluation in patients with previously identified opthalmologic lesions or vision symptoms at the baseline evaluation. More frequent assessment, including those treated with vigabatrin, is of limited benefit and not recommended unless new clinical concerns arise.
TABLE 6: Presenting time of Clinical Manifestations [2, 32, 39-41]

<table>
<thead>
<tr>
<th>Clinical Feature</th>
<th>Time of onset</th>
</tr>
</thead>
<tbody>
<tr>
<td>Present at birth</td>
<td>Hypopigmented macules</td>
</tr>
<tr>
<td></td>
<td>Cortical tubers</td>
</tr>
<tr>
<td></td>
<td>SEN</td>
</tr>
<tr>
<td></td>
<td>SEGA</td>
</tr>
<tr>
<td></td>
<td>Cardiac Rhabdomyomas</td>
</tr>
<tr>
<td></td>
<td>Renal cysts</td>
</tr>
<tr>
<td>Late onset</td>
<td>Facial angiofibromas</td>
</tr>
<tr>
<td></td>
<td>Fibrous cephalic plaques</td>
</tr>
<tr>
<td></td>
<td>Shagreen patches</td>
</tr>
<tr>
<td></td>
<td>Confetti lesions</td>
</tr>
<tr>
<td></td>
<td>Renal angiomyolipomas</td>
</tr>
<tr>
<td></td>
<td>Retinal hamartomas</td>
</tr>
<tr>
<td></td>
<td>Dental pits</td>
</tr>
<tr>
<td></td>
<td>LAM</td>
</tr>
</tbody>
</table>

TABLE 7: Clinical Manifestations by age [41]

<table>
<thead>
<tr>
<th>Presenting period of life</th>
<th>Clinical presentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetal period</td>
<td>Seizures</td>
</tr>
<tr>
<td></td>
<td>Cardiac arrhythmias</td>
</tr>
<tr>
<td>Neonatal period</td>
<td>Wolf-Parkinson-White syndrome – on ECG</td>
</tr>
<tr>
<td></td>
<td>Hydrops fetalis</td>
</tr>
<tr>
<td></td>
<td>Hypomelanotic macules</td>
</tr>
<tr>
<td></td>
<td>Multiple renal cysts</td>
</tr>
<tr>
<td></td>
<td>Cardiac rhabdomyomas</td>
</tr>
<tr>
<td>Infancy</td>
<td>Infantile spasms</td>
</tr>
<tr>
<td></td>
<td>Retinal hamartomas</td>
</tr>
<tr>
<td></td>
<td>Hypomelanotic macules</td>
</tr>
<tr>
<td></td>
<td>Developmental delay</td>
</tr>
<tr>
<td>Early childhood</td>
<td>Autism</td>
</tr>
<tr>
<td></td>
<td>Seizures</td>
</tr>
<tr>
<td></td>
<td>Hypomelanotic macules</td>
</tr>
<tr>
<td>Late childhood</td>
<td>SEGA</td>
</tr>
<tr>
<td></td>
<td>Facial angiofibroma</td>
</tr>
<tr>
<td></td>
<td>Ungual fibroma</td>
</tr>
<tr>
<td>Adolescence</td>
<td>Shagreen patches</td>
</tr>
<tr>
<td>Adult</td>
<td>LAM, Renal angiomyolipoma</td>
</tr>
</tbody>
</table>

1.6. Phenotypic differences between TSC1 and TSC2

Locus heterogeneity is a phenomenon where a disorder may due to mutations in more than one gene or locus [42]. When different mutations within the same gene at the same chromosome locus give rise to a single disease phenotype, it is defined as the allelic heterogeneity [42]. TSC has locus heterogeneity as TSC1 at 9q34 and TSC2 at 16p13.3
rise to the same disease. It is also an example for allelic heterogeneity as there are many gene mutations giving rise to the phenotype.

It is clear that there are overlapping clinical features among patients with \textit{TSC1} mutations and \textit{TSC2} mutations, but patients with \textit{TSC1} mutations depicts a significantly less severe phenotype when compared to patients with \textit{TSC2} mutations [19]. Some phenotypes such as grade 2-4 polycystic kidneys and angiomyolipomas, liver angiomyolipomas, retinal hamartomas and forehead plaques are virtually not seen in patients with mutations in the \textit{TSC1} gene [19]. According to recent studies, patients with \textit{TSC1} mutations have lower seizure frequencies, lower percentage of severe mental retardation, smaller number of subependymal nodules and cortical tubers and a lesser frequency of facial angiofibromas when compared to patients with \textit{TSC2} mutations [8]. \textit{TSC2} mutations are associated with early presentation of epilepsy, mainly infantile spasms [43], but recent studies report that missense mutations located in exon 23 to 33 of \textit{TSC2} are associated with significantly less infantile spasms [29]. It is documented that the earlier the onset of seizures and the presence of infantile spasms, the poorer the prognosis. This group of patients are commonly reported with antiepileptic drug resistance, moderate to severe mental retardation and neuro cognitive phenotypes such as autism spectrum disorders which are significant in \textit{TSC2} mutations [44].

Cystic kidneys can be present in patients with both \textit{TSC1} and \textit{TSC2} mutations. The more severe, early onset polycystic kidney disease results due to a contagious gene micro deletion syndrome resulting from a deletion in \textit{TSC2} gene along with deletions in the adjoining \textit{PKD1} gene [27]. It is also noted that symptomatic LAM is more frequently associated with \textit{TSC2} mutations [32].

Among familial cases almost equal distribution between \textit{TSC1} and \textit{TSC2} can be seen while among sporadic cases \textit{TSC2} mutations are about five times common than \textit{TSC1} mutations [8]. \textit{TSC1} mutations as well as the familial \textit{TSC2} mutations are less severe in phenotype when
compared to *de novo TSC2* mutations. It is suggested this is due to more offspring in less severely affected patients with *TSC1* mutations [22]. Patients with no mutations in either gene had less severe phenotype than both *TSC1* and *TSC2* mutations [22]. In a study it was found that intellectual disability was more frequent in patients with *TSC2* mutations when compared to patients with *TSC1* mutations [45].

A broad spectrum of mutations has been identified in both genes varying from point mutations to large deletions, duplications and insertions. The mutations in *TSC1* are mostly point mutations such as nonsense mutations, small insertions and small deletions resulting in truncated proteins or inactivation of the protein synthesis. The mutation spectrum of *TSC2* comprises large deletions and rearrangements, small insertions and small deletions as well as point mutations [22].

1.7. **Common mutation types in TSC**

**Nonsense mutation** is a change in a single nucleotide that changes a single amino acid which eventually results a stop codon, resulting a truncated protein, usually an incomplete or a nonfunctional protein [42].

![DNA and Protein Diagram](Fig11.png)

Fig 11: Replacement of cytosine by thymine has created a stop codon TGA result a truncated protein

C= Cytosine, T= Thymine, A= Adenine, G= Guanine
Missense mutation is a change in a single nucleotide that changes only a single amino acid which changes the protein and the protein may or may not become nonfunctional due to the substitution of the different amino acid. Missense mutations account for more than half of all mutations [42].

Fig 13: Replacement of adenine by cytosine changes the amino acid from Histidine to Proline, but does not result in a truncated protein
Fig 14: Sanger sequencing TSC2 Exon 38 c.5024C>T missense mutation

**Splice site mutation** is a DNA change at a splicing site. During DNA transcription, intronic regions of DNA are spliced and only the exonic regions are present during the process of resulting mature messenger RNA (mRNA) from precursor mRNA. An insertion or a deletion of several nucleotides that disturbs a splice site may result in an incomplete splicing leading to aberrant protein product.
**In-frame mutation** is a mutation that does not change the triplet reading frame. Reading frame is a sequence of mRNA that translates into an amino acid chain, three bases (codon) at a time. A mutation that does not cause a shift in the triplet reading frame, but insertion/deletion of few amino acids either three or its multiples can change codons and there by the amino acid sequence resulting abnormal protein products.

**Frame shift mutation** changes the coding frame by insertion or deletion of several nucleotides that are not multiples of three, eventually disturbs the codons resulting in a completely different translation, thereby a completely different protein. Most frame shift mutations result in a premature stop codon downstream to mutation [42].
Fig 16: Sanger sequencing TSC2 Exon 24 c.2764_2765 deletin of TT resulting a frame shift mutation.

**Indels** result from either insertion of few nucleotides or deletion of few. Insertion is an addition of one or more nucleotides. Deletion is a loss of DNA. Net result is a change in total number of nucleotides [42]. This results in deletion or insertion of amino acids or even a change in the reading frame leading to a different protein or a truncated protein. This protein may be non functional or with a change of function. Large deletions or insertions may result in unequal cross over during cell division leading to severe disease phenotype [42].
Fig 17: MLPA results, deletion of Exon 33 to 36 (above) and haplo insufficiency of TSC2 (below)

Frequencies of different types of mutations found in TSC1 and TSC2 were documented in a study done in 2005 [22]. Figure 18 shows the results of this study.
Fig 18: Different types of mutations in TSC1 and TSC2 genes
2. Justification of a study on Tuberous Sclerosis Complex in a Sri Lankan cohort

2.1. Importance of this study

Tuberous Sclerosis Complex is no more considered a rare disorder. Recent studies estimate an incidence rate between 1 in 6,000 to 1 in 10,000 live births with a population prevalence of 1 in 20,000 [2].

A study done in 2009 and few case reports have confirmed the presence of TSC in Sri Lanka [46-48]. However, a study regarding the mutations in TSC genes in Sri Lankan patients has not been conducted. Therefore genotype-phenotype correlation among the Sri Lankan patients is not yet known.

From the view of the health care system, phenotyping the TSC patients in Sri Lanka is necessary for healthcare planning with a multidisciplinary approach. Phenotyping TSC is a practically challenging process because of its numerous and diverse clinical signs, its variable expressivity and the age related nature of some clinical manifestations. Phenotyping patients with TSC in Sri Lanka will provide them with information on their disease and prognosis. This process will also contribute to the development of a management protocol which is both according to the international guidelines and also suitable for the Sri Lankan health care system.

Genotyping $TSC1$ and $TSC2$ is also a challenging process as there are said to be more than 1000 disease causing mutations and the number of new mutations are constantly increasing. Although there has been a steady increment in understanding the phenotypic and genotypic variation associated with TSC during the last two decades, there is paucity with regard to the genotype of the Sri Lankan patients. Obtaining three generation family history is beneficial in
diagnosing family members with milder phenotype. Genetic testing is also beneficial for the patients as they can get a better knowledge with regard to the disease with pre-test and post-test counselling apart from the genetic diagnosis. We will be able to counsel them on diagnosis, prognosis, reproductive options and future management plans. Awareness of the parents will probably lead to better care for the affected children; especially in those with epilepsy. Patients with a genetic diagnosis will also be able to participate in research and clinical trials leading to new treatment. Academically, genotyping is beneficial as we can correlate the phenotype with the genotype and compare it with the rest of the world. We will also be able to look for founder mutations specific to Sri Lankan patients and novel mutations that are clinically significant. This will be beneficial for the general knowledge of TSC.

Further development of this project can lead to the establishment of an advocacy group such as Tuberous Sclerosis Alliance group in USA, so that both the patients and clinicians can benefit from management of the TSC patients in Sri Lanka as well as rest of the world.

2.2. Objectives

The main objective of the work presented in this thesis is to clinically and genetically characterize TSC patients in Sri Lanka. The second objective is to correlate the genotype with phenotype and to establish a genotype-phenotype spectrum in Sri Lankan patients.

Objectives:

1. To describe the clinical phenotype of TSC in a cohort of Sri Lankan children
2. To study the mutations in TSC1 and TSC2 in Sri Lankan children with TSC
3. To identify the pattern of mutations in TSC1 and TSC2 giving rise to infantile spasms and other seizure types
4. To identify *PKD1* gene deletions in TSC patients with polycystic kidney disease and to compare with the known deletions

5. To compare Sri Lankan genotypes with genotypes from patients with TSC from other countries

**Ethical considerations**

The ethical review committee of Faculty of Medicine, University of Colombo, Sri Lanka has approved the study.

The study was conducted according to the Declaration of Helsinki (2008) and is a collaborative effort between the Human Genetics Unit, Faculty of Medicine, Colombo, patients with TSC, paediatric neurologists, paediatric cardiologists, dermatologists and paediatricians at the tertiary care hospitals in Sri Lanka and the Department of Medical Genetics, University of Oslo, Norway, Norwegian Epilepsy Center, Sandvika and the Norwegian center for rare epilepsy-related disorders, Oslo University Hospital. The study has social value as this is the first effort to correlate phenotype with genotype in Sri Lankan children with TSC and therefore contributes to the general knowledge in the field. The study was designed to ensure scientific validity. The study was opened to all patients with a clinical diagnosis of TSC who falls into the age group of 0 – 18 years.

Appropriate measures were taken to ensure that consent is obtained in an ethical manner from all study participants. Written informed consent was obtained from parents or guardians (proxy consent) using information sheets (Appendix 3) and consent forms (Appendix 3) in Sinhala, Tamil and English languages according to their preference. The patients and their parents/guardian were interviewed privately to ensure privacy and were able to discuss the study privately with the principal investigator without the presence of others.
Parents/guardians were able to make a decision to participate their children to the study without external interference.

The data collection booklet (Appendix 3) was designed to ensure confidentiality of information gathered. Soon after collecting the personal information, the identification page was removed and filled separately. The only identification number in the rest of the booklet is a coded subject study number which cannot be linked to an individual without the page containing the personal information which was kept by the principal investigator under lock and key. The electronic database containing the clinical data only had the subject study number, thus ensuring confidentiality. The database and the computer containing the database were password protected.

Photographs of some clinical manifestations were captured by the principal investigator only after proper proxy consent was obtained. Full anonymity was maintained by covering the eyes where the face is exhibited. Name of the patient was not mentioned in images such as the 2D echocardiograms. The small risk of complications, such as bruising or infection after the venaepuncture was minimized as it was performed under aseptic conditions by the principal investigator or a trained nurse.

Benefits to the patient included improved knowledge on the disease and also in the cases where we could establish a genetic diagnosis, they receive a molecular diagnosis. All the parents of the participants will be counseled and advised on family planning, pre-conception genetic testing and life style modification of their children with TSC according to the test results.
The samples and data obtained will be stored for further studies in TSC until 2024. Thereafter the remaining samples will be anonymised and discarded by the laboratory under the supervision of the investigator. Appropriate consent has been obtained for this purpose and such studies would be subjected to ethics review prior to conduct.
3. Material and methods

This study was conducted in a paediatric population age ranging from 0 – 18 years. All the patients presented to paediatric neurology units, paediatric cardiology units at LRH and other teaching hospitals were recruited into the study. Patients were recruited from October 2013 to March 2014.

3.1. Study population

Inclusion and exclusion criteria for the study were as below:

Inclusion criteria

1. Clinical diagnosis of TSC; two major criteria or one major with two or more minor criteria according to Table 2
2. Ability to provide proxy consent from parent/guardian
3. Ability to obtain a valid history of epilepsy and/or polycystic kidney disease with TSC
4. Sri Lankan origin

Exclusion criteria

Subjects with TSC with other reasons for epilepsy such as head injury, epileptic encephalopathy etc.

Patients’ parents were contacted via phone/mail and those who gave informed proxy consent were recruited into the study. Data for a total of 44 patients were included in to the study and recorded in the data base.
Recruitment of Patients

Identification and recruitment of the patients with Tuberous Sclerosis Complex was conducted in the following manner;

1. Identification from the database at the professorial Paediatric Neurology Unit at Lady Ridgeway Hospital for Children (LRH), Colombo.

2. Sending a call letter to all the paediatric neurologists, paediatric cardiologists, dermatologists and paediatricians at the tertiary care centers around the country requesting to refer patients with TSC into the study.

3. Retrieving the archives of Human Genetics Unit, Faculty of Medicine, Colombo from 2005 to 2013 for patients with TSC.

4. Paying home visits for the children who are not visiting the hospitals, but being registered at neurology, cardiology or dermatology clinics at tertiary care centers.

3.2. Registration of the Patients

A data base was established including gender, presenting features, three generation pedigree, clinical manifestations, investigation findings (MRI, CT, EEG, ultra sound scans, genetic diagnosis), social status and treatment regimes. Each index case was registered in this data base. The 44 patients were from 42 families as two patients were twins and two were siblings.

3.3. Clinical Evaluation

Detailed medical history was obtained from parents/guardian of each patient. To minimize recall bias, the history was obtained from two adults closely in contact with the patient whenever possible. Family history was obtained both from the mother and the father whenever possible. A thorough medical examination was done with emphasis on diagnostic
criteria of TSC by the principal investigator and all previous and relevant investigations were evaluated. The findings were reassessed by the supervisors.

Diagnosis of epilepsy was classified according to International League Against Epilepsy (ILAE) classification 2010 [49]. Infantile spasms were confirmed by referring the clinical notes, EEG findings and detailed history from parents/guardian. According to the ILAE proposals, failure of adequate trials of two or more tolerated and appropriately chosen anti epileptic drug schedules is considered as patients with intractable epilepsy [50].

**ILAE classification 2010**

Classification is essential to understand, diagnose and manage epilepsy. Depending on the etiology the treatment regime and management plan differs. I.e. Vigabatrin is the drug of choice in infantile spasms in patients with TSC.

The International League Against Epilepsy (ILAE) was established in 1909 with a mission of supporting health professionals, patients and their care givers, governments and public with all aspects related to epilepsy.

ILAE has revised the classification and terminology for organization of seizures and forms of epilepsy in 2010 (Appendix 2). This new classification has revised the concepts, terminology and approaches of the previous classifications published in 1981 on epileptic seizures and in 1989 on epileptic syndromes [51].

With new classification all seizures fall into generalized, focal or unknown type. The terminology and concepts have also changed towards highlighting genetic aetiology instead of idiopathic epilepsy. TSC falls into structural-metabolic aetiology of seizures. Infantile spasms are classified as brief contractions lasting 70-200ms involving any group of muscles. Other seizures in TSC are usually focal in origin. The ILAE classification is freely available on line at [http://www.ilae.org](http://www.ilae.org).
Electroencephalogram

Electroencephalogram (EEG) is one of the main diagnostic tests used in epilepsy and is also useful in diagnosis of some brain disorders such as tumors, stroke, coma, encephalopathy and brain death [52]. EEG records the changes in electrical activity in different regions of the brain with the help of small probes attached to the scalp [53].

In this study, the EEG report prior to treatment and the subsequent EEG reports were taken in consideration when classifying seizures in the patients. The EEG pattern in patients with TSC and IS not necessarily similar to the EEG pattern in patients with West syndrome (IS, hypsarrhythmia in EEG and developmental delay) [29, 54]. Therefore, apart from the EEG reports, the clinical notes describing the seizure semiology and the history from the parents and or guardians were also considered in determining the seizure types.

Neuro imaging

Magnetic resonance imaging (MRI) is the standard recommended imaging technique to diagnose cortical tubers and other cortical dysplasias, SEN and SEGAs according to international guidelines [1]. In this study population MRI scanning was available only in 17 patients. In 25 patients, computerized tomography (CT) was available and 2 patients who were below 6 months of age had ultrasound scan brain and were awaiting CT examination of the brain.

Ultrasound scan of the abdomen

All patients were screened with ultrasound scan (USS) abdomen to look for angiomyolipomas in abdominal viscera and renal cysts at the time of diagnosis. Subsequent scans were done according to the international guide lines depending on the state of the first scan [1]. In this study, we reviewed the report from the most recent scan confirm or exclude the relevant diagnostic criteria.
Two Dimensional Echocardiogram

At least one Two Dimensional Echocardiogram (2D Echo) was available in all 44 patients done at the time of diagnosis. The first 2D Echo findings were considered in this study in order to identify the earliest possible findings as cardiac rhabdomyomas may be congenital in TSC.

3.4. Genetic Testing

DNA extraction

At recruitment, 5ml of venous blood was collected in K/EDTA tubes from each patient. The samples were stored at -80°C at the Human Genetics Unit, until the DNA extraction was carried out. DNA was extracted using the QIAamp DNA Blood Mini Kit (250) by QIAGEN® (Venlo, Netherlands). Extracted DNA was stored at -20°C until it was shipped to Oslo, Norway. DNA was quantified using NanoDrop 8000 UV-Vis Spectrophotometer (Thermo Scientific).

Polymerase Chain Reaction

Before sequencing the genes, it is necessary to amplify the number of copies of the target sequence with a Polymerase Chain Reaction (PCR). Using this biochemical technology, single or limited numbers of copies of DNA are amplified in to many folds resulting in thousands to millions of copies of the DNA sequence that we are interested in.

The method is based on two primers annealing to complementary sequences at either end of the target sequence. DNA polymerase synthesizes DNA, elongates the primers and makes two new duplicates of the original template. In order to do this, PCR is dependent on a DNA template, specific primers, DNA polymerase enzyme, deoxynucleotide triphosphates (dNTPs) and thermal cycling [55].
**Primer design**

The reference sequence used for \(\text{TSC1}\) and \(\text{TSC2}\) were [NM_000368.4](http://www.ensembl.org/) and [NM_000548.3](http://www.ensembl.org/) respectively, and were downloaded from Ensembl (http://www.ensembl.org/). Primers are short oligonucleotides that are complementary to the end of a target sequence, and have to be unique for each target sequence. The primer design for \(\text{TSC1}\) and \(\text{TSC2}\) was carried out at the Department of Medical Genetics at the University Hospital in Oslo using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). Full primer sequence is attached in appendix 4.

**Sanger sequencing**

Once the target DNA was amplified, the sequencing process can start. Sanger sequencing is a DNA sequencing method based on selective incorporation of chain terminating dideoxynucleotides (ddNTDs) by DNA polymerase during in vitro DNA replication [56]. The technology is used to determine the order of the nucleotides in a DNA sequence, and also, as in this project, detect single nucleotide variation (SNV) and small deletions or insertions.

Then the sequencing reaction is run on an automated Applied Biosystems 3730xl DNA Analyzer for electrophoresis and data analysis. During electrophoresis, DNA molecules in the reaction are injected into a capillary array. The injected DNA molecules are then separated
through the array according to size. Each fragment is detected by the fluorescent tag, present on each ddNTD that were incorporated during the sequencing reaction. It appears as a colored peak on data analysis software. Different ddNTDs give four different colors depending on the attached nucleotide. The data analysis software interprets raw data peaks and automatically generates the final sequence of DNA template.

**Multiplex Ligation dependent Probe Amplification**

Multiplex Ligation dependent Probe amplification (MLPA®) is a high-throughput method to determine copy number variations (CNVs) in genomic sequences [57]. With MLPA we can detect larger deletions and rearrangements than with Sanger sequencing, i.e. these two methods fulfill each other when searching for a genetic cause for disease.

In the MLPA method it is not the sample DNA that is amplified during PCR reaction, but MLPA probes that hybridize to the target DNA sequence [58]. Each MLPA probe consists of two oligonucleotide probes that hybridize adjacent to target. Only the probes that have ligated are amplified by PCR. One pair of primers is used per MLPA PCR reaction. The relative number of fragments present after PCR reaction depends on the relative amount of the target sequence present in a DNA sample. MLPA® comes as a commercial kit solely produced by MRC – Holland (Amsterdam, the Netherlands) [59]. Samples that showed negative results and the samples that are inconclusive after Sanger sequencing were analyzed by MLPA.
3.5 Analysis of Results

Mutation analysis done by referring Leiden Open Variation database (LOVD) (www.lovd.nl/TSC1, www.lovd/TSC2), Human Gene Mutation (HGMD) database (http://www.hgmd.org/) and ClinVar database (http://www.ncbi.nlm.nih.gov/clinvar/). Gene protein effects were analyzed using Mutilizer 2.0.beta – 31 database (https://mutalyzer.nl). For the mutations that were not reported in these databases, a literature search was done using PubMed (http://www.ncbi.nlm.nih.gov/pubmed/) and Google scholar (http://scholar.google.no/).
4. Results

The total number of patients in the study population was 44 from 42 families. Among them, 34 (77.3%) were Sinhalese, 6 (13.6%) were Sri Lankan Tamil and 4 (9.1%) were Moor. There were 24 (54%) males and 20 (46%) females, aged 9 days to 18 years with a mean age of 6.5 years and a median age of 4.5 years (standard deviation +/- 2.4). Among the patients at least one clinical feature was present at birth in 31 (70.4%) patients while late presentations were noted in 13 (29.6%) patients.

The most common clinical feature present at birth was hypopigmented macules, which was the presenting clinical sign in 26 (84%) patients. Three patients (10%) presented with cardiac murmurs or arrhythmia and one patient (3%) presented with gross ascites on day one of life due to renal failure. All these 4 patients also had hypopigmented macules in addition to their main presenting complaint. One patient (3%) presented with a cephalic plaque at birth.

Neurobehavioral phenotypes were not taken into account as psychiatric assessment was not being done as a routine part of evaluation of patients with TSC in Sri Lanka.
4.1 Phenotype of the sample population

Table 8, 9 and 10 summarises the phenotype of 44 patients in the study population.
TABLE 8: Skin manifestations of the study population

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<th>FH</th>
<th>Skin manifestations</th>
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<td><strong>%</strong></td>
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Pt No = Patient number, FH = Family History, Hypopig:macules = hypopigmented macules, Fibrous ce:plaques = fibrous cephalic plaques, Y = present, N = absent, % = percentage
### TABLE 9: Brain manifestations of the study population

<table>
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<th>Pt No</th>
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<th>Generalized</th>
<th>Focal</th>
<th>Intractable</th>
<th>FH</th>
<th>Tubers</th>
<th>SEN</th>
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Pt No = Patient number, FH = Family History of seizures, Y = present, N = absent, % = percentage
TABLE 10: Other manifestations of the study population

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### Other manifestations

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Pt No = Patient number, Y = present, N = absent, % = Percentage, G1, G2, G3 & G4 = Grading of AML and RC according to Dabora et al [19].(Appendix 2)

**4.2 Genotype of the study population**

As two pairs of patients were twins and siblings, the mutations of one pair are considered as one. Therefore the total number of mutations considered was 42. With Sanger sequencing 20 mutations were detected in TSC2 and with MLPA another 7 deletions were detected adding the total number to 27 mutations (64.2%). In TSC1, 8 mutations were detected with Sanger sequencing, while one mutation was detected by MLPA. The total mutations found in TSC1 were 9 (21.5%). In the whole sample population mutation detection rate was 85.7%. No mutation was detected in 6 patients (14.3%).
Fig 22: mutation pattern in Sri Lankan study population
Table 11 shows the genotype of the study population.

**TABLE 11: Genotype of TSC patients in this study**

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<th>Location</th>
<th>Mutation</th>
<th>Effect</th>
<th>Predicted effect</th>
<th>Inheritance by Clinical Evaluation</th>
<th>Reports</th>
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<td>p.Arg458*</td>
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</tbody>
</table>

Highlighted in yellow – novel mutations, con.gene del = contagious gene deletion, ?Familial = presence of clinically affected family members, but presence of reported data with De novo cases, De novo = parents tested, no mutations found

There were some normal variants previously reported in studies as well as in databases. In this study, in TSC1, they were c.965T>C [68], c.1335A>G [62] and c.1460C>G [66]. In TSC2, they were c.1100G>A, c.1578C>T, c.2580T>C, c.5017G>C [68], c.4536C>T and c.5161-10A>C [62].

There were some variants which are apparently neutral as they were present in more than 6 patients apart from well documented pathogenic mutations, though there is no reported data strong enough to conclude they are normal variants. In TSC1, they were c.1218C>T, c.1333+13C>T and c.3387C>T. In TSC2 they were c.1600-14C>T, c.2546-12C>T, c.3544A>G, c.5202T>C and c.5397G>C.

4.3 Treatment regimens

There were 6 (13.6%) patients who were not on any drug treatment. Two of these had never experienced seizures and four patients had been seizure free for a long time. Three patients were on antihypertensive (Captopril) treatment in addition to antiepileptic drugs and one patient used deperiprone for iron chelation as he also suffered from β Thalassemia major.

Following is the breakup of the pattern of drug treatment in this study population.
TABLE 12: Treatment pattern in the study population

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of patients</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monotherapy (On one drug)</td>
<td>6</td>
<td>13.6</td>
</tr>
<tr>
<td>Polytherapy 2 drugs</td>
<td>18</td>
<td>40.9</td>
</tr>
<tr>
<td>3 drugs</td>
<td>9</td>
<td>20.4</td>
</tr>
<tr>
<td>4 drugs</td>
<td>4</td>
<td>9.0</td>
</tr>
<tr>
<td>5 drugs</td>
<td>1</td>
<td>2.2</td>
</tr>
<tr>
<td>Other drugs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antihypertensives</td>
<td>3</td>
<td>6.8</td>
</tr>
<tr>
<td>Iron chelation</td>
<td>1</td>
<td>2.2</td>
</tr>
</tbody>
</table>

TABLE 13: Drugs used in managements of the patients with TSC

<table>
<thead>
<tr>
<th>Name of the drug</th>
<th>Number of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Valproate</td>
<td>19</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>14</td>
</tr>
<tr>
<td>Topiramate</td>
<td>14</td>
</tr>
<tr>
<td>Clobazame</td>
<td>13</td>
</tr>
<tr>
<td>Vigabatrin</td>
<td>11</td>
</tr>
<tr>
<td>Lamotrigine</td>
<td>3</td>
</tr>
<tr>
<td>Captopril</td>
<td>3</td>
</tr>
<tr>
<td>Deperiprone</td>
<td>1</td>
</tr>
</tbody>
</table>

Commonly used drugs for management of these patients are noted above in Table 13

![Drug use in study population](image)

Fig 23: Drug treatment pattern in the study population
5. Discussion

Tuberous Sclerosis Complex (TSC) is an autosomal dominant disorder characterized by development of hamartomas in almost all organ systems of the body. Other characteristic features of TSC are epilepsy, intellectual disabilities, autism spectrum disorders and organ failure [62]. TSC is no more considered a rare genetic disorder with a birth incidence of 1/6000 to 1/10,000 and a population prevalence of 1 in 20,000. It is estimated that nearly one million individuals worldwide are affected with TSC, involving all ethnic groups irrespective of their gender [2, 27].

It is difficult to evaluate TSC clinically due to its highly variable phenotype with multisystem involvement and age dependent appearance of some clinical features. Even though the inheritance is autosomal dominant, about two-thirds of the cases are sporadic [62].

It is relatively challenging to diagnose TSC genetically due to the large size of the two genes responsible for TSC, with highly heterogeneous mutations scattered all over the two genes, the TSC1 and TSC2. In this study we have used Sanger sequencing of all the exons in both genes as the first line of molecular testing to evaluate point mutations and small deletions, insertions and duplications. In failing to detect the above, MLPA has been done to look for large deletions or insertions. These two methods provide a sensitive strategy in molecular diagnosis of TSC.

During the last two decades, there has been a steady increment in understanding the phenotypic and genotypic variation associated with TSC. Knowledge has expanded to understand the tumor suppressor effects of TSC genes on the mTOR pathway, seeking for new treatment options for the disease [69, 70]. This study is an initial attempt to look at the possible genotypic composition of a cohort of patients from Sri Lanka with regard to TSC.
5.1 The phenotype and genotype of patients with TSC in Sri Lanka

The most common skin manifestation was hypopigmented macules found in 88.6%, followed by facial angiofibromas (47.7%), fibrous cephalic plaques (34%) and shagreen patches (29.5%). No confetti skin lesions were noted in the study group. These findings were similar to the published data except for facial angiofibromas and shagreen patches which were less prevalent in this population [2, 3, 28]. Among 44 patients, 39 (88.6%) suffered from epilepsy. In this study, evidence of infantile spasms (IS) was noted in 19 (43.1%), focal seizures (FS) in 36 (81.8%) and intractable epilepsy noted in 17 (38.6%) patients is comparable to previously reports [44]. Only five patients were seizure free and six patients had total seizure control with monotherapy, making the total of 25% of the patients who had seizure control. This is lower than reported other cohorts [44]. This may be due to sample selection bias, as the patients were recruited only from the tertiary care centers, where only the difficult to manage patients end up. There were three patients with a family history of epilepsy, but no family history of TSC. This might be due to sub-clinical TSC running in the family, which remains to be discovered.

When considering the major clinical criteria diagnosed by neuro-imaging techniques, cortical tubers were present only in 28 patients (63.6%), which is lower than compared to other reported data [2]. MRI is the procedure of choice in diagnosing brain manifestations in TSC and this technique more accurately localizes cortical tubers [71]. In this study MRI were available in only 17 patients and 14 of them reported presence of cortical tubers. Subependymal nodules (SEN) were reported in 30 patients (68.1%) which is also less than what reported previously [2], though it is reported that CT scanning is more accurate in detecting calcified SEN [71]. Among the 25 patients with available CT scans, 19 showed calcified SEN. There were seven patients with no available imaging reports, and these patients may have been added on to the presence of tubers and SEN if they were available.
Subependymal giant cell astrocytomas (SEGAs) were reported in 5 – 15% of the patients with TSC [2, 27] and the findings were similar in this study population.

A study reported that 55% of a children cohort with TSC with a mean age of 6.9 years had some renal abnormality [72]. In this study population (mean age 6.5 years) only 12 patients (27.3%) had renal angiomiolipomas, renal cysts or both.

Presence of lymphangioleiomyomatosis (LAM) was not investigated as this was a paediatric population and the only patient who was 18 years was a male.

According to previously reported data approximately 30% of the TSC mutations are familial, while the remaining 70% are de novo [8]. In this study 38.6% of the patients had a family history of TSC where one or more family members were clinically affected. Genotyping the parents may confirm a higher percentage of familial TSC in this population. Among nine patients with TSC1 mutations, 55.5% had clinical familial history. In patients with TSC2 mutations, only 33.3% were found with familial mutations. Hence, sporadic mutations are more often reported in TSC2 mutations than with TSC1 mutations. This agrees with the previous study that there is relative paucity of TSC1 mutations in sporadic TSC when compared to TSC2 mutations [73].

**5.2. Pattern of mutations**

To determine the pattern of mutations, the classification system for sequence variants at Ullevaal hospital (Appendix 5) was taken into account.

**Classification system for sequence variants**

This tool is being used at the department of Medical Genetics, University hospital, Ullevaal, Norway to classify mutations according to the possibility of being pathogenic. In this classification system, word pathogenic is referred to destructive for protein production and/or function. There are 5 categories in this classification system. The variants can be checked with databases, available literature and reported data with population based studies for
categorization. The well documented normal variants fall into category 1 and well documented pathogenic mutations fall into category 5. The uncertain variants are categorized into other 3 categories, depending on the evidence based manner, the most possible normal variants fall into category 2, while most possible pathogenic variants fall into category 4. The category 3 is the variants of uncertain significance (VUS) with lack of documentation. These are the most unpredictable variants that need functional studies to determine the exact effect caused by the mutation. This classification system is a reliable method to gain an idea of the novel mutations. The classification is developed by the expertise at the University hospital, Ullevaal, Norvay.

There were nine identified neutral variants which had been reported in previous studies as well as in databases. These normal variants fall into category 1 according to the classification. These variants were found in more than 6 patients in the study, yet it can be confirmed by checking with normal Sri Lankan population as the reported data are from different populations.

There were eight variants falling into category 2. As there is no reported data to confirm these are normal variants, it will be interesting to check with normal Sri Lankan population for confirmation.

The number of novel mutations was 22. Among them there were three missense mutations and a splice site mutation which falls into category 3, the variants of uncertain significance (VUS). The patient with deletion in TSC2 exon 1 up stream and three patients with one or few base pair deletions in TSC2 also belong to category 3. In these eight mutations, a further study into mRNA and protein functional status is needed to come to a conclusion, though the patients exhibit a severe clinical phenotype of TSC. Reporting of the same mutations resulting in TSC phenotype can add on more power to confirm these mutations as pathogenic.
There are 4 nonsense mutations and 4 frame shift mutations that can be classified as category 4. The four patients with large deletions in \( TSC2 \) involving at least one exon and the patient with large deletion in \( TSC1 \) also belong to category 4 as the predicted protein truncation effect is high with these mutations [29]. The only duplication reported is predicted to result in truncated protein, 42 amino acids downstream to the mutation in exon 33. Although these variants are completely novel, it is highly likely that they are pathogenic. Yet, it will be interesting to study protein functions to verify them as protein truncating mutations. Reported data on same mutation presenting in TSC phenotype will add on power to confirm these as pathogenic mutations.

The remaining 20 mutations are well documented pathogenic variants, which fall into category 5. Among them there are two patients with contagious gene syndrome, involving total deletion of \( TSC2 \) and \( PKD1 \) genes. The extent of the deletions are yet unknown. They might contain other neighboring genes such as \( NTHL1 \) and \( SLC9A3R2 \) in the deleted region. It will be interesting to perform a karyotyping followed by break point analysis in these two patients to look for the exact site of deletion and the genes involved.

The most common mutation in this study population is \( TSC2 \) Ex38 c.5024C>T missense mutation reported in 3 families. These families are all from western province, Colombo district. There is no strong evidence to confirm that this is a founder mutation. However this can be studied by performing haplotype analysis of the nearby region. There are two other mutations in \( TSC1 \) that are present in two families, a nonsense mutation and a missense mutation which are found in different ethnic groups. They carry a remote possibility of being founder mutations in Sri Lankan patients with TSC.

### 5.3. Infantile Spasms and genotype – phenotype correlation

Among the 19 patients with IS, 16 patients (84.2%) were found to have mutations in \( TSC2 \) while two patients (10.5%) had mutations in \( TSC1 \) and one patient (5.3%) had no mutations
in either gene. In this study, a relatively high percentage of TSC2 mutations are associated with IS when compared to previously reported data [29]. As our sample size is small, this might be coincidental. Only one of the patients with IS in our cohort had a missense mutation in the central domain of TSC2 (exon 23 – 33), which is less than expected, if the location of the mutation were to be unconditionally spread throughout the gene (p=0.052). This agrees with the findings of van Eeghen and colleagues, which show that missense mutations in the central TSC2 are less likely to cause IS [29].

Focal epilepsy (FE) was reported in 89.4% of the patients who had IS, and 47.3% of them were resistant to drugs. This reveals that the patients with IS and IS together with FE are more prone to be resistant to drugs [44]. 98.1% of the children who are resistant to antiepileptic drugs have had IS together with FS and 88.2% experienced the first episode of convulsion during the first six months of their life. This confirm that IS is a risk factor for intractable epilepsy and that seizure onset within the first year of life with IS and/or focal seizures is characteristic of drug resistant epilepsy in patients with epilepsy [43, 44].

In three patients (6.8%), initial presentation was febrile convulsions, and these patients later developed IS. TSC presenting with febrile convulsions as the first episode of seizures has been reported previously [74], but the association to the development of IS has not been described. To establish whether febrile convulsions are a presenting feature of TSC with IS, further investigation is needed.

5.4. PKD1 deletions

According to published data, 2% of the patients with TSC are found to be with contagious gene deletions syndrome which includes the deletion of whole or part of the PKD1 gene located only 48 base pairs away from TSC2 gene on chromosome 16p13.3 region [27]. In this study population, 3 (6.8%) patients were found with TSC2 and PKD1 deletion, whereas 2 of
these (4.5%) had complete deletion of TSC2, resulting in haplo-insufficiency of TSC2. In addition to these two patients, patients with missense mutations and deletions in the GAP domain (exon 34-38) of the TSC2 gene (as described by Maheshwar et al [75]), lead to grade 3 or grade 4 renal manifestations. The relationship between mutations in GAP domain of TSC2 and renal involvement of TSC is yet mainly unknown.

5.5. Overall genotype

The Sri Lankan population with TSC is relatively similar with regards to genotype and phenotype to the other reported patients with TSC. The frequencies of different types of mutations in TSC1 and TSC2 are mainly as reported in other studies [22], and the findings of this study is shown in figure 19.

There are 6 patients with no variants in either TSC1 or TSC2 genes. The cause of TSC in these patients is so far not known. Could there be a third locus giving rise to the TSC phenotype? If so, that hitherto unknown gene should probably be acting on the mTOR signaling pathway, in order to give rise to the same phenotype. This is an interesting area to be explored. We have only sequenced the coding regions of TSC1 and TSC2. May be the disease causing mutations are within one of the genes, but in an intronic region, promoter
region or a regulator region as there are well documented pathogenic mutations reported in non-coding regions causing TSC [76]. Another possible explanation at least in the sporadic cases, is somatic mosaicism that can be missed by testing only the peripheral lymphocytes [77, 78].

Patient 13 is diagnosed β- thalassemia major apart from his novel de novo mutation in TSC1 exon 18, c.2356C>T which is a nonsense mutation that fall into category 4. There are around 2000 reported patients with β- thalassemia major in Sri Lanka [79], therefore the estimated prevalence is 1 in 10,000 individuals. Considering this high predicted prevalence, this can be concluded as an incidental event.
6. Conclusion

Clinical characteristics of TSC in Sri Lankan children were similar to the previously described features of the TSC phenotype. There were 22 novel mutations in the \textit{TSCI} and \textit{TSC2} genes reported in this study population. Among them, 14 mutations had a high potential of being pathogenic. There were six patients with TSC phenotype, but where no mutations were found in coding regions of either gene, which is opened for further investigation.
7. Limitations of the Study

The sample population was gathered from tertiary care centers around the country, basically on referrals. As only the severely affected patients encounter these centers, the patients who were not followed up by these clinicians have been missed. Therefore selection bias is the major limitation in this study.

Psychiatric assessment has not been done routinely at the time of diagnosis for the patients with TSC in Sri Lanka. Even though cognitive disability and neuropsychiatric phenotype is common in TSC, neurobehavioral phenotype could not be investigated.

Parents of most of the patients with novel mutations were not genotyped. It will be interesting to genotype the parents to obtain the inheritance pattern of novel mutations.

Only the coding regions were sequenced in TSC1 and TSC2. Therefore mutations in noncoding regions, promoter regions, modulators and enhancers are being missed.
8. Recommendations

At the beginning of this study, pre test genetic counseling was offered to all the parents of the participants by the principal investigator. During that time only eight parents knew the genetic factor of TSC. It will be much easier for post test counseling as there is some prior knowledge regarding the genetic predisposition among the parents. Genetic counseling will be a benefit to the patients as an outcome of this study.

MRI is an important investigation to diagnose cortical tubers, SEN and SEGA which are hallmarks in clinical diagnosis of TSC [71]. According to recommendations of the international TSC consensus conference 2012, every patient should undergo MRI of the brain at the time of establishment of the diagnosis of TSC [1]. It is recommended to have at least one MRI scan, probably at the time of diagnosis for every patient with TSC in Sri Lanka, though the resources are limited.

Vigabatrin is considered the drug of choice for IS in TSC [80]. Only 11 patients out of 19 are being treated with Vigabatrin. This is due to unavailability of the drug in centers other than the Lady Ridgeway Hospital for children. If the drug can be made available, the patients with IS will benefit from this. The mTOR inhibitors are not available in Sri Lanka yet. Apart from patients with epilepsy and cognitive impairment, patients with SEN, SEGA and renal angiomyolipomas could benefit from this drug [81, 82]. This will help reduce the polytherapy and control intractable seizures. Patients with facial fibromas will also benefit with systemic or topical application of mTOR inhibitors [83].

For the patients with drug resistant epilepsy, modified ketogenic diet is a good option [27], though it is difficult to offer this diet for the children with intractable seizures, as it needs close monitoring at dietitians’ departments. It is worthwhile to use this diet as it may prevent polytherapy while gaining better seizure control.
Vagal nerve stimulation is considered in TSC patients with drug resistant epilepsy, where the surgical management is not feasible [84]. Vagal nerve stimulation is not a good choice in Sri Lanka, as the high expense might be unbearable for most patients. If it can be implemented through the Government and offered free of charge, this will benefit the patients who need this method of treatment.
9. Future Prospective

Next generation sequencing is a technique that can sequence large regions of DNA at a much lower cost than Sanger sequencing. Using this technique in diagnostics, one might consider to sequence the complete region of the gene, including intronic regions, promoters, regulatory elements and untranslated regions (UTR) [85]. This might also be used to sequence the patients where no mutations in coding regions of TSC1 and TSC2 are found, as one might suspect that there are mutations outside the coding part of the genes that causes TSC in these patients.

As there are 22 novel mutations it could be interesting to do mRNA studies to investigate the transcripts of and different transcripts of TSC1 and TSC2 in the patients. Furthermore, it could be beneficial to perform functional studies to determine the effect of novel mutations. i.e to support or exclude pathogenicity of the mutations.

Development of new databases with variant data for South Asian population will be beneficial for researches, geneticists and patients, as it will improve the variant evaluation and the classification of the variants in both research and diagnostics.
10. References


11. Appendixes

Appendix 1: List of abbreviations

TSC – Tuberous Sclerosis Complex

TS – Tuberous Sclerosis

*TSC1* – Tuberous Sclerosis Complex 1 gene

*TSC2* – Tuberous Sclerosis Complex 2 gene

mTOR – mammalian Target Of Rapamycin

GTP – Guanidin Triphosphate

Rheb – Ras homolog enriched in brain

MRI – Magnetic Resonance Image

SEN – Subependymal Nodules

SEGA – Subependymal Giant cell Astrocytomas

IS – Infantile Spasms

EEG – Electroencephalogram

*PKD1* – polycystic kidney disease 1 gene

ADPKD – Autosomal Dominant Polycystic Kidney Disease

LAM – Lymangioleiomyomatosis

CR – Cardiac Rhabdomyomas

AH – Astrocytic Hamartoma

TAND – TSC-Associated Neuropsychiatric Disorder

GFR – Glomerular Filtration Rate

CT – Computed Tomography
HRCT – High-Resolution Computed Tomography

ACTH – Adrenocorticotropic Hormone

ECG – Electrocardiogram

DNA – Deoxyribonucleic Acid

mRNA – messenger Ribonucleic Acid

LRH – Lady Ridgeway Hospital for Children

ILAE – International League Against Epilepsy

USS – Ultra Sound Scan

2D echo – Two Dimensional echocardiogram

PCR – Polymerase Chain Reaction

dNTPs – deoxynucleotide Triphosphates

SNV – Single Nucleotide Variation

MLPA – Multiplex Ligation dependent Probe Amplification

CNV – Copy Number Variation

VUS – Variants of Uncertain Significance

FE – Focal Epilepsy

UTR – Untranslated Regions
Appendix 2: Clinical Diagnostic Criteria

Grading of clinical manifestations

<table>
<thead>
<tr>
<th>Clinical features</th>
<th>Grade</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>seizures</td>
<td>3</td>
<td>Severe: no speech intelligible to a stranger</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Moderate: some speech, no reading</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Mild: reading at less than 3rd grade level</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>None: reading above 3rd grade level</td>
</tr>
<tr>
<td>mental retardation</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hypomelanotic maculex (&gt;2)</td>
<td>present</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>facial angiofibroma</td>
<td>grade: 0, 1, 2, 3</td>
<td>grade 0: none; grade 1: macular (flat) lesions only on cheek; grade 2: papular lesions, &lt;3 mm diameter; grade 3: papular lesions, &gt;3 mm diameter; and/or extending to below mouth</td>
</tr>
<tr>
<td>shigreen patch</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>uogmal fibromas</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>forehead plaque</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Radiographic studies or other evaluation - please circle appropriate term or give number; please give dates of all studies

8. SENS                        # periventricular lesions seen on CT/MRI, date of exam:
9. SEGA                        present      absent
10. cortical tubers            # seen on MRI (do not use CT); date of exam:
11. kidney cysts               grade: 0, 1, 2, 3, 4 | grade 0: none; grade 1:1-2 small (<2 cm) cysts; grade 2: >2 small (<2 cm) cysts; grade 3: >2 and at least one >2 cm; grade 4: classic polycystic kidney disease- multiple cysts with renal enlargement
12. kidney angiomyolipoma      grade: 0, 1, 2, 3 | grade 0: none; grade 1: one or more, all <1 cm diameter
13. liver angiomyolipoma       grade: 0, 1, 2, 3 | grade 2: multiple, one or more >1 cm, and all <4 cm in diameter; grade 3: multiple, one or more >4 cm
14. retinal hamartomas         present      absent
15. cardiac rhabdomyomas       present      never
16. lymphangiomas              severe (clinical symptoms) | mild (abn, chest X-ray) | absent
17. other                      |                |                                                  |

Demographic

18. Ethnic background:

19. Family history- draw a family tree and/or provide a list of all immediate relatives of all members of family with TS.

Name: relationship age medical condition potentially related to TS
ILAE Proposal for Revised Terminology for Organization of Seizures and Epilepsies 2010

Classification of Seizures

Generalized seizures
- Among within and rapidly engaging bilaterally discharging networks
  - Tonic-Clonic
  - Absence
  - Atonic
  - Myoclonic
    - Myoclonic-abortive
    - Myoclonic-atactic
    - Myoclonic-tonic
  - Typical
  - Atypical

Focal seizures
- Originating within networks limited to one hemisphere
  - Characterized by one or more features:
    - Aura
    - Motor
    - Autonomic
    - Awareness/responsiveness altered (dyscognitive) or retained

Unknown
- Insufficient evidence to characterize as focal, generalized, or both
  - Epileptic spasms
  - Others

May evolve to
- Bilateral convulsive seizure

Changes in terminology and concepts

<table>
<thead>
<tr>
<th>New Term and Concept</th>
<th>Examples</th>
<th>Old Term and Concept</th>
</tr>
</thead>
<tbody>
<tr>
<td>Etiology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genetic: genetic defect directly contributes to the epilepsy and seizures are the core symptom of the disorder.</td>
<td>Channelopathies, Glu1 deficiency, etc.</td>
<td>Idiopathic: presumed genetic</td>
</tr>
<tr>
<td>Structural/metabolic: caused by a structural or metabolic disorder of the brain</td>
<td>Tuberous sclerosis, cortical malformations, etc.</td>
<td>Symptomatic: secondary to a known or presumed disorder of the brain</td>
</tr>
<tr>
<td>Unknown: the cause is unknown and might be genetic, structural or metabolic</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Terminology

Self-limited: tendency to resolve spontaneously with time
- Benign
- Catastrophic

Pharmacoresponsive: highly likely to be controlled with medication
- Complex partial
- Simple partial

Focal seizures: seizure semiology described according to specific subjective (aura), motor, autonomic, and dyscognitive features

Evolving to a bilateral convulsive seizure: e.g. tonic, clonic, tonic-clonic
- Secondary generalized

References:
ILAE Proposal for Revised Terminology for Organization of Seizures and Epilepsies 2010

**Electroclinical Syndromes and Other Epilepsies Grouped by Specificity of Diagnosis**

### Electroclinical syndromes

**Neonatal period**
- Benign neonatal seizures
- Benign familial neonatal epilepsy (BFNE)
- Ohtahara syndrome
- Early Myoclonic Encephalopathy (EME)

**Infancy**
- Febrile seizures
- Febrile seizures plus (FS+)
- Benign infantile epilepsy
- Benign familial infantile epilepsy (BFIE)
- West syndrome
- Dravet syndrome
- Myoclonic epilepsy in infancy (MEI)
- Myoclonic encephalopathy in nonprogressive disorders
- Epilepsy of infancy with migrating focal seizures

**Childhood**
- Febrile seizures
- Febrile seizures plus (FS+)
- Early onset childhood occipital epilepsy (Panayiotopoulos syndrome)
- Epilepsy with myoclonic atonic (previously atonic) seizures
- Childhood absence epilepsy (CAE)
- Benign epilepsy with centrotemporal spikes (BECTS)
- Autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE)
- Late onset childhood occipital epilepsy (Gastaut type)
- Epilepsy with myoclonic absences
- Lennox-Gastaut syndrome (LGS)
- Epileptic encephalopathy with continuous spike-and-wave during sleep (CSWS)
- Landau-Kleffner syndrome (LKS)

### Variable age at onset

- Familial focal epilepsy with variable foci (childhood to adult)
- Progressive myoclonic epilepsies (PME)
- Reflex epilepsies

### Distinctive constellations/surgical syndromes

- Mesial temporal lobe epilepsy with hippocampal sclerosis (MTLE with HS)
- Reformussen syndrome
- Gelastic seizures with hypothalamic hamartoma
- Hemiconvulsion-hemiplegia epilepsy

### Nonsyndromic epilepsies**

- Epilepsies attributed to and organized by structural/metabolic causes
  - Malformations of cortical development
    - Hemimegalencephaly, heterotopia, etc.
  - Neurocutaneous syndromes (tuberous sclerosis complex, Sturge-Weber, etc.)
  - Tumor, infection, trauma, angioma, antenatal and perinatal insults, stroke, etc

### Epilepsies of unknown cause

* This arrangement of electroclinical syndromes does not reflect etiology.
* Not traditionally diagnosed as epilepsy
* Sometimes referred to as electrical status epilepticus during slow sleep (ESES)
** Terms of epilepsies not meeting criteria for specific syndromes or constellations

This Proposal is a work in progress....

We welcome your thoughts on this proposal. Please visit our Classification & Terminology Discussion Group at: [http://community.ilae-epilepsy.org/home/](http://community.ilae-epilepsy.org/home/) to login and register your comments.
Appendix 3: Documents used for subject recruitment
This appendix contains the English, Sinhala and Tamil versions of the documents which were used for subject recruitment

1. Information leaflet for the parents/guardians of the study participants used to recruit patients with Tuberous Sclerosis Complex
2. Consent form used for recruitment patients with Tuberous Sclerosis Complex
3. Data Collection booklet (Only in English, as it was filled by the principal investigator)
INFORMATION SHEET

A STUDY OF TUBEROUS SCLEROSIS IN A COHORT OF SRI LANKAN PATIENTS

This study is conducted by me, Dr. Samudita Senaratne, Msc student in Clinical Genetics at Human Genetics Unit, Faculty of Medicine, University of Colombo. I would like to invite you to take part in the research study titled “A STUDY OF TUBEROUS SCLEROSIS IN A COHORT OF SRI LANKAN PATIENTS”. This study is conducted in collaboration with University of Oslo, Norway by me, under the supervision of

Prof. Vajira H.W. Dissanayake, at the Human Genetics Unit, University of Colombo

Dr. Karl Otto Nakken, at the National Center for Epilepsy, Norway

Dr. Jithangi Wanigasinghe, at the Department of Pediatrics, Faculty of Medicine, University of Colombo and

Dr. Dulika Sumathipala, at the Human Genetics Unit, University of Colombo.

1. Purpose of the study

Genes determine how we look and also determine our body functions. Genetic information in our body is carried by genes which contain in structures called chromosomes within the nucleus of the cell. When there are abnormalities in genes it might lead into genetic disorders that run along generations in a family.

Tuberous Sclerosis Complex (TSC) is a genetic disorder that causes noncancerous tumors in many different organs, namely in the brain, skin, eyes, heart, kidney and lungs. The aspects of TSC that most strongly affect quality of life are generally associated with brain including fits, delay in development, reduced thinking ability and a psychiatric illness called autism. However many people with TSC are living independent and virtually healthy lives. The incidence and severity of the various aspects of TSC can vary widely between individuals, even between identical twins.

Research into genetic defects of TSC patients in Sri Lanka has not been done before. Therefore a need to determine genetic defect of Sri Lankan patients with TSC arises.

Purpose of the study is to look at the different presentations of the patients with Tuberous Sclerosis Complex (TSC) to get an idea of the different presentations of the disorder in the Sri Lankan children. Gene testing will be done to detect the genetic defect and to relate it with the disease features of the child.

2. Voluntary participation

Taking part of your child in this study is voluntary. You are free to not add your child at all or to withdraw from the study at any time despite consenting to take part earlier. There will be no loss of medical care or any other available treatment for your child’s illness or condition to which he/she is otherwise entitled. If you decide not to participate you may withdraw him/her from the study at any time by informing us.
3. **Duration, procedure of the study and participant’s responsibilities**

The study will be conducted over 1 year. We require your permission to ask you questions, examine your child, have access to his/her medical records such as the clinic book, and investigation reports. We also need your permission to publish the data collected in a scientific journal. We will not mention your child’s name or any other information that your child could be identified when we publish the results. We may also need to take 5ml of venous blood from your child to do the genetic testing. After testing for the two genes which are responsible for TSC, if there is left out blood samples it will be stored at -80°C for further research into TSC in Sri Lanka and kept at least for a time period of ten (10) years.

4. **Potential Benefits**

Taking part in this study will help you to know the cause or genetic defect that has made your child develop Tuberous Sclerosis Complex. This will contribute to the increasing of knowledge about Tuberous Sclerosis Complex in Sri Lankan patients. It will help us to come to a genetic diagnosis. It will contribute to the knowledge of the disorder in Sri Lankan patients.

5. **Risks, hazards and discomforts**

Blood will be drawn from your child to detect the genetic defect causing Tuberous Sclerosis. Approximately 5ml of blood will be taken for testing from your child. The risk to your child by The electronic database containing the data will have only the subject study number. The computer containing the database would be password protected. Participating in the study is the risk of pain, bruising and infection at the needle prick site. These will be minimized by performing blood drawing under aseptic conditions by trained nurse or a medical technician specialized in blood drawing (phlebotomist).

6. **Reimbursements**

There will be no payment for you or your child for participating in the study, but you will be given a copy of the molecular genetic test results done on your child.

7. **Confidentiality**

Confidentiality of all records is guaranteed. Information that you or your child can be identified will not be released or published. The data collection booklet is designed to ensure confidentiality of information gathered. These data will never be used in such a way that you or your child could be identified in any way in any public presentation or publication without your permission.

8. **Termination of study participation**

You may withdraw participation of your child in this study at any time, with no penalty or effect on medical care or loss of benefits. Please notify us as soon as you decide to withdraw your consent. However it will not be possible for you to withdraw once the results are sent for publication or once the results are published.

9. **Clarification**

If you have questions about any of the tests / procedures or information please feel free to ask any of the persons listed below by calling 011 2689 545.
<table>
<thead>
<tr>
<th>Dr. Samudita Senaratne</th>
<th>Prof. Vajira H.W. Dissanayake</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSc Student</td>
<td>Medical Geneticist</td>
</tr>
<tr>
<td>Human Genetics Unit</td>
<td>Human Genetics Unit</td>
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<td>Faculty of Medicine</td>
<td>Faculty of Medicine</td>
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<td>Colombo</td>
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<tr>
<td>Tel: 077 7697 480</td>
<td>011 2689 545</td>
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<td>011 3165 805</td>
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</tbody>
</table>
කිසදු ඕනෑම කමම න් ඉහතත්තේ වේෂණකරෝසස් මානස්කරා වන අපකේ රජරුු දයරුා වෙනුවෙන් බර්තනය වේ උඳු වූ අන්වීකරණය හැකියේ?

 строки උඳු වූ. යම් මුකුතාකම - අපකේ වේෂණකරෝසස් මානසකරා පමණක් ප්රතිපාදකරන අස්ථානයේය?

ස්තවච්චා කෛනසක් ඉක්නක් බිලක් අධ්යපදුරු ඇදරුා ඉහතත්තේ වේෂණකරෝසස් මානසකරා පමණක් ප්රතිපාදකරන අස්ථානයේය?

කෛනසක් ඉක්නක් බිලක් අධ්යපදුරු ඇදරුා ඉහතත්තේ වේෂණකරෝසස් මානසකරා පමණක් ප්රතිපාදකරන අස්ථානයේය?

න් ඉහතත්තේ වේෂණකරෝසස් මානසකරා පමණක් ප්රතිපාදකරන අස්ථානයේය?

1. ප්රාදේශීය නටබුන්

විදේශ කොහේමක් විශේෂ දිගින් පෙළමුව කළ වන හැකිය. මොය වේෂණකරෝසස් මානස්කරා පමණක් ප්රතිපාදකරන අස්ථානයේය?

විදේශ කොහේමක් විශේෂ දිගින් පෙළමුව කළ වන හැකිය. මොය වේෂණකරෝසස් මානස්කරා පමණක් ප්රතිපාදකරන අස්ථානයේය?

විදේශ කොහේමක් විශේෂ දිගින් පෙළමුව කළ වන හැකිය. මොය වේෂණකරෝසස් මානස්කරා පමණක් ප්රතිපාදකරන අස්ථානයේය?

විදේශ කොහේමක් විශේෂ දිගින් පෙළමුව කළ වන හැකිය. මොය වේෂණකරෝසස් මානස්කරා පමණක් ප්රතිපාදකරන අස්ථානයේය?

මොය වේෂණකරෝසස් මානස්කරා පමණක් ප්රතිපාදකරන අස්ථානයේය?

2. ප්රාදේශීය පරිදියන

කොහේමක් විශේෂ වේෂණකරෝසස් මානස්කරා පමණක් ප්රතිපාදකරන අස්ථානයේය?

කොහේමක් විශේෂ වේෂණකරෝසස් මානස්කරා පමණක් ප්රතිපාදකරන අස්ථානයේය?

ප්රාදේශීය පරිදියන කොහේමක් විශේෂ වේෂණකරෝසස් මානස්කරා පමණක් ප්රතිපාදකරන අස්ථානයේය?
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9.මිළිය ආරම්භය

මෙහෙය යන්ත පෙළමාර්ග විසින් පිළිබද වේකවර්ත විය විදිය ආරම්භය වන විදියේ බැහැ බිමේව වේකවර්ත වේකවර්ත මැටි ප්‍රශ්නයක් දක්වා පහයුභයක් පෙරහැ අන්තිරිය පෙරදුරක්.

මහාචාර්ය උප. උප. යුනාරත්න ආහර්භනා - මහාචාර්ය ආහර්භනා - මහාචාර්ය ආහර්භනා - මහාචාර්ය ආහර්භනා, මහාචාර්ය ආහර්භනා, මහාචාර්ය ආහර්භනා, මහාචාර්ය ආහර්භනා. ආහර්භනා, 0112689545

0777697480, 0113165805, 0113165805, 0113165805
தகவல் பத்திரம்

இலங்கையில் டியுபபரஸ் ஸ்கலலரோசிஸ் (Tuberous Sclerosis) ல் யினோல் போதிக்கப்பட்ட லோயோளிகள் பபருங்குழுவில் இந்தகயப் பற்றிய ஒரு பபரும் ஆய்வு.

மனித மரபியல் பிரிவு, கசொழும்பு மருத்துவப் பிறக்கத்தில் மருத்துவம் மாறுருவியொளியொன்று எனும் தலைப்பில், மாணவியும் இருக்கும் Dr. சமுதினேல் சசசனொரத்னு ஆற்றன் இருந்து ஆய்வு மாறுசன்னாராலைத்

"தியுபபரஸ் ஸ்கலலரோசிஸ் (Tuberous Sclerosis) வடிவமாய் பாலிகாமல் வட்டாராயில் வெவ்வேறுபட்டுலக்காட்டும் என்று குறிப்பிட்டம், 

மனித மரபியல் பிரிவில் ஒசையொன்று விளக்கம் தரும் பதின் ஆய்வு என்று பாலிகாமல் வெவ்வேறு

டியுபபரஸ் ஸ்கலலரோசிஸ் (Tuberous Sclerosis Complex) ஒரு மாறுருவியொளியொன்று. இது முழுநிலைமாது, 

புரப்பலரயொழியம் அழத்தொளியும் அலைவிய அலைவிய டுட்டிலை உண்டொக்கும்.

இசநொயின் தொக்கும் மற்றும் தீவிரம் இவ்வசவொருவரில்லடசய, ஒத்த இரட்ட்டைற்றில் கூட மொறுபடைொம். 

எனவச, இசநொயின் சவறுபொடுப்பற்பேித்தல் அேிவசத 

டியுபபரஸ் ஸ்கலலரோசிஸ் (Tuberous Sclerosis Complex) ஒரு மாறுருவியொளியொன்று.
ஆய்வினுளைய பண்பாடு. மருத்துவ காரணிகளைச் செலுத்தி மருத்துவ சுற்றுலா பல்லில் அதன் தொடர்புடன் தொடர்க்கப்பட்டுள்ளது.

கல்விக்கான பண்பாடு

இறுதி அப்பால் பலரும் குறிப்பிட்டுதலுக்கு பல்கலைக்கழகத் தலைமையில் வலம் விடப்பட்டது. பலரும் குறிப்பிட்டுதலுக்கு பல்கலைக்கழகத் தலைமையில் இறுதி அப்பாலின் முதலிய விளையாட்டுடன் தொடர்புபடுத்தப்படும். அனைத்து பொருள் பெருமையை கையாளவிள்ளையான பல்கலைக்கழகத் தலைமையில், பலரும் இறுதி அப்பாலின் முதலிய விளையாட்டுடன் தொடர்பு பொருள்கூடியதாக இருக்கும்.

அப்பாலின் கல்வி, பல்கலைக்கழகம் பல்கலைக்கழகம் கல்விக்கான

இறுதி அப்பால் வலளவைக் காட்சிகளை தொடர்பிட்டு பலரும் பல்கலைக்கழகத் தலைமையில். 100 முதல் குறிப்பிட்டுதலுக்கு பல்கலைக்கழகத் தலைமையில் இறுதி அப்பாலின் முதலிய விளையாட்டுடன் தொடர்புபடுத்தப்பட்டது.

தொடர்புபாடு

இறுதி அப்பால் பலரும் பல்கலைக்கழகத் தலைமையில். 100 முதல் குறிப்பிட்டுதலுக்கு பல்கலைக்கழகத் தலைமையில் இறுதி அப்பாலின் முதலிய விளையாட்டுடன் தொடர்புபடுத்தப்பட்டது. பலரும் பல்கலைக்கழகத் தலைமையில் இறுதி அப்பாலின் முதலிய விளையாட்டுடன் தொடர்புபடுத்தப்பட்டது.

அப்பாலான், இறுதி மறியான்று பல்கலைக்கழகத்தில்

அப்பாலான் பல்கலைக்கழகத் தலைமையில் 5 முதல் குறிப்பிட்டுதலுக்கு பல்கலைக்கழகத் தலைமையில் இறுதி அப்பாலின் முதலிய விளையாட்டுடன் தொடர்புபடுத்தப்பட்டது. அப்பாலான் பல்கலைக்கழகத் தலைமையில் இறுதி அப்பாலின் முதலிய விளையாட்டுடன் தொடர்புபடுத்தப்பட்டது.
பசலவு

இந்த ஆய்வில் பங்கு பகறுவதற்கு உங்ளொல் அல்ைது உங்ள் குைந்லதக்கொண்டு அதன் முடிவு பிரசுரிக் பொதும்பின்னசரொ அல்ைது பிரசுரிக் அனுப்பிய பின்னசரொ இந்த ஆய்விலிருந்து விைக் ிக்க முடியொது.

பிரிவியக்கம

பயப்பாடை வரலாற்றுக்கான பிரிவியக்கான பவ்ஸ்வையாளருக்கிடையில் பங்காக்கம் கான்னாங்கன அண்டவராக உண்மையாகும் மொத்தொது பலகையாளரின் வெளியில் போராடிய பாரத்தினமான காலத்துடன் இருக்கும் குறிப்பிட்டியால், கம்பாரியாளர்கள் காண்யாளர்களுக்கு பாரத்தினமான காலத்துடன் இருக்கும் குறிப்பிட்டியால், கம்பாரியாளர்கள் காண்யாளர்களுக்கு பாரத்தினமான காலத்துடன் இருக்கும் குறிப்பிட்டியால் கம்பாரியாளர்கள் காண்யாளர்களுக்கு பாரத்தினமான காலத்துடன் இருக்கும் குறிப்பிட்டியால்.

பாம்பிரிவியக்கம

இன்றான நூற்றாண்டில் பிரிவியக்கான பிரிவியக்கான சாகவான காலமை, கம்பாரியாளரின் அண்டவரால் பாரத்தினமான பொருண்டினருக்கிடையில் கைதொளில்ளியாளரின் முடிவுக்குரிய துறைமுகத்துடன் இருக்கும் குறிப்பிட்டியால், இந்தியாவில் அமையும் பொருண்டினர் பொருண்டினர்க்கு பாரத்தினமான காலத்துடன் இருக்கும் குறிப்பிட்டியால், இந்தியாவில் அமையும் பொருண்டினர் பொருண்டினர்க்கு பாரத்தினமான காலத்துடன் இருக்கும் குறிப்பிட்டியால்.

பிரிவியக்கம

சம்பாத்திய பரிசார்க்களான பொருண்டினருக்கு புறநிலை பிரிவியக்கம் அண்டவராக கம்பாரியாளர்கள் பொருண்டிலிருந்து பொருண்டிலும் பொருண்டிலிடம். 011 - 2689 545 எனும் தனித்துவமான திசைத்தொண்டியல்ய நிறுவனம் நிறுவனத்தில் வரலாற்று திசைத்தொண்டியல்ய நிறுவனாடலின் கையேற்றத் திசைத்தொண்டியல்ய நிறுவனத்தில்.
Dr. சமுதிதொசசனொரத்தின்
மருத்துவமரபியல் முதுநிலை மொணவியமனிதமரபியையர்,
மருத்துவபீடம், கொழும்புபல்லைக்கு முன்னேரும்.
(0777697480, 0113165805)

சபரொசிரியர். வஜிர H.W.திசொநொயங்கான, 
மருத்துவமரபியையர்,
மனிதமரபியையர்,
மருத்துவபீடம் பிரிவு,
முற்றுகற்பெடை,
முன்னேரும் பல்லைக்கு முன்னேக
(0112689545)
CONSENT FORM

A STUDY OF TUBEROUS SCLEROSIS IN A COHORT OF SRI LANKAN PATIENTS

To be completed by the parent/guardian

The parent/guardian should complete the whole of this sheet himself/herself.

1. Have you read the information sheet? (Please keep a copy for yourself) YES/NO

2. Have you had an opportunity to discuss this study and ask any questions? YES/NO

3. Have you had satisfactory answers to all your questions? YES/NO

4. Have you received enough information about the study? YES/NO

5. Who explained the study to you?
................................................................................................................................................

6. Do you understand that you are free to withdraw your child from the study at any time, without having to give a reason and without affecting your child’s medical care? YES/NO

7. Sections of your child’s medical notes, including those held by the investigators relating to his/her participation in this study may be examined by other research assistants. All personal details will be treated as STRICTLY CONFIDENTIAL. Do you give your permission for these individuals to have access to your child’s records? YES/NO

8. Do you agree to have leftover blood samples and DNA is stored for future research into Genetics? YES/NO

9. Do you agree for the samples to be sent abroad? YES/NO
10. Have you had sufficient time to come to your decision?  YES/NO

11. Do you agree in your child take part in this study?  YES/NO

Parent’s/ Guardian’s name (BLOCK CAPITALS)

.................................................................................................................................

Parent’s/ Guardian’s signature..................................................................................Date...........................

To be completed by the investigator

I have explained the study to the above parent/guardian and he/ she has indicated his/her willingness in his/her child take part.

Name (BLOCK CAPITALS)

.................................................................................................................................

Signature of investigator.................................................................Date..........................
අසාංකීය තැනීම විශේෂව

විශේෂයේ මෙහෙයින් ආරාමිකයන්ගේ මෙහෙයින් නැස්මාරුි ප්‍රකාශකරණයක් ඉදිරිපත්

100 පරිසරියන් පිළිබද නැගැලී මහන්නේ.

10 /මෙ /මෙහෙයින් පැටුමා පිළිබද පිළිබද නැගැලී.

1. දැනටින් විශේෂයේ නැගැලීමවද පෙනියා? (මෙහෙයින් සහ කිත්කරගායම)

2. පිනි නැවැත්තේ නාවික පැයක් සම්පුර්ණ වියක් සහ නැවැත්තේ පිළිබදයි? (මෙ/නෙ)

3. පිටි ලෙස නැවැත්තේ දැනටි විශේෂයන්ගේ මෙහේරුද? (මෙ/නෙ)

4. දැනටි පිළිබද පුරාණය ආරාමිකයන්ගේ පිළිබද පිළිබද පිළිබදයි? (මෙ/නෙ)

5. කමමේ පිළිබද වැඩිසළමක් සහ කමමේ පිළිබදයි? (මෙ/නෙ)

6. ලෙස පිශීෂයේ මෙහෙයින් කමමේ පිළිබද පුරාණය ආරාමිකයන්ගේ පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදවක් සම්බන්ධව යින්වන්නේ පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව පිළිබදව
8. දාන්ත්‍රික ආභාංක විශේෂ විශේෂ පිළිබඳ කොටසේ සතුන් අධ්‍යයනවාසී ලියෝ විස්තර ක්‍රමව අතිනික්‍රමව අදායමක් විශේෂ විශේෂත්වයක්?

9. ආභාංක විශේෂ විශේෂ විශේෂ විශේෂත්වයක් විස්තර ක්‍රමව අතිනික්‍රමව අදායමක් විශේෂ විශේෂත්වයක්?

10. දාන්ත්‍රික විශේෂ විශේෂ විශේෂ විශේෂත්වයක් විස්තර ක්‍රමව අතිනික්‍රමව අදායමක් විශේෂ විශේෂත්වයක්?

11. දාන්ත්‍රික විශේෂ විශේෂ විශේෂ විශේෂත්වයක් විස්තර ක්‍රමව අතිනික්‍රමව අදායමක් විශේෂ විශේෂත්වයක්?

ාය (ඉ/න/වාර්තාප [=] ) .................................................................

දිනා ............................................................................................

දිනා ............................................................................................

කාර්යාන්තර ග්‍රාමීය විශේෂත්වයක් විස්තර ක්‍රමව අතිනික්‍රමව විශේෂ විශේෂත්වයක්.

කාර්යාන්තර ග්‍රාමීය විශේෂත්වයක් විස්තර ක්‍රමව අතිනික්‍රමව විශේෂ විශේෂත්වයක්.

ඉදිරිපත් අතිනික්‍රමව විශේෂත්වයක් ..................................................

ඉදිරිපත් අතිනික්‍රමව විශේෂත්වයක් ..................................................

ඉදිරිපත් අතිනික්‍රමව විශේෂත්වයක් ..................................................

ඉදිරිපත් අතිනික්‍රමව විශේෂත්වයක් ..................................................

ඉදිරිපත් අතිනික්‍රමව විශේෂත්වයක් ..................................................
ஆய்வு அடத்துவதற்கு எதிர்கரும்: ................................................

சுருக்கம் பொதுமான

தொண்டைலகிகளின் புதியபடி சுரண்குச்சுறுக்கள் (Tuberous Sclerosis) நிறுவிச்சிதியான் பாறைத்தொகுப்பு
தொண்டைலகிகளின் கலம்புரில் என்னுடைய விளக்கம் விளக்கம் கலைத்தியல் பாறை
பொதுமான ஆய்வை.

பொருளைச் சார்ந்த வலைத் திறனைந்து விளக்கப்படும் வைத்து.

நடுவத்திவன் பாறைத்தொகுப்பு பாறைத்தொகுப்பு விளக்கப்படும் வைத்து.

இந்த ஆய்வைப் பற்றியுள்ள முதலாக்கத்தின் ஒரு பிரதிலிபி நீங்கள் லவத்திருக்கும் அல்லாம்/இல்லை
இந்த ஆய்வைப் பற்றியுள்ள உங்களுக்குச் சந்தர்ப்பம் முடித்தது என்று காட்டுகிறீர்கள்?

இந்த ஆய்வைப் பற்றியுள்ள உங்களுக்கு குளைந்ததை
அளிக்கப்படும் மருத்துவசசலவயில் எவ்விதபொதிப்பு பொருட்பாடு?

இந்த ஆய்வின் நெடுவரை எந்தகையோரும் கதரிவிக்கும் அல்லது
உங்கள் குளைந்ததை அளிக்கப்படும்?

எந்தவிதொருளிலும் கையிலும் அல்லது
உங்கள் குளைந்ததை முடியும் என்பலதப்பை
புரிந்துகிறீர்கள்?

..........................................................
உங்களுக்கு ஆய்வொளியில் இருபது பதிவுகள் பயன்படுகின்றன. பங்குகபற்றும் மற்றும் உங்களுக்கு குறிப்பிடும் பதிவுகள் என்றும், இந்தவரையில் குறிப்பிடப்பட்ட பதிவுகள் என்றும், ஆய்வொளியில் முதல் பதிவுகள் தம்மிடும் ஆய்வொளியில் விளக்கம் செய்யப்பட்டுள்ளன. 

முதல் பதிவு குறிப்பிடும் பதிவுகளை முதல் பதிவுகளை எவர் விளக்க செய்தான்? 

இரண்டாம் பதிவு குறிப்பிடும் பதிவுகளை எவர் விளக்க செய்தான்? 

இரண்டாம் பதிவு குறிப்பிடும் பதிவுகளை எவர் விளக்க செய்தான்?

ஆம்/இல்லை

பங்குகபற்றுபவர் / பொதுவைருக்கு விளக்கம்: .................................................................

பங்கு (பங்குபடுத்தப்பட்டது): ................................................................. தின்ப...

ஆய்வொளியில் சான்றாக பயன்படுத்தப்பட்டோ இல்லை:

தாரம. இறை ஆய்வொளியில் பங்கு படுத்தி பொருள் குறிப்பிட்டோ இல்லை: தாரமானால் பங்குபடுத்தப்பட்ட இறைஆய்வொளியில் பங்கு படுத்தி மற்றும் தையும் செய்யப்பட்டோ.

ஆய்வொளியில் சான்றாக பயன்படுத்தப்பட்டோ: .............................................

பங்கு (பங்குபடுத்தப்பட்டது): .................................................................
DATA COLLECTION FORM

A STUDY OF TUBEROUS SCLEROSIS IN A COHORT OF SRI LANKEN PATIENTS

Subject Study Number

Name of subject

Age

Name of parent guardian

Address

Telephone number (Home)

(Mobile)

E mail

Referring physician

Date of referral

Hospital Ward

Clinic No/BHT No

Data Protection and Confidentiality

After completion of this page, ensure that the subject study number is entered on all pages of this booklet. Then detach this page and store separately from the remainder of the booklet.
Date of entry to study

Date on consent form

Date of birth

1. Sex            Male            Female

2. Age of onset

2.1 Features present at birth (i).................................
   (ii).................................
   (iii).................................

2.2 Features appeared after birth (i)..........................Noticed at........yrs.
   (ii)..........................Noticed at........yrs.
   (iii)..........................Noticed at........yrs.

3. Consanguinity     Yes            No

3.1 Family tree

I

II

III

IV

V
3.2 Additional Information

<table>
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<tr>
<th>Location in pedigree</th>
<th>Clinical/other info</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

4.0 Clinical Manifestations

4.1 Skins

<table>
<thead>
<tr>
<th>Hypomelanotic Macules**</th>
<th>Absent</th>
<th>Present</th>
<th>#........</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrous cephalic plaques**</td>
<td>Absent</td>
<td>Present</td>
<td>#........</td>
</tr>
<tr>
<td>Ungual Fibromas**</td>
<td>Absent</td>
<td>Present</td>
<td>#........</td>
</tr>
<tr>
<td>Shangreen Patch**</td>
<td>Absent</td>
<td>Present</td>
<td>#........</td>
</tr>
<tr>
<td>Confetti skin lesions*</td>
<td>Absent</td>
<td>Present</td>
<td>#........</td>
</tr>
<tr>
<td>Angiofibromas**</td>
<td>Grade:</td>
<td>0 1 2 3</td>
<td></td>
</tr>
</tbody>
</table>

[0 – None, 1 – Macular(Flat)lesions, 2 – Papular lesions < 3mm, 3 – Papular lesions > 3mm and / or extending to below mouth]

4.2 Brain

<table>
<thead>
<tr>
<th>Seizures</th>
<th>Never</th>
<th>Ever Present</th>
<th>Chronic</th>
</tr>
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<tbody>
<tr>
<td>Type of Seizures</td>
<td>Infantile spasms</td>
<td>Generalized</td>
<td></td>
</tr>
<tr>
<td>Partial</td>
<td>Intractable</td>
<td>Family history</td>
<td></td>
</tr>
<tr>
<td>Cortical Tubers**</td>
<td>Absent</td>
<td>Present</td>
<td>#........</td>
</tr>
<tr>
<td>Sub Ependymal Nodules**</td>
<td>Absent</td>
<td>Present</td>
<td>#........</td>
</tr>
</tbody>
</table>
Subject Study Number

Sub Ependymal Giant cell □ Absent □ Present #........
Astrocytomass**

4.3 Kidney

Renal Angiomyolipomas** Grade: 0 1 2 3
[0 – None, 1 – One or more, all < 1 cm, 2 – Multiple, One or more > 1cm, all < 4cm, 3 – Multiple, One or more > 4cm]

Renal Cysts*
Grade: 0 1 2 3 4
[0 – None, 1 – 1 to 2 small (< 2cm), 2 – More than 2 small(< 2cm), 3 – More than 2 and at least one > 2cm, 4 – Classic polycystic kidney disease, multiple cysts with renal enlargement]

4.4 Liver

Hepatic Angiomyolipomas** Grade: 0 1 2 3
[0 – None, 1 – One or more, all < 1 cm, 2 – Multiple, One or more > 1cm, all < 4cm, 3 – Multiple, One or more > 4cm]

4.5 Eye

Retinal Hamartomas** □ Absent □ Present #........
Retinal Acromic Patches* □ Absent □ Present #........

4.6 Heart

Cardiac Rhabdomyomas** □ Ever Present □ Never

4.7 Lung

Lymphangiomatosis** □ Severe □ Mild □ Absent

4.8 Mouth and Teeth

Dental Enamel Pits* □ Absent □ Present #........
Intra Oral Fibromas* □ Absent □ Present #........

** - Major Criteria No.............
* - Minor Criteria No...............
### 5 Investigation Findings

<table>
<thead>
<tr>
<th>Investigation</th>
<th>Date</th>
<th>Findings and conclusions</th>
</tr>
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<tbody>
<tr>
<td>MRI Scan</td>
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<tr>
<td>CT Scan</td>
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<tr>
<td>Ultra Sound Scan</td>
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<td>EEG</td>
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<td>2D Echo</td>
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<td>Biopsy Histology</td>
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<tr>
<td>Other</td>
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### 6 Behavioral / Neuropsychiatric phenotypes

**Mental Retardation**

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<thead>
<tr>
<th>Grade:</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
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</thead>
</table>

[0 – None (reading above 3\textsuperscript{rd} Grade), 1 – Mild (reading at < 3\textsuperscript{rd} Grade), 2 – Moderate (some speech no reading), 3 – Severe (no speech intelligible to a stranger)]
Subject Study Number

Autism

☐ Severe  ☐ Mild/Moderate  ☐ Absent

CARS 2

60

[Childhood Autism Rating Scale 2]

7 Genetics

7.1 Pre test Counseling  ☐ Done  ☐ Not done

7.2 Genetic Testing  ☐ Done  ☐ Not done

☐ TSC 1 sequencing  ☐ Pos  ☐ Neg  ☐ Not tested

☐ TSC 1 MLPA  ☐ Pos  ☐ Neg  ☐ Not tested

☐ TSC 2 sequencing  ☐ Pos  ☐ Neg  ☐ Not tested

☐ TSC 2 MLPA  ☐ Pos  ☐ Neg  ☐ Not tested

7.3 Mode of Inheritance  ☐ de novo  ☐ Inherited  ☐ Not known

7.4 Post test Counseling  ☐ Done  ☐ Not done

8 Social Status

Education level of parents

Father  Grade:  0 1 2 3 4

Mother  Grade:  0 1 2 3 4

[0 – Not gone to school, 1 – Schooling up to Grade 5, 2 – Schooling up to Grade 10, 3 – Schooling up to Grade A/L, 4 – Graduate]

Employment  ☐ Father  ☐ Mother  ☐ Both

Monthly income Rs..............................

Who looks after the child.................................................................
Schooling  □ Yes  □ No
If schooling  □ Normal school  □ Special school

9 Treatments

9.1 Drug Treatments  (i)……………………………………………………………………
                     (ii)……………………………………………………………………
                     (iii)……………………………………………………………………

9.2 Surgical Treatments (i)……………………………………………………………………
                      (ii)……………………………………………………………………
                      (iii)……………………………………………………………………

Final Diagnosis…………………………………………………………………………………………
Subject Study Number

Patient Date

<table>
<thead>
<tr>
<th>Label</th>
<th>Volume</th>
<th>Storage</th>
<th>Comments</th>
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</thead>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>1.</td>
<td>1.</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>2.</td>
<td>2.</td>
<td></td>
</tr>
</tbody>
</table>

COMMENTS

Record reasons for missing data and any additional relevant comments. ENSURE THAT ANONYMITY IS PRESERVED.

The booklet should be signed when ALL available data have been entered and cross checked with relevant data recorded elsewhere in this booklet.

Signed................................................................. Date.........................................................

Investigator/Research Assistant

References:

Appendix 4: Genetic testing details & primer sequences

Appendix 4 contains the genetic testing in detail and the primer sequences, probes used in Sanger sequencing and MLPA techniques.

**PCR procedure in detail**

In the first step of the PCR, original double stranded DNA (dsDNA) is denatured applying a high temperature of 95°C so that the hydrogen bond between the two complementary DNA threads are separated into single stranded DNA (ssDNA). The ssDNA works as a template for the target sequence, and when the reaction is cooled down to 60°C, the primers are able to anneal to the ssDNA.

At the next step, DNA polymerase begins synthesizing new DNA strands from the end of the primer; this part of the PCR is called extension and happens at a temperature of 72°C.

See table 1 for the PCR program.

**Table 1: PCR Program**

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme activation</td>
<td>95°C</td>
<td>10min</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C</td>
<td>30sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>60°C</td>
<td>30sec</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>1min</td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C</td>
<td>7min</td>
</tr>
<tr>
<td>Soak</td>
<td>10°C</td>
<td>∞</td>
</tr>
</tbody>
</table>

As PCR cycles through these thermal steps, newly synthesized DNA is used as templates for replication, setting in a chain reaction where the DNA template is exponentially amplified. In this project we have used the AmpliTaq 360DNA polymerase kit form Applied Biosystems.

After PCR the products were purified with Agencourt’s AMPure XP beads using a Biomek FXP automated system (Beckman Coulter). The AMPure XP beads utilize Agencourt’s solid-phase paramagnetic bead technology and removes excess primers, nucleotides, salts and enzymes. ([https://www.beckmancoulter.com](https://www.beckmancoulter.com)).
Primer design

The primers are 20-25 bp long and have a M13 tail at the 5’end. The forward primer is tailed with the M13 F sequence 5’- tgtaaaacgacggccagt-3’ and the reverse primer is tailed with M13 R sequence 5’- caggaaacagctatgacc-3’. This tailing makes it possible to use the same complementary primers for sequencing the target sequence after PCR. The PCR primers are also designed with the same melting temperatures, which allow a better workflow as one can use the same PCR program for all the reactions. Table 2 and 3 shows the primer sequence in the upper case, and the M13 tailing in lower case for TSC1 and TSC2 respectively.

Table 2: Primer sequence of coding regions in TSC1

<table>
<thead>
<tr>
<th>Exon</th>
<th>Primer pair</th>
<th>Primer sequence (’5-&gt;3’)</th>
<th>Design</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>TSC1_3F</td>
<td>tgtaaaacgacggccagtTGCATGATTCTTACTTCATGTGTGC</td>
<td>RSA000572713</td>
</tr>
<tr>
<td>3</td>
<td>TSC1_3R</td>
<td>caggaaacagctatgacceCCCTCTTCTATAAAACTCGCCAAAGA</td>
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<td>4</td>
<td>TSC1_4F</td>
<td>tgtaaaacgacggccagtGAACAATGTCATCAGTGGCGCA</td>
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<td>TSC1_5R</td>
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<td>-------------</td>
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<td>TSC2_19R_v2</td>
<td>caggaaacagctatgaccGAGCTCAGTGAGTGAGCTTCGCC</td>
<td>RSA000023560</td>
</tr>
<tr>
<td>20</td>
<td>TSC2_20F</td>
<td>tgtaaaacgcggccagtAGTGGAGTGAGTGAGCTTCGCC</td>
<td>RSA000023560</td>
</tr>
<tr>
<td>20</td>
<td>TSC2_20R_v2</td>
<td>caggaaacagctatgaccGAGCTCAGTGAGTGAGCTTCGCC</td>
<td>RSA000023560</td>
</tr>
<tr>
<td>21</td>
<td>TSC2_21F</td>
<td>tgtaaaacgcggccagtAGTGGAGTGAGTGAGCTTCGCC</td>
<td>RSA000023560</td>
</tr>
<tr>
<td>21</td>
<td>TSC2_21R</td>
<td>caggaaacagctatgaccGAGCTCAGTGAGTGAGCTTCGCC</td>
<td>RSA000023560</td>
</tr>
</tbody>
</table>
### Sanger sequencing procedure in detail

For sequencing, a mixture of target DNA template, short DNA primer complementary to target DNA, DNA polymerase, 4 nucleotides and 4 dideoxynucleotides with fluorescent tags are used.

The process starts with heating the mixture 96°C until the two complementary DNA strands separate. Then the temperature lowers to 50°C and the primers bind to the template. In the next step the temperature is raised up to 60°C and the enzyme binds to DNA to create a copy of the template DNA. Detailed sequencing program is presented in Table 4.
Table 4: Sequencing program

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>96°C</td>
<td>10 sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>60°C</td>
<td>5 sec</td>
</tr>
<tr>
<td>Extension</td>
<td>50°C</td>
<td>4 min</td>
</tr>
<tr>
<td>Soak</td>
<td>10°C</td>
<td>∞</td>
</tr>
</tbody>
</table>

The sequence of newly created copy of the template DNA is complementary to the original DNA template. DNA polymerase incorporates both nucleotides and fluorescently tagged ddNTDs. When the ddNTDs is incorporated, the reaction is terminated. The ddNTDs can be incorporated at any position in the strands, and the strands will be terminated at different positions resulting in a mixture of strands with many different lengths. Once the sequencing reaction is completed, the mixture is purified using Agencourt’s CleanSEQ® magnetic beads and the unwanted material is removed with mainly the same principle as Agencourt’s AMPure beads (https://www.beckmancoulter.com).

**Multiplex Ligation dependent Probe Amplification procedure in detail**

At the first step DNA was denatured and incubated for 20 hours with a mixture of MLPA probes for hybridization. We used the SALSA MLPA probekit P124-C1- 0112 TSC1 and the SALSA MLPA probekit P046-C1- 1011 TSC2, both products from MRC-Holland, and consisting of minimum one probe pair per exon (www.mlpa.com). A thermal cycler was set up to run MLPA program with a denaturation temperature of 98°C, then cooling down to 25°C, again denaturation at 95°C and hybridization at 60°C. During this overnight process, the probes are hybridized to immediately adjacent target sequences. The ligation reaction was started by adding the ligation mix to each sample.

The ligation program was run at 54°C for 15 minutes. Only the probes that were hybridized to immediately adjacent target sequences could be ligated during this ligation reaction. After the ligation reaction, we used PCR to amplify the number of probes. It was performed using Veriti 96 well thermal cycler by Applied Biosystems with MLPA – PCR program. See Table 5 for details on the program.
Table 5: MLPA PCR program

<table>
<thead>
<tr>
<th></th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Denaturation</strong></td>
<td>95°C</td>
<td>30sec</td>
</tr>
<tr>
<td><strong>Annealing</strong></td>
<td>60°C</td>
<td>30sec</td>
</tr>
<tr>
<td><strong>Extension</strong></td>
<td>72°C</td>
<td>1min</td>
</tr>
<tr>
<td><strong>Final extension</strong></td>
<td>72°C</td>
<td>20min</td>
</tr>
<tr>
<td><strong>Soak</strong></td>
<td>15°C</td>
<td>∞</td>
</tr>
</tbody>
</table>

Ligated probes were amplified with this PCR. The number of probe ligation products is a measure of the number of target sequences in the sample. The amplification products were separated using capillary electrophoresis. The amplified products which were separated generate a signal, and these results were interpreted using Gene Marker® software program. (For full probe sequence go to www.mlpa.com).
Appendix 5: Classification of sequence variants

Classification of sequence variants at Dept. of Med. Gen., Ullevål

Pathogenic = destructive for protein production and/or function

Well documented normal variant

Not well documented, but likely to be normal variant

Not documented uncertain variant (VUS – variant of unknown significance)

Not documented, but likely to be pathogenic variant

Well documented pathogenic variant

Documented neutral variants:

Reports in databases with ”normal controls” with data on frequency.

Available databases:


NHLBI exome sequencing project (http://evs.gs.washington.edu)

1000 genomes (http://browser.1000genomes.org)
Frequency must be considered in context of population size.

relevant disease (penetrance, expressivity, age of onset)

mode of inheritance (dominant, recessive)

Papers reporting variants in normal controls.

Seen in our own patient material.

Found in "in house" controls.

Recurrent mutations in class 5.

When X-linked inheritance with homogeneous expressivity and complete penetrance is found in healthy father, it can be concluded that the variant is neutral.

**Not well documented, but likely to be normal variant:**

Synonymous variants that are likely to be neutral, but lack sufficient documentation in order to belong to group 1.

Exception from rule:

The variant changes one of the last three or three first bases of an exon.

The variant introduces a new splice site (tested in prediction tools in Alamut).

Intron variants outside consensus splice site, which are probably neutral variants, but lack sufficient documentation in order to belong to group 1.

If prediction tools predicts that a de novo splice site has been made, next step could be to analyse mRNA, given that information exists on where the protein is expressed.

Missense variants that are reported to be neutral (e.g. do not segregate with disease).

Class 1 and 2 will not be sent out in report, only in rare occasions where the disorder has 100% penetrance and when the mutation is de novo.
Variants of uncertain significance (and lack of documentation):

Usually missense and splice site variants (without documentation), however, separate considerations might move them to class 2 or 4.

Missense:

Generally:

Pathogenicity clues from AlaMut is included in VUS evaluation form, but value of these clues is very uncertain.

Conservation between species. NCBI HomoloGene

Individual evaluation according to protein:

Does the amino acid change involve:

Cysteins involved in S-S-bindings?

Binding sites for co factors, such as Ca^{2+}?

An AA that is known to be modified (glycosylated, phosphorylated etc)?

An AA which is involved in a binding site for other proteins?

(UniProt/litteratur)

Is the variant located in a well characterised and important domain?

(UniProt, litteratur)

Are there known/reported other pathogenic variants in same location?

Are there known/reported other pathogenic variants close to the location?

(HGMD, PubMed, Google)

Variant in consensus splice site:

Consensus splice site [(n)_{20},XXX---------------XXXnnnnnn]:

donor splice site: 3 last bases of exon (X) and 6 first bases of intron (n)

acceptor splice site: 20 last bases of intron (n) and first bases in exon (X)
Prediction tools (AlaMut): MaxEntScan
NNSPLICE
GeneSplicer
Human Splicing Finder

4. Not documented, but likely to be pathogenic variant;

Nonsense og frame shift variants
Premature stop codon (PSC) is likely to activate nonsense mediated mRNA decay (NMD).

NB: NMD will only be activated if PSC is appr. 50 bp upstream om last exon-intron boundary. If PSC is located in a C-terminal tail of the protein, it can lead to a truncated, but functional protein.

Splice site
Variants in +1,+2---- -2,-1 can be regarded as pathogenic (very few exeptions).

Other issues
De novo variants in genes with good association to phenotype can be regarded as pathogenic.

Class 3 and 4 variants are to be reported. If a class 3 variant is identified in addition to a variant from class 4 or 5, it may be omitted from the report.

Consider to check for cosegregation /familystudies, or mRNA analyses.

Well documented pathogenic variants
When previously identified and evaluated in the department and/or fulfills the following:
Well documented in publications:
Previously identified in at least 2 patients, and described in 2 independent papers

De novo inheritance in patient

Well described in >1 publication

Well described in 1 publication, and supported by other studies (family studies or functional studies)

Reported in a locus specific database and/or in HGMD database alone, is not sufficient. Published documentation is necessary.

If the variant is ever identified in healthy controls, the frequency has to be according to knowledge on mode of inheritance, penetrance and expressivity for the relevant disease.