mTOR inhibition as a therapeutic strategy in tuberous sclerosis or sporadic lymphangioleiomyomatosis

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Tables and figures

Summary

Tuberous sclerosis is an autosomal dominant multisystem disorder characterised by the development of benign tumours in many organs, including the brain, skin, kidneys and heart, seizures and intellectual disability.

The condition results from mutations in either of two genes, *TSC1* (encoding TSC1) or *TSC2* (encoding TSC2). Loss of functional TSC1 or TSC2 leads to activation of mTORC1 (mammalian target of rapamycin complex 1), a key regulator of multiple cellular processes including cell growth and division.

Lymphangioleiomyomatosis (LAM) is a lung disorder which can lead to respiratory failure and is characterised by the proliferation of abnormal 'LAM' cells LAM occurs in patients with tuberous sclerosis and can also occur as a sporadic disorder due to acquired mutations in TSC2. Renal angiomyolipomas are benign tumours which affects80% of patients with tuberous sclerosis and 40% of patients with sporadic LAM.

Sirolimus is an inhibitor of mTORC1 and is used in clinical practice as an immunosuppressant. Preclinical studies suggest that TSC1 or TSC2 deficiency renders cells sensitive to mTOR inhibition.

This thesis describes a phase 2 trial to assess the safety and efficacy of 2 years of treatment with sirolimus for renal angiomyolipomas in patients with tuberous sclerosis or LAM. Response of angiomyolipoma was the primary efficacy end point. Effects of sirolimus on lung function and neurocognitive function are also reported.

Our data show an angiomyolipoma response rate, by RECIST criteria, of 50% in the intention to treat population. There was little change in lung function. Recall but not recognition memory tended to improve. Adverse events were common and consistent with the known toxicities of sirolimus. Our findings suggest that mTOR inhibition is a potential therapeutic strategy in the treatment of tuberous sclerosis and lymphangioleiomyomatosis.

Acknowledgments

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Roles

The trial protocol was written by DMD, JS, SJ, AT, CK, PdV, DM, KP, JC and TD. DMD specifically wrote the sections dealing with assessment of response, trial statistics, pharmacovigilance and trial management.

DMD and JS obtained the required permissions to conduct the trial, with assistance from SJ, AT, JC, CK and AS. DMD co-ordinated and monitored the trial. Pharmacovigilance was performed by DMD in conjunction with JS.

Patients were managed by DMD, JS, SJ, AT, CK and AS. DMD managed the patients seen in Cardiff in conjunction with JS.

Imaging analysis was performed by KP, JC and TD. Cognitive function testing was performed by DM and the cognitive function data analysed by DM, PdV and JS.

Genotyping of the patients with tuberous sclerosis was performed by the Wales Gene Park.

Abbreviations used

ADPKD	autosomal dominant polycystic kidney disease
AE	adverse event
AMIPB	Adult Memory and Information Processing Battery
AML	angiomyolipoma
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
AR	adverse reaction
Atg13	autophagy-related gene 13
ATM	ataxia telangiectasia mutated
ATP	adenosine triphosphate
ATR	ataxia telangiectasia and Rad3 related
Bcl-2	B-cell lymphoma 2
BHD	Birt-Hogg-Dube
Bnip3	Bcl-2/adenovirus E1B 19-kDa interacting protein 3
C_{max}	maximum plasma concentration
CANTAB	Cambridge Neuropsychological Test Automated Batteries
CDK1	cyclin dependent kinase 1
CHMP	Committee for Medicinal Products for Human Use
COPD	chronic obstructive pulmonary disease
CPK	creatine phosphokinase
CR	complete Response
CT	computerised tomography
CTA	clinical trials authorisation
CTCAE	National Cancer Institute's Common Terminology Criteria for

	Adverse Events
CYP3A4	cytochrome P450 3A4
DAPK	death-associated protein kinase,
Deptor	DEP-domain containing mTOR-interacting protein
DLCO	diffusion lung capacity for carbon monoxide
DNA-PKs	DNA-dependent protein kinase
E6AP	E6-associated protein
4E-BP	eukaryotic initiation factor 4E-binding protein
EGCG	epigallocatechin gallate
EGFR	epidermal growth factor receptor
ER	endoplasmic reticulum
EU	European Union
FAK	focal adhesion kinase
FAT	FRAP-ATM-TRRAP
FEV1	forced expiratory volume in 1 second
FGFR	fibroblast growth factor receptor
FIPP200	focal adhesion kinase [FAK] family interacting protein of 200 kD
FMR1	fragile X mental retardation–1
FRB	FKBP12-rapamycin binding
FVC	forced vital capacity
FKBP12	FK506-binding protein
GAP	GTPase-activating protein
GEF	guanine nucleotide exchange factors
GFR	glomerular filtration rate
GSK3	glycogen synthase kinase 3

GTP	guanosine triphosphate
HEAT	Huntington, EF3, A subunit of PP2A, TOR
HERC1	homologous to the E6-AP (UBE3A) carboxyl terminus domain and RCC1 (CHC1)-like domain (RLD) 1
Hg	mercury
HIF	hypoxia inducible factor
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
hVps34	human vacuolar protein sorting mutant 34
IDED	Intra-Dimensional Extra-Dimensional shifting task
IKK	Ikappa beta kinase
IQ	intelligence quotient
IRS	insulin receptor substrate
KO	knock out
LAM	lymphangioleiomyomatosis
LD	longest diameter
LDH	lactate dehydrogenase
LOH	loss of heterozygosity
MAPK	mitogen activated protein kinase
MDRD	Modification of Diet in Renal Disease
mGluR	group 1 metabotropic glutamate receptor
MLPA	multiplex ligation-dependent probe amplification
mLST8	mammalian lethal with SEC13 protein 8
MREC	multi-centre research ethics committee
MRI	magnetic resonance imaging
MAP4K3	mitogen-activated protein kinase kinase kinase kinase 3

Mcl-1	myeloid cell leukemia sequence 1
MEF	mouse embryonic fibroblast
MMPH	multifocal micronodular pneumocyte hyperplasia
mSIN1	mammalian stress-activated protein kinase interacting protein
mTOR	mammalian target of rapamycin
mTORC1	mTOR complex 1
mTORC2	mTOR complex 2
NADE	p75NTR-associated cell death executor
NF1	neurofibromatosis type 1
OMIM	on line Mendelian inheritance in man
PA	phosphatidic acid
PAM	protein associated with Myc
PARP	poly(ADP-Ribose) Polymerase
PD	progressive Disease
PDGFR	platelet-derived growth factor receptor
PEC	perivascular epithelioid cell
PEComa	perivascular epithelioid cell tumour
PET	positron emission tomography
PIKK	phosphoinositide 3-kinase related kinase
PKCα	protein kinase Cα
PKD	polycystic kidney disease
PL-D1	phospholipase-D1
PML	promyelocytic leukemia
PPARγ	peroxisome proliferator-activated receptor-γ
PR	partial Response

PRAS40	proline rich AKT substrate 40kDa
PROTOR1	protein observed with Rictor-1
PtdIns (3,4,5)P3	phosphotidylinositol (3,4,5)-triphosphate
PTEN	phosphatase and tensin homolog
Raptor	regulatory associated protein of mTOR
RECIST	Response Evaluation Criteria in Solid Tumors
REDD1	regulated in development and DNA damage response
Rheb	ras homologue enhanced in brain
Rictor	rapamycin-insensitive companion of mTOR
RSK	ribosomal S6 kinase
SAE	serious adverse event
SD	stable Disease
SEGA	subependymal giant cell astrocytoma
SEN	subependymal nodule
SGK1	serum- and glucocorticoid-induced protein kinase 1
S-LAM	sporadic LAM
SMW	Spatial Working Memory task
SoC	Stockings of Cambridge
SPC	summary of product characteristics
SREBP1	sterol regulatory element binding protein 1
Ste20	(sterile20)-related kinase mammalian protein kinase
t_{max}	time after absorption maximum drug plasma concentration
TCTP	translationally controlled tumour protein
TGFβ	transforming growth factor beta
TLC	total lung capacity

TNFα	tumour necrosis factorα
TSC	tuberous sclerosis
TSC-LAM	tuberous sclerosis associated LAM
ULK	unc-51-like kinase
VHL	Von Hippel-Lindau

Chapter 1: The clinical features of tuberous sclerosis and lymphangioleiomyomatosis

1.1 Tuberous sclerosis

Tuberous sclerosis (TSC) is an autosomal dominant, multisystem disorder affecting approximately 1 in 6,000 individuals [1]. The condition affects multiple organs and has a variable phenotype, both in terms of the type and severity of features seen in patients. The main features of the condition [2] are set out below:

1.1.1 Neurological

The most common, and often the most disabling features of tuberous sclerosis, are seizures, intellectual disability and neurobehavioral abnormalities, such as autistic spectrum disorder. The characteristic macroscopic brain lesions associated with tuberous sclerosis are cortical and subcortical tubers, subependymal nodules in the periventricular zone lining the lateral ventricles and subependymal giant cell astrocytomas. Tubers are developmental abnormalities of the cerebral cortex characterized histologically by a loss of the normal six-layered structure of the cortex and by dysmorphic neurons, large astrocytes, and a unique type of cell known as a giant cell [3]. Subependymal giant cell astrocytomas are tumours of mixed glial and neuronal lineage. Growth of these lesions can block circulation of the cerebrospinal fluid, leading to progressive ventricular dilatation and increased intracranial pressure. Subependymal nodules are asymptomatic hamartomas arising from the walls of the lateral and third ventricles.

White matter abnormalities seen by imaging in patients with tuberous sclerosis include superficial white matter abnormalities associated with cortical tubers, radial white

matter bands and cyst like white matter lesions [4]. Superficial white matter abnormalities are related to almost all cortical tubers and reflect reduced myelin or increased gliotic reaction. Radial white matter bands reflect developmental migration defects of neurons and glial cells. White matter cyst like lesions are located in deep white matter and although the pathogenesis of cyst like lesions remains unclear, they are considered to reflect cystic degeneration of white matter or dilated perivascular spaces. Diffusion tensor imaging has shown that white matter structures that appear normal on conventional magnetic resonance imaging (MRI) may be accompanied by microstructural changes such as gliosis and myelinisation defects [5].

Around 80% of children with tuberous sclerosis have seizures [6] and the commonest presentation of the condition is with seizures, particularly infantile spasms, in infancy or early childhood [7]. Many types of seizure have been reported. The seizures are often refractory to treatment and may require the use of multiple antiepileptic drugs. Infantile spasms, which are associated with a poor cognitive prognosis, occur in 20-30% of children with tuberous sclerosis.

The distribution of intelligence quotient (IQ) scores of individuals with tuberous sclerosis suggests the existence of two distinct subgroups [6]. Approximately 30% are profoundly impaired with very low IQs (too low to be assessed by standardized measures of cognitive function); the remaining 70% have normally distributed IQs with a slight reduction in mean IQ relative to unaffected individuals. This second group is affected by specific cognitive deficits, such as deficiency in long term memory skills, attentional skills and executive skills [8-10].

Neurodevelopmental disorders, including attention deficit hyperactivity disorder, aggressive /disruptive behaviour and autistic spectrum disorder, are often seen in people

with tuberous sclerosis. Approximately 20-60% of patients with tuberous sclerosis meet criteria for autistic spectrum disorder, with the criteria being met in 17% of those with an IQ in the normal range [8]. In adults, psychiatric features such as depression and anxiety are also frequently encountered [11].

1.1.2. Renal

Renal features of tuberous sclerosis include angiomyolipomas (AMLs), oncocytomas, simple cysts, polycystic kidney disease and renal cell carcinoma [12]. AMLs are found in approximately 80% of tuberous sclerosis patients. They are usually multiple and bilateral and often asymptomatic but AMLs can cause flank pain, impair renal function, and spontaneously bleed causing haematuria and occasionally life threatening haemorrhage [13]. A retrospective study of 102 patients with renal AMLs (both sporadic and tuberous sclerosis associated) with a median follow up of 4 years showed no evidence of spontaneous regression of any lesions [14].

Current conventional management options are observation or active intervention by either surgical resection or selective arterial embolisation. Some investigators have suggested that prophylactic renal selective arterial embolisation is the management of choice in all patients with symptomatic lesions or asymptomatic lesions of >4cm, as they may be at higher risk of rupture/haemorrhage. However, a recent study used digital subtraction angiography to classify AMLs into three grades (grade 1, minimal vascularity; grade 2, moderate vascularity; grade 3, marked vascularity) and found that none of the asymptomatic grade 1 tumours caused bleeding [15]. Hence, it might be that the extent of vascular tissue in an AML is the important predictor of subsequent haemorrhage, rather than the size of the overall tumour.

Renal cysts are common in tuberous sclerosis and usually asymptomatic but a polycystic kidney phenotype occurs in those patients with a contiguous deletion of *TSC2* and *PKD1* genes, who often progress to renal failure in early adult life [16]. The overall incidence of renal cell carcinomas in patients with tuberous sclerosis is thought to be similar to that in the general population but renal cell carcinomas associated with tuberous sclerosis tends to occur in younger patients [17]. A variety of types of renal cell carcinoma, including clear cell, papillary, and chromophobe carcinoma, have been reported in patients with tuberous sclerosis.

1.1.3 Pulmonary

Lymphangioleiomyomatosis (LAM) is a rare disorder of the lungs and lymphatics which can occur sporadically or in association with tuberous sclerosis [18]. LAM almost exclusively affects females and radiographic studies suggest it affects 40% of female patients with tuberous sclerosis, although many of these women are asymptomatic [19]. LAM is discussed further in section 1.2.

Focal proliferations of type II pneumocytes, termed multifocal micronodular pneumocyte hyperplasia (MMPH), may also be a pulmonary manifestation of tuberous sclerosis but is not thought to be clinically significant [20].

1.1.4 Skin

Several types of skin lesion can occur in tuberous sclerosis [2]. Hypomelanotic macules are often present at birth or develop during infancy and are best seen in ultraviolet light using a Wood's lamp. Facial angiofibroma are flesh coloured or red papules, typically occurring over the nose, nasolabial folds, cheeks and chin, usually developing by the age of 5 years. Shagreen patches, which are elevated irregular brown or flesh coloured lesions, typically develop in the lumbar sacral area during childhood. Ungual fibromas

are flesh-coloured or pink nodules that occur on the finger or toe nail beds. A linear depression in the nail can suggest the presence of a subungual fibroma. Gingival fibromas can also occur.

1.1.5 Cardiac

Cardiac rhabdomyomas are an early manifestation of tuberous sclerosis, appearing at 22 to 28 weeks of gestation [21]. They usually regress during childhood and are normally asymptomatic but can be associated with obstructive heart failure or arrhythmias. Cardiac rhabdomyomas can be an unexpected finding on routine antenatal scans where they are associated with a substantial risk of tuberous sclerosis, especially if the lesions are multiple.

1.1.6 Ophthalmological

The commonest ophthalmologic manifestations of tuberous sclerosis are retinal hamartomas [22]. They seldom affect vision.

1.1.7 Neuroendocrine tumours

Case reports have documented the occurrence of neuroendocrine tumours of the pituitary, pancreas and parathyroid, as well as phaeochromocytomas in patients with tuberous sclerosis. It is unclear if this is just coincidental or there is an association between neuroendocrine tumours and tuberous sclerosis [23].

1.1.8 Gastrointestinal polyps

Gastrointestinal polyps (generally hamartomatous) may be common in patients with tuberous sclerosis [24-27]. The prevalence of gastrointestinal polyps is uncertain but may be underestimated because they are usually asymptomatic. Gould *et al* documented that 14 of 18 (78%) patients with tuberous sclerosis had intestinal polyps (25).

1.1.9 Skeletal involvement

Bone manifestations of tuberous sclerosis include cyst like lesions, hyperostosis of the inner table of the skull, osteoblastic changes, periosteal new bone formation, and scoliosis [28].

1.1.10 Clinical assessment and diagnostic criteria

The diagnosis of tuberous sclerosis is usually based on clinical and radiological features using the criteria shown in table 1 [29]. These are divided into major and minor criteria. As many of the features associated with tuberous sclerosis can occur sporadically, the presence of one major feature suggests but does not establish the diagnosis. The minor features are even less specific. No single feature is diagnostic and many show age related penetrance.

Table 1: Diagnostic criterion for tuberous sclerosis [29]

Major Features

- Facial angiofibromas or forehead plaque
- Nontraumatic ungual or periungual fibromas
- Hypomelanotic macules (three or more)
- Shagreen patch (connective tissue nevus)
- Multiple retinal nodular hamartomas
- Cortical tuber [1]
- Subependymal nodule
- Subependymal giant cell astrocytoma
- Cardiac rhabdomyoma, single or multiple
- Lymphangiomyomatosis [2]
- Renal angiomyolipoma [2]

Minor Features

- Multiple randomly distributed pits in dental enamel
- Hamartomatous rectal polyps
- Bone cysts
- Cerebral white matter radial migration lines [1]
- Gingival fibromas
- Nonrenal hamartoma
- Retinal achromic patch
- "Confetti" skin lesions
- Multiple renal cysts

Definite tuberous sclerosis: Two major features or one major feature plus two minor features

Probable tuberous sclerosis: One major feature plus one minor feature

Possible tuberous sclerosis: One major feature or two or more minor features

1. If the cerebral cortical dysplasia and cerebral white matter migration tracts occur together they should be counted as one rather than two features of TSC.

2. When both lymphangioleiomyomatosis and renal angiomyolipomas are present, other features of tuberous sclerosis must be present before a definite diagnosis is made.

1.1.11 Clinical management and surveillance

Clinical management requires a multi-disciplinary approach tailored to the specific problems of the affected individual. Ideally, patients should be seen by specialists with an interest in the condition. For some areas there are disease specific deviations from standard practice, such as in anti-epileptic drug selection, and there is a risk of over treatment, for example, unnecessary kidney resection due to atypical AMLs being mistaken for renal carcinomas.

In individuals suspected of having tuberous sclerosis the following initial assessments should be considered to establish the diagnosis and to identify complications amenable to treatment [30]:

- Medical history and three generational family history
- Clinical examination, including fundoscopy and skin examination under ultaviolet light
- Cranial imaging e.g. MRI
- Renal ultrasound
- Echocardiography in infants
- Electroencephalography, if seizures are present
- Neurodevelopmental and behavioural evaluation
- Chest CT and spirometry for adult females, in the presence of respiratory symptoms

After initial evaluation the following surveillance has been suggested in patients with tuberous sclerosis:

- Renal ultrasonography every one to three years, replaced with renal MRI if multiple or large angiomyolipomas are present
- Cranial MRI every one to three years for children and adolescents
- Electroencephalography, if seizures are problematic
- Neurological, developmental and behavioural evaluations
- Echocardiography, if cardiac symptoms indicate
- Chest CT, if chest symptoms indicate

However, the value of performing 'routine' cranial and renal imaging in the absence of symptoms or pathology is debatable. An alternative approach is a clinical evaluation at regular intervals (e.g. annually) with a focus on neurological, renal, skin, pulmonary, cardiac, developmental and psychological problems, with investigations directed accordingly and interim assessment if clinical problems develop.

1.2 Clinical features of lymphangioleiomyomatosis (LAM)

LAM is a rare disorder, occurring almost exclusively in premenopausal women. The clinical features result from the accumulation of 'LAM' cells in the pulmonary interstitium and the lymphatic system and cystic degeneration of the lung, generally leading to progressive respiratory failure [18, 31].

Sporadic LAM (S-LAM) is a rare condition with a prevalence of approximately 1 in 1,000,000 [32] in the whole population. However, LAM is much more common in patients with tuberous sclerosis (TSC-LAM). Radiological evidence of LAM is present in 40% of adult females with tuberous sclerosis although only 2-3% will develop symptoms of LAM [33, 34].

The disease tends to present between the menarche and menopause, with the mean age of onset being 34 years. Respiratory problems tend to dominate the clinical course, with the most common being progressive dyspnoea and pneumothorax [35-37]. Other respiratory problems seen include cough, haemoptysis, chyloptysis and chylous pleural effusions. Extrapulmonary manifestations of LAM are lymphadenopathy, lymphangioleiomyomas (large cystic masses which most commonly occur in the abdomen, retroperitoneum and pelvis), chylous acites and, in approximately half of patients, renal AMLs [38-40].

The differential diagnosis of LAM includes Langerhans' cell histiocytosis, COPD (particularly α1-antitrypsin related COPD), sarcoidosis, Sjogren syndrome, follicular bronchiolitis and lymphocytic interstitial pneumonitis, chronic hypersensitivity pneumonitis and Birt-Hogg-Dube syndrome.

The chest radiograph is often normal in early disease but may show a pneumothorax or pleural effusion. The most common findings in established disease are reticulonodular shadowing and cysts or bullae with overexpansion of the lungs. The disease has a characteristic high resolution CT appearance with multiple thin-walled cysts scattered throughout the lung fields with normal intervening parenchyma.

Pulmonary function tests may be normal in early disease although the majority of patients have airflow obstruction with a decreased FEV1. The lung volumes are generally preserved and patients may have an increased total lung capacity (TLC) because of lung hyperinflation and an increase in residual volume may be seen because of air trapping. Poor gas exchange results in reduced diffusion capacity (DLCO) [36].

The gold standard for the diagnosis of LAM is a tissue biopsy of the lung or involved lymphatics. Tissue biopsy is not necessary to establish the diagnosis of LAM in patients with a characteristic chest CT appearance and other manifestations of the disease such as AMLs. All patients with LAM should undergo a careful examination for evidence of tuberous sclerosis.

Patients with sporadic LAM generally develop progressive airflow obstruction. In a US study of a cohort of over 300 LAM patients a mean decline of FEV1 of 75ml a year and of DLCO of 0.69 mL/min/mm Hg was reported [41]. A European study suggested that the forced FEV1 declined at a rate of 118mL per year [32]. There are no prospective studies on survival but retrospective data suggest the 10-year survival is in the order of 55-71% [37]

Current treatment options for LAM are limited. Two small retrospective studies have tried to assess the efficacy of progesterone, one showed a significant reduction in the rate of decline in DLCO in treated, compared with untreated, patients, the other showed no difference in lung function between treated and untreated patients [32, 42]. Lung transplantation is a potential option for patients with severe LAM (typically when FEV1 and/or DLCO are below 40% of predicted). In a retrospective series of 34 patients with LAM who underwent a lung transplant survival was 65% at five years [43].

1.3 Histopathology of AMLs and LAM

AMLs and LAM lesions are considered perivascular epithelioid cell (PEComa) tumours, mesenchymal tumours that share a histologically and immunohistochemically distinctive cell type, the perivascular epithelioid cell or PEC [44]. These cells are characterised by a perivascular location and a epithelial or spindle shape morphology, resembling smooth muscle cells with variable accumulation of lipid, which can mimic adiopocytes. PEComas contain blood vessels, in which the PECs appears to comprise the muscular wall [45]. Great variation in the relative proportion of epithlioid, spindle and lipid distended cells occurs. Immunohistochemically, PEComas express myogenic and melanocytic markers, such as gp100, MelanA/Mart1, micropthalmia transcription factor, and smooth muscle markers, including smooth muscle actin and, less commonly, desmin [45].

PECs are present in a group of lesions including AML, LAM tissue, clear-cell "sugar" tumor of the lung and extrapulmonary sites, clear-cell myomelanocytic tumor of the

falciform ligament/ligamentum teres and abdominopelvic sarcoma of perivascular epithelioid cells.

AML is the commonest PEComa and occurs both sporadically and in association with tuberous sclerosis. Classic AMLs have a triphasic histology with tortuous blood vessels, myoid appearing PEC and lipid distended PEC [46]. Blood vessels with walls composed of PECs and vessels with normal smooth muscle walls are seen in AMLs [47].

In the lung, LAM consists of a proliferation of myoid appearing PECs distributed around bronchial lymphatics, interlobular septae and pleura. LAM of lymph nodes or the thoracic duct is characterised by perilymphatic proliferation of PECs [44].

1.4 The genetic basis of tuberous sclerosis and LAM

1.4.1 *TSC1* and *TSC2*

TSC1 (OMIM #605284) and *TSC2* (OMIM #191092) were identified in 1997 and 1992, respectively, [48, 49] as the genes mutated in tuberous sclerosis. The *TSC1* gene on chromosome 9 consists of 23 exons, the first two of which are untranslated and the second is alternatively spliced. Hamartin or TSC1, the protein product of *TSC1* consists of 1164 amino acids. The *TSC2* gene on chromosome 16 consists of 41 coding exons and a noncoding leader exon, of which exons 25, 26 and 31 are alternatively spliced. The protein product, tuberin, or TSC2 is a full length isoform of 1807 amino acids. A region spanning residues 1517-1674, encoded by exons 34-38 contains a GTPase activating domain. TSC2 also contains a calmodulin binding domain and an oestrogen receptor binding domain.

Studies of the *TSC1* and *TSC2* genes in patients with tuberous sclerosis have revealed a wide spectrum of mutations, but there are no particular regions within the genes in which mutations occur at a high rate. Tuberous sclerosis associated *TSC2* mutations include missense, nonsense and frameshift deletions/insertions and splice junction mutations. Also, significant numbers of large (exonic and whole-gene) deletions have been reported [50-54]. *TSC1* mutations in patients with tuberous sclerosis are primarily small deletions and insertions and nonsense mutations. Pathogenic *TSC1* missense changes are rare. However, recent studies of *TSC1* missense variants in bladder cancers [55] and in patients with TSC [56, 57] have shown that TSC1 amino acid substitutions can be pathogenic.

Approximately 10-20% of patients who meet the diagnostic criteria for TSC do not have any identifiable mutations. These patients appear to have a distinct phenotype from patients in whom a mutation has been identified with a lower incidence of neurological features and renal findings than those with *TSC2* mutations and a lower incidence of seizures but a higher incidence of both renal angiomyolipomas and pulmonary lymphangioleiomyomatosis compared with tuberous sclerosis patients with *TSC1* mutations [58].

Mutations in *TSC2* are about 5 times more common than mutations in *TSC1* in the sporadic tuberous sclerosis population, whereas the ratio is approximately 1:1 in large families with multiple generations affected. Patients with mutations in *TSC2* are more likely to have a higher number and/or more severe clinical features than those with mutations in *TSC1* [51, 54].

1.4.2 Modulation of mTORC1 and mTORC2 activity by TSC1/TSC2

The proteins encoded by *TSC1* and *TSC2* form a complex within cells. TSC1 stabilises TSC2, possibly by inhibiting its interaction with HERC1 ubiquitin ligase [59]. One of the best defined functions of the TSC1/TSC2 complex is in the regulation of mTOR, which in mammalian cells exists in two functionally distinct complexes, mTORC1 and mTORC2.

mTORC1 is a key mediator of multiple cell functions. TSC2's GAP (GTPase-activating protein) domain stimulates the intrinsic GTPase activity of the small G-protein, Rheb, enhancing the conversion of the active GTP bound form of Rheb to the inactive GDP form. Rheb, when it is GTP-bound [60] and farnesylated [61], activates mTORC1,

possibly by inhibiting FKBP38, an endogenous inhibitor of mTOR [62]. In addition, Rheb-GTP stimulates phospholipase-D1 (PL-D1) to generate phosphatidic acid, a positive effector of mTORC1 activation. Thus, loss of functional TSC1/2 leads to overactivation of mTORC1. The contribution of Rheb's regulation of PL-D1 and FKBP38 to mTORC1 activation, relative to Rheb's direct binding to mTOR, remains to be fully defined.

Translationally controlled tumour protein (TCTP) has been suggested to act as a Rheb guanine nucleotide exchange factor (GEF) [63] but this has not been consistently demonstrated [64, 65].

The TSC1/TSC2 complex appears to also promote mTORC2 activation in a Rheb independent manner by an unknown mechanism [66].

1.4.3 Molecular pathology of AMLs and LAM

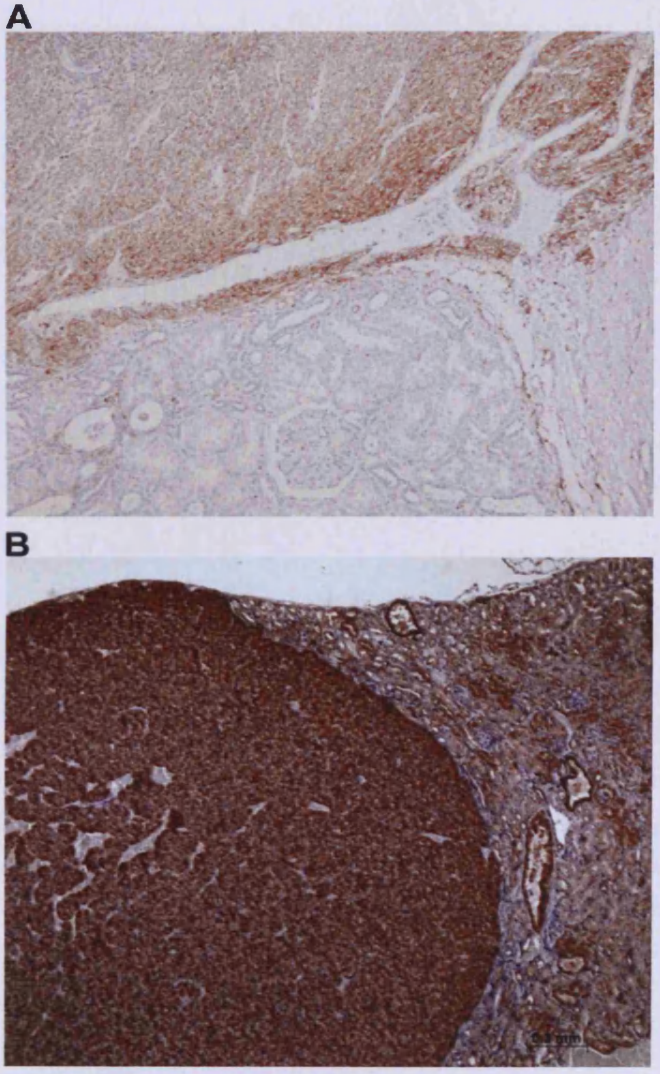
Mutation of both alleles of either *TSC1* or *TSC2* appears to be required for the formation of most lesions in tuberous sclerosis, consistent with the two-hit tumour suppressor gene model of Knudson [67]. Many second hit mutations are large deletions and LOH in either *TSC1* or *TSC2* has been reported in most tuberous sclerosis associated angiomyolipomas, SEGA's and cardiac rhabdomyomas but only rarely in cortical tubers [68-70]. Haploinsufficiency may play a role in some of the pathology associated with tuberous sclerosis and it has also been suggested that inappropriate phosphorylation of TSC2 may mimic a second hit in brain and cardiac lesions [71-73].

Patients with sporadic LAM do not have germline mutations in *TSC1* or *TSC2*. It has been suggested that histologically benign appearing LAM cells arise from a renal angiomyolipoma or an alternative unknown site and migrate to the lung in a process that

has been termed 'benign metastasis' [74]. Genetic analysis has demonstrated identical *TSC2* mutations and LOH at the *TSC2* locus in paired lung LAM tissue and renal angiomyolipomas but not in normal lung tissue [75-77]. In samples from a patient with recurrent sporadic LAM post lung transplantation, the same mutation in *TSC2* was found in LAM cells from both the native and transplanted lung [78]. LAM cells have been isolated from the circulatory system of patients with LAM, indicating that these migratory cells have the potential to survive in the circulation [79].

Evidence of mTORC1 pathway activation is consistently seen in lung LAM tissue [80] and in angiomyolipomas [81] and other tuberous sclerosis related lesions [72, 82] (Fig. 1).

Figure 1: mTORC1 pathway activation in human and mouse TSC-associated renal tumours



*Immunohistochemical staining with an anti-phospho- ribosomal protein S6 antibody (Ser240/Ser244) of (**A**) a renal angiomyolipoma from a patient with tuberous sclerosis showing phospho-S6-positive cells in the tumour (upper part of section) and (**B**) phospho-S6-positive cells in a renal cell carcinoma (left) and scattered small cysts (right) from the kidney of a Tsc1+/– mouse. Figure courtesy of Dr C. Bonnet (Institute of Medical Genetics, Cardiff University).*

Chapter 2: The pharmacology of sirolimus

2.1 Introduction

Sirolimus, (Rapamune, Wyeth Pharmaceuticals), is a macrolide antibiotic first discovered in 1975. It was isolated from a strain of *Streptomyces hygroscopicus* cultured from a soil sample from Rapa Nui, one of the Easter Islands in the South Pacific. Hence, the original name of the drug, rapamycin [83-85]. Sirolimus is used in clinical practice as an immunosuppressant in organ transplantation [86] and in drug eluting stents as an inhibitor of restenosis after angioplasty [87]. Analogues of rapamycin (rapalogs) are currently used for the treatment of renal cancer and mantle cell lymphoma [88].

Sirolimus forms an intracellular complex with the peptidyl-prolyl cis-trans isomerase FKBP12 [89]. This drug/receptor complex binds to mTOR at a location N-terminal to the kinase domain. Binding of FKBP12-sirolimus leads to inhibition of mTOR function in a poorly understood manner.

Sirolimus is available as 1 mg and 2 mg coated tablets or as a 1mg/ml oral solution. In the context of renal transplantation the usual dosage regimen is a 6 mg oral loading dose followed by 2 mg once daily. When used with cyclosporin the dose is titrated to obtain whole blood trough levels of 4 to 12 ng/ml. As a single agent the sirolimus dose is adjusted to obtain whole blood trough levels of 12 to 20 ng/ml.

2.2 Pharmacokinetics

Following administration of the oral solution, sirolimus is rapidly absorbed, with a time to peak concentration of 1 hour in healthy subjects receiving single doses. The half-life

in renal transplant patients was 62±16h. Pharmacokinetic parameters for sirolimus obtained from 19 renal transplant patients receiving cyclosporin, corticosteroids and daily doses of 2 mg sirolimus solution in a phase III clinical trial, were; C_{max} 12.2±6.2 ng/ml, t_{max} 3.01±2.40h, AUC_t 158±70 ng·h/ml. Mean sirolimus whole blood trough levels were 7.2 ng/ml (range 4.0 to 11 ng/ml) [90].

2.3 Interactions with other medications

Sirolimus is extensively metabolised by the CYP3A4 isozyme in the intestinal wall and liver. Sirolimus is also a substrate for the multidrug efflux pump, P-glycoprotein, located in the small intestine. Strong inhibitors of CYP3A4 (such as ketoconazole, voriconazole, itraconazole, telithromycin and clarithromycin) decrease the metabolism of sirolimus and may markedly increase sirolimus levels. Strong inducers of CYP3A4 (such as rifampicin or rifabutin) increase the metabolism of sirolimus and may markedly decrease sirolimus levels. Co-administration of sirolimus with strong inhibitors of CYP3A4 or inducers of CYP3A4 is not recommended. Grapefruit juice affects CYP3A4 mediated metabolism and should therefore be avoided [90].

2.4 Adverse reactions

The most commonly reported adverse drug reactions (occurring in >10% of patients) associated with sirolimus are thrombocytopenia, anaemia, pyrexia, hypertension, hypokalaemia, hypophosphataemia, urinary tract infection, hypercholesterolaemia, hyperglycaemia, hypertriglyceridaemia, abdominal pain, lymphocoele, peripheral oedema, arthralgia, acne, diarrhoea, oral mucositis, constipation, nausea, headache, increased blood creatinine, and increased blood lactate dehydrogenase (LDH) [90] (table 2).

Table 2: Toxicities associated with sirolimus (adapted from sirolimus summary of product characteristics [90])

Class	Very common (≥ 1/10)	Common (≥ 1/100 to <1/10)	Uncommon (≥ 1/1000 to <1/100)
Infections	Urinary tract infection	Sepsis Pneumonia Pyelonephritis Fungal, viral, and bacterial infections	
Neoplasms benign and malignant		Skin Cancer	Lymphoma / post transplant lymphoproliferative disorder
Blood and lymphatic system disorders	Thrombocytopenia Anaemia	Thrombocytopenic purpura/haemolytic uraemic syndrome Leukopenia Neutropenia	Pancytopenia
Metabolism and nutrition disorders	Hypokalaemia Hypophosphataemia Hypercholesterolaemia Hyperglycaemia Hypertriglyceridaemia		
Nervous system disorders	Headache		
Cardiac disorders		Tachycardia	Pericardial effusion
Vascular disorders	Lymphocele	Deep vein thrombosis	Pulmonary embolism

	Hypertension		
Respiratory, thoracic, and mediastinal disorders		Pneumonitis Pleural effusion Epistaxis	Pulmonary haemorrhage
Gastrointestinal disorders	Abdominal pain Stomatitis Diarrhoea Constipation Nausea		Pancreatitis
Hepatobiliary disorders		Abnormal liver function tests	
Skin and subcutaneous tissue disorders	Acne like rash		
Musculoskeletal and connective tissue disorders	Arthralgia	Osteonecrosis	
Renal and urinary disorders		Proteinuria	Nephrotic syndrome
General disorders	Peripheral oedema Pyrexia	Impaired healing	
Other investigations	Blood lactate dehydrogenase increased Blood creatinine increased		

Chapter 3: The mTOR pathway

3.1 Introduction

The ability to sense and integrate diverse signals is essential for cells to modulate physiological responses. Eukaryotic cells have the capacity to sense signals from their growth environment such as the availability of nutrients and also receive additional signals in the form of growth factors, cytokines and cell-adhesion molecules. A complex intracellular signalling network integrates the diverse inputs and directs appropriate cellular responses. The mTOR signalling pathway (Fig. 2) plays a central role integrating the growth factor, nutrient and stress signals in the control of multiple cellular functions, allowing a cell to balance catabolic and anabolic processes.

The serine/threonine kinase, mTOR, is a member of the phosphoinositide 3-kinase related kinase (PIKK) family, along with ATM, ATR, DNA-PKs and hSMG1. The domain structure of mTOR consists of multiple HEAT repeats (for Huntington, EF3, A subunit of PP2A, TOR) at the NH2 terminus domain, the FAT domain (FRAP-ATM-TRRAP), the FRB (FKBP12-rapamycin binding) domain, the kinase domain and another FAT domain close to the C-terminus.

Figure 2: The TSC/mTOR signalling pathway

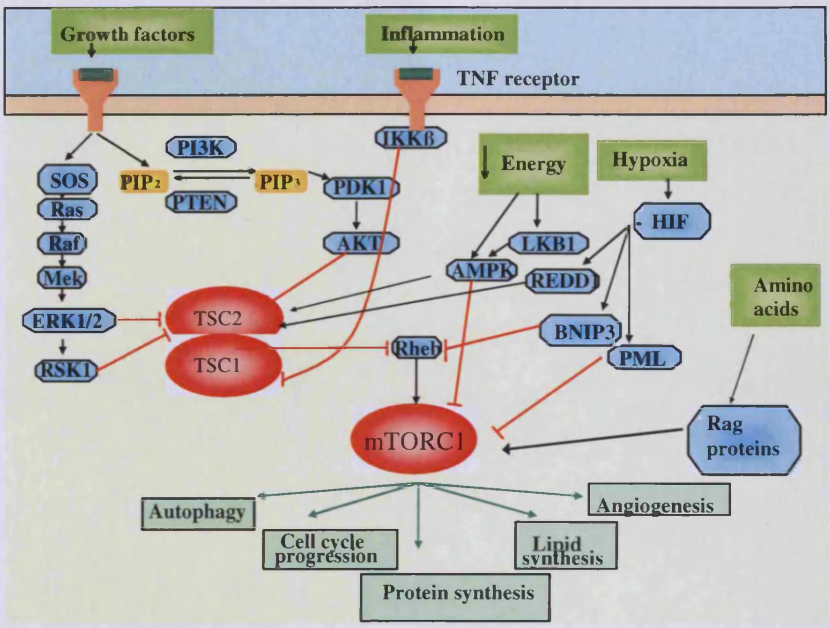


Figure 2: mTORC1 integrates multiple signals in the control of key cell processes.

In mammalian cells, mTOR exists in two functionally distinct complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 is comprised of mTOR, Raptor (regulatory associated protein of mTOR), mLST8 (mammalian lethal with SEC13 protein 8), also known as GβL, proline rich AKT substrate 40kDa (PRAS40) and DEP-domain containing mTOR-interacting protein (Deptor) [61, 91-97]. Within mTORC1, Raptor contributes to mTOR substrate specificity by binding to substrates via TOR signalling [61, 91-98] motifs on the proteins. The role of mLST8 is unclear whilst PRAS40 and Deptor have been characterised as negative regulators of mTORC1. The best characterised mTORC1 substrates are the ribosomal protein S6 kinases (S6K1 and S6K2) and the eukaryotic initiation factor 4E-binding proteins (4E-BP1, 4E-BP2 and 4E-BP3). The second mTOR containing protein complex, mTORC2, is comprised of 6 different proteins, several of which are common to mTORC1 and

mTORC2: mTOR, rictor (rapamycin-insensitive companion of mTOR), mammalian stress-activated protein kinase interacting protein (mSIN1), protein observed with Rictor-1 (PROTOR1), mLST8/Gbl and Deptor [91, 92, 94, 99-105].

3.2 Functions of the TSC1/TSC2/mTORC1 pathway

3.2.1 Control of cell growth, macromolecule synthesis and metabolism

Promotion of cell growth (an increase in cell mass) is an evolutionary conserved mTORC1 function which extends to the control of organ and organism size [106, 107]. In model systems loss of *TSC1/2* is associated with an increase in cell and organ size [108-110] and treatment with sirolimus results in a reduction in cell size [106].

mTORC1 plays a key role in the regulation of the synthesis of macromolecules such as proteins and lipids which are required for cell growth [111]. mTORC1 regulates the activity of the translational machinery as a whole and also specifically controls the translation of a subset of messenger RNAs (mRNAs) that are thought to promote cell growth and proliferation. mTORC1 regulates cap-dependent translation by phosphorylating 4E-BP1 preventing its binding to eIF4E [112]. Phosphorylation of S6K1 by mTORC1 leads to increases mRNA synthesis, cap-dependent translation and elongation and translation of ribosomal proteins. The activation of mTORC1 also leads to promotion of ribosomal biogenesis by stimulating the transcription of ribosomal RNA [113].

mTORC1 regulates glycolysis, sterol and lipid biosynthesis and the pentose phosphate pathway [111]. mTORC1 activates sterol regulatory element binding protein 1 (SREBP1) and peroxisome proliferator-activated receptor-γ (PPARγ), two transcription

factors that control the expression of genes encoding proteins involved in cholesterol, fatty acid, triglyceride and phospholipid synthesis. Inhibition of mTOR with sirolimus reduces the activity of PPARγ and constitutive activation of mTORC1 via loss of *TSC2* increases PPAR-γ activity and expression of SREBP1, promoting adipogenesis [114, 115].

An unbiased metabolomic screen in *Tsc1* or *2* deficient MEFs showed an increase in the intermediates of glycolysis and the pentose phosphate pathway [111]. Much of the glycolytic response downstream of mTORC1 is mediated by increased HIF1α activity. However, cells lacking *TSC2* have reduced membrane localization of the glucose transporters Glut1, Glut2, and Glut4 following growth factor stimulation [116]. In concordance with this, LAM tissue and tuberous sclerosis associated AMLs do not exhibit abnormal uptake of [18F]2-fluoro-2-deoxyglucose during PET scanning [117].

3.2.2 Control of stress responses, apoptosis and autophagy

Autophagy is a catabolic pathway for the degradation of cellular components, including organelles and long-lived proteins via regulated lysosomal degradation. Autophagy is up-regulated in mammalian cells under certain conditions of cellular stress such as nutrient restriction and hypoxia. Under stress the degradation of organelles and proteins through autophagy provides substrates to sustain anabolic processes and energy production. Activation of mTOR inhibits autophagy while inhibition of mTOR with sirolimus induces autophagy. A mechanism by which mTORC1 controls autophagy is via phosphorylation of autophagy-related gene 13 (Atg13), focal adhesion kinase family interacting protein of 200kDa (FIP200) and the mammalian Atg1 homologues unc-51-like kinase 1 and 2 (ULK1 and ULK2). Binding of Atg13 stabilises ULK and facilitates

the phosphorylation of FIP200 by ULK leading to an activation of autophagy [118]. *TSC1-/-* MEFs exhibit abrogated autophagy which remains sirolimus inducible [119].

mTOR has a complex effect on apoptosis, having pro and anti-apoptotic effects depending on factors such as cell type and environment [120, 121]. *TSC1/2* deficient cells exhibit increased sensitivity to some apoptosis inducing stimuli e.g. glucose deprivation [122-124] with sirolimus having a protective effect but decreased sensitivity to other apoptotic inducing stimuli e.g. hypoxia [125]. The sensitisation of *TSC1/2* deficient cells to some proapoptotic stimuli may be explained by inhibition of AKT activity by a negative feedback mechanism induced by the hyperactive mTORC1 or as a result of inflexibility of metabolism and stress responses. The resistance of *TSC1/2* deficient cells to specific apoptotic stimuli may be attributable to increased HIF1α activity, *Mcl-1* expression, sustained glycogen synthase kinase 3 beta inhibition [126] or aberrant AMPK [127] and p27 function [128, 129]. . Loss of *TSC2* in a murine *c-myc* driven lymphoma model led to accelerated oncogenesis due to defective apoptosis despite compromised AKT phosphorylation [130] and *TSC1/2* deficient cells exhibit resistance to anoikis [131].

3.2.3 Cell cycle progression

In mammalian cells mTORC1 promotes transition from G1 to S and entry into mitosis. In many cell types these transitions are blocked by sirolimus. Sirolimus prevents the mitogen induced decrease in levels of p27, a cyclin-dependent kinase inhibitor, and decreases levels of cyclin D1 and D3. Phosphorylation of Raptor during mitosis facilitates cell cycle transit through G2/M, with the cyclin-dependent kinase 1 (cdk1/cdc2) and glycogen synthase kinase 3 (GSK3) pathways probably being the two

mitosis-regulated protein kinase pathways involved [132]. CDK1 also phosphorylates TSC1 during G2/M [133]. Mitotic phosphorylated raptor promotes translation by internal ribosome entry sites on mRNA during mitosis and is associated with sirolimus resistance [134].

The effect of activated mTOR signalling on cell cycle progression may be context dependent. Thus, while increased mTOR signalling promotes proliferation under favourable conditions, increased mTOR signalling can promote senescence under some conditions in the context of conflicting signals [135, 136]. Depending on the cell type and other factors, p53 activation can result in apoptosis or reversible cell cycle arrest (quiescence) or irreversible cell cycle arrest (senescence). Activation of p53 increases the expression of several genes, including *TSC2* whose protein products inhibit mTOR activity. In some cell lines activation of p53 and the consequent inhibition of mTOR leads to quiescence. *Tsc2(-/-)* MEFs display early senescence that is rescued by loss of p53 [137]. However, if *Tsc2* is knocked down by siRNA, activation of p53 leads to senescence [138].

3.2.4 Mitochondrial biogenesis

Mitochondrial biogenesis and metabolism are both regulated by mTORC1. The expression of a number of genes involved in mitochondrial biogenesis, cellular mitochondrial DNA content mitochondrial membrane potential, oxygen consumption, and ATP synthetic capacity correlates with mTORC1 activity [139, 140]. $Tsc2^{-/-}$ MEFs have increased mitochondrial DNA content, oxygen consumption and expression of genes encoding mitochondrial components and transcription factors which promote mitochondrial biogenesis, all of which are down regulated by sirolimus [140].

3.2.5 Stem cell maintenance

Excessive mTORC1 activity leads to stem cell depletion, which is at least in part mediated by the induction of stem cell senescence and can be reversed by sirolimus [141, 142]. Conditional *Tsc1* deletion in the haematopoietic stem cells in mice leads to a reduction in the self renewal of haematopoietic stem cells and decreased haematopoiesis, which can be rescued by sirolimus [143, 144]. In the *Drosophila* ovary, disruption of either the *Tsc1* or *Tsc2* gene in the germline stem cell leads to precocious differentiation and loss. The germline stem cell loss can be rescued by treatment with sirolimus [145]. Restriction of stem cell depletion may partially account for the increase in longevity seen in mice following treatment with sirolimus [146].

3.3.6 Cell motility

The evidence regarding the role of the *TSC* genes in cell motility is conflicting; with some studies suggesting loss of TSC1/2 promotes motility [147-149] and others suggesting TSC1/2 loss inhibits motility [150]. This may reflect cell type dependent effects or variation in assay methods used.

3.2.7 Epithelial-mesenchymal transition

TSC1/2 deficient cells exhibit a reduction in plasma membrane located E-cadherin, in a sirolimus sensitive manner, and consequently reduced cell-cell adhesion and loss of contact inhibition. Non adherent *TSC1/2* deficient cells acquire the molecular and functional characteristics of epithelial-mesenchymal transition, rendering them resistant to anoikis [131].

3.2.8 mTORC1 or sirolimus insensitive functions of TSC1/2

A number of other functions of the TSC1/TSC2 complex appear to be mTORC1 independent or sirolimus insensitive. The TSC1/TSC2 complex inhibits Wnt induced β-catenin transcriptional activity and promotes the degradation of β-catenin [151]. AMPK is activated in *TSC2*-null cells via Rheb independently of its regulation of mTORC1 signalling [152]. Loss of *TSC2* leads to up regulation of notch signalling in a sirolimus insensitive manner [153]. *TSC2* interacts with the ezrin-radixin-moesin family of actin-binding proteins Rho and Rac1 to regulate cell adhesion and cell motility, as well as influencing the actin cytoskeleton, in a sirolimus insensitive manner [154, 155]. The tuberous sclerosis proteins also regulate the formation of primary cilia via a sirolimus insensitive mechanism [156]. Additionally, Rheb inhibits B-Raf and C-Raf kinase activity [157, 158] and regulates the interaction of FKBP38 with Bcl-2 and Bcl-XL in a sirolimus insensitive manner [159].

3.2.9 The regulation of the mTORC1/TSC pathway

mTORC1 acts as a signal integrator for four major regulatory inputs - growth factors, energy, oxygen and amino acids.

3.2.10 Growth factors

Growth factors stimulate mTORC1 through the activation of intracellular signalling pathways. For example, the binding of insulin to its cell surface receptor promotes the tyrosine kinase activity of the receptor, the recruitment of insulin receptor substrate 1 (IRS1), the production of phosphotidylinositol (3,4,5)-triphosphate [PtdIns (3,4,5)P3] and the recruitment and activation of AKT at the plasma membrane. Activated AKT phosphorylates several downstream substrates and stimulates mTORC1 through two interconnected mechanisms, inhibiting both PRAS40 and TSC2. AKT directly phosphorylates TSC2 on a number of residues, including Ser939, Ser981 and Thr1462, inhibiting the ability of TSC2 to repress mTORC1 activation, possibly by disruption or increased degradation of the TSC1/TSC2 complex or by altered subcellular localisation, due to altered interaction with the cytosolic anchor protein 14-3-3. TSC2 bound by 14-3-3 in response to AKT phosphorylation is sequestered away from membrane-bound TSC1 and Rheb [160].

PRAS40 has been described as either an mTORC1 inhibitor, mTORC1 substrate or both. Activated AKT phosphorylates PRAS40, resulting in the dissociation of PRAS40 from mTORC1, possibly mediated by the binding of 14-3-3 to phosphorylated PRAS40 [161-163].

Mitogen activated Ras-ERK signalling has been shown to trigger the activation of mTORC1 signalling via ERK- and RSK-dependent phosphorylation of TSC2 at Ser664 and Ser1798 respectively [72, 164]. RSK also directly targets the mTORC1 complex by phosphorylation of Raptor and directly promoting mTOR1 kinase activity [165].

3.2.11 Energy

Depletion of intracellular energy leads to inhibition of mTORC1. The energy status of a cell is signalled to mTORC1 by AMP-activated protein kinase (AMPK), a master sensor of intracellular energy status. When intracellular ATP levels decline and AMP levels increase, the binding of AMP to AMPK primes it for activation by upstream kinases including the tumour suppressor LKB1 [166]. Activated AMPK then interacts with multiple cellular metabolic targets to maintain intracellular ATP homeostasis by up-regulation of catabolic processes which generate ATP and down regulation of ATP consuming processes.

AMPK inhibits mTORC1 in two ways. AMPK phosphorylates TSC2 on at least two residues (Ser1387 and Thr127), which may activate its GAP activity [123]. Additionally, AMPK directly phophorylates Raptor inhibiting mTORC1 kinase activity [167]. The AMPK dependent phosphorylation at Ser1387 primes TSC2 for further phosphorylation by glycogen synthase kinase 3β (GSK3β) within the context of Wnt signalling. This provides an opportunity for cross talk between Wnt signalling and energy sensing pathways. Wnt which inhibits GSK3β appears to activate mTORC1 by overcoming AMPK mediated activating effects in the TSC1-TSC2 complex [168].

The transcription factor REDD1 (regulated in development and DNA damage response) is induced in response to energy stress, hypoxia, DNA damage, glucocorticoids and oxidative stress. A REDD1 mediated decrease in mTORC1 signalling in response to hypoxia is TSC2 dependent and can be inhibited by direct activation of AMPK by AMP mimetics. REDD1 has been shown to bind to 14-3-3 proteins and may activate the TSC1-TSC2 complex by releasing TSC2 from its association with inhibitory 14-3-3 proteins[169]. TSC1/2 deficient MEFs are highly susceptible to apoptosis upon glucose starvation with sirolimus having a protective effect [123, 124].

3.2.12 Oxygen levels

Hypoxia inhibits mTORC1 by a number of mechanisms. As oxygen is required for aerobic ATP production via mitochondrial oxidative phosphorylation, hypoxia causes energy stress and AMPK mediated phosphorylation and activation of TSC1/TSC2 [170]. Hypoxia induced HIF1 activity leads to induction of REDD1 and hence of TSC1/TSC2 complex activity [171, 172]. Hypoxia also results in TSC1/2 independent inhibition of mTORC1 via the protein promyelocytic leukemia (PML) [173] which negatively regulates the association of mTOR with Rheb and Bnip3 which mediates a decrease in Rheb GTP levels [174].

In response to hypoxia, TSC deficient cells fail to down regulate mTORC1 activity, exhibit an exaggerated and prolonged increase in HIF activity, continue to proliferation at a rate much higher that their wild type counterparts in a manner that is, at least partially, insensitive to sirolimus and are protected against hypoxia induced apoptosis [125, 169, 171, 172, 175].

3.2.13 Amino acids

Amino acids, particularly the branched chain amino acid leucine, represent a strong signal that positively regulates mTORC1. The primary amino acid sensor is unknown but candidate downstream mediators have emerged, including the Ste20 (sterile20)-related kinase mammalian protein kinase, MAP4K3 (mitogen-activated protein kinase kinase kinase kinase 3) [176] and hVps34 (human vacuolar protein sorting mutant 34) [177]. Intracellular amino acid activation of mTORC1 is known to be independent of TSC1/2 as the mTOR remains sensitive to amino acid deprivation in cells that lack TSC1 or TSC2 [177]. A protein complex called Ragulator, which interacts with Rag GTPases, is necessary for amino acid dependent mTORC1 activation and mediates a translocation to lysosomal membrane surfaces.[178-180].

3.2.14 Other regulators of TSC1/2 and of the mTOR pathway

In addition to the key signals described above, other cellular conditions and signalling pathways regulate mTORC1 activity including genotoxic stress, oxidative stress, endoplasmic reticulum stress, phosphatidic acid and cytokines. Over 50 proteins have been shown to interact with TSC1 or TSC2 or both and the functional significance of many of these interactions remains to be fully elucidated [181, 182]. TSC1 contains phosphorylation sites for the kinases Cdk1 [133], GSK3 [151] and IKKbeta [183]. TSC2 is phosphorylated by Erk [72], Akt [184-186], GSK3 [168], DAPK1 [187], the p38 activated kinase MK2 [188], AMPK [123], RSK1 [164] and FAK [189].

Genotoxic stress reduces mTORC1 activity by a number of mechanisms. Activation of p53 in response to DNA damage activates AMPK which in turn activates TSC2 [190]. Additionally, p53 negatively regulates mTORC1 by increasing the transcription of

PTEN and TSC2 [190, 191]. Oxidative stress in the form of reactive oxygen species leads mTORC1 repression due to ATM mediated activation of TSC2 via LKB1/AMPK [192].

The endoplasmic reticulum is an organelle involved in the folding and post-translational modification of proteins, as well as calcium and sterol lipid biosynthesis. A number of physiological and pathological stimuli can interrupt the protein folding process and subsequently cause accumulation of unfolded or misfolded proteins in the ER - a condition referred to "ER stress". The ER stress response is a series of coordinated cellular responses to reduce ER stress but in conditions where ER stress is prolonged or excessive cell death results [193]. ER stress down regulates mTORC1 activity and TSC1/2 deficiency leads to a truncated ER stress response and increased sensitivity to ER stress inducing agents, which is not relieved by sirolimus [119, 194-196].

Phosphatidic acid (PA) is generated by phospholipase D mediated hydrolysis of phosphatidylcholine. PA binds to the FKBP12-rapamycin binding domain of mTOR in a manner that is competitive with FKBP12-rapamycin [197] and is required for formation of mTORC1 and mTORC2 complexes [198]. Inflammatory mediators can induce mTORC1 activity. The pro-inflammatory cytokine tumour necrosis factor α (TNFα) stimulates mTORC1 signalling through IKKβ mediated inhibition of TSC1-TCS2 by phosphorylation of TSC1 at Ser487 and Ser511 [183].

TSC2 binds to and is phosphorylated by DAPK1 leading to mTORC1 inhibition [187]. DAPK plays an important role in a diverse range of signal transduction pathways involved in cell proliferation, apoptosis, autophagy and immune responses. DAPK

activity is responsive to growth factors, TNF-α, TGF-β, IFN-γ, DNA damage and transformed oncogenes [199].

TSC2 is phosphorylated and inhibited by FAK, a mediator of integrin signalling, suggesting a role for TSC2 in the regulation of mTOR signalling by cell adhesion [189]. FIP200 binds to and inhibits the kinase domain of FAK and also binds TSC1 [200].

Primary cilia are cell surface organelles that act as sensory antennae for various input signals. Bending of the cilium promotes relocalization of LKB1 to close proximity to its substrate AMPK, whose activation inhibits mTORC1 signaling [201]. PC-1, the product of *PKD1*, regulates the cellular localisation of TSC2 [202]. Activation of mTORC1 is seen in several rodent models of polycystic kidney disease and in renal cysts from humans with autosomal dominant polycystic kidney disease (ADPKD) [156, 203, 204]. Treatment with sirolimus has been shown to alleviate the ADPKD phenotype in murine models [205-207]

TSC2 is inactivated by ubiquitinisation and subsequent degradation. TSC2 is ubiquinated by the putative E3 ubiquitin ligases HERC1[59], PAM [208] and E6AP [209].

The mTOR pathway is regulated by a number of tumour suppressor genes associated with tumour predisposition syndromes. Loss of *NF1*, *PTEN*, *LKB1* and *BHD* activity is associated with up regulation of mTORC1 in cell culture, animal models and in tumours derived from patients with germline mutations in these genes. Sirolimus abrogated cell growth in cell culture and animal models of neurofibromatosis type1, Cowden's disease,

Peutz-Jeghers syndrome and Birt-Hogg-Dube syndrome and is currently being investigated in clinical trials in these conditions [210-221]. (ClinicalTrials.gov Identifiers -NCT01031901, NCT00634270, NCT00722449, NCT00971789, NCT00811590, NCT00722449, NCT00971789).

3.2.15 mTORC2

In contrast to mTORC1, for which many regulatory signals and cellular functions have been defined, relatively little is known about mTORC2. The best characterised function of mTORC2 is the phosphorylation of AKT on Ser473, leading to its activation [222, 223].

mTORC2 also phosphorylates SGK1 (serum- and glucocorticoid-induced protein kinase 1) [224], regulates the phosphorylation of PKCα (protein kinase Cα) [223] and has been suggested to influence cell morphology and cytoskeleton reorganisation, through the Rho family small GTPases, including Rho, RAC and Cdc42 [100, 101]

The signalling pathways that regulate mTORC2 are not well characterised. Growth factors increase mTORC2 kinase activity and AKT phosphorylation at Ser473 [66, 222, 225]. PtdIns(3,4,5)P3 can directly stimulate intrinsic mTORC2 kinase activity [226]. The TSC1/TSC2 complex, whilst inhibiting mTORC1 signalling, promotes mTORC2 activity. Thus, loss of the TSC1-TSC2 complex results in elevated mTORC1 signalling and attenuated mTORC2 signalling. Loss of mTORC2 activity in cells lacking the TSC1-TSC2 complex, coupled with mTORC1-mediated feedback mechanisms, leads to strong attenuation of the growth factor-stimulated phosphorylation of AKT on S473 [66]. mTORC2 substrates are affected by loss of the TSC1-TSC2 complex in cell culture models and kidney tumours from both Tsc2 (+/-) mice and tuberous sclerosis patients [227].

3.2.16 Feedback loops in the mTOR pathway

Intracellular signalling pathways are regulated by crosstalk and feedback mechanisms. A number of feedback loops affecting mTOR signalling have been described. One of

the best characterised is inhibition of the PI3K pathway by mTORC1. Activation of mTORC1 induces a negative feedback loops that attenuates signalling through the PI3K/Akt pathway, mediated by the phosphorylation and inhibition of IRS-1 by S6K [228, 229]. Functionally similar loops mediate the inhibition of PDGFR [230] and the ERK/MAPK [231] pathway in response to mTOR activity.

Tsc2-/- MEFs exhibit mTORC1 activation and attenuated signalling associated with PDGFR down regulation [137] and S6K1 mediated down regulation of insulin-/insulin-like growth factor receptor substrate 1 (IRS-1) [228] and are strongly defective in growth factor (insulin and IGF) stimulated PI3K-Akt signalling [137, 232-235]. The baseline and serum stimulated activation of Akt is markedly reduced in $Tsc2^{-/-}$ MEFs and heterozygous *Tsc* knockout mouse models can be restored by treatment with sirolimus [228, 236, 237]. Inhibition of mTORC1 signalling by sirolimus and other rapalogs reduced the feedback inhibition of Akt in a range of cancer cell lines *in vitro* as well as in cancer tissue derived from patients under treatment [238-241].

These negative feedback loops may have a number of consequences for tuberous sclerosis pathology and the therapeutic use of mTOR inhibitors. The benign nature of tumours in TSC may be partly due to inhibition of pro-survival pathways. It has been suggested that treatment with sirolimus may be attenuated by up regulation of these and other pathways, or even that malignant progression could be promoted.

3.2.17 Mechanism of action of sirolimus

mTORC1 is acutely sensitive to sirolimus but not all mTORC1 outputs are sirolimus sensitive [242-246]. The mechanism by which sirolimus inhibits mTORC1 remains to be completely defined but sirolimus weakens the interaction between mTOR and

Raptor [247] and reduces mTORC1 intrinsic kinase activity [248]. mTORC2 is not acutely sensitive to sirolimus but prolonged treatment with the drug can, in some cell types, inhibit mTORC2 activity, possibly by sequestering mTOR in inhibited mTOTC1 complexes [249] and suppressing the phosphorylation of Akt on Ser473. Moreover, high concentrations of sirolimus also block mTORC2 activity [250].

The mechanism by which sirolimus exerts effects may depend on the cell type and the mutation spectra present. In some cell lines sirolimus induces apoptosis but in others the effect is predominantly cytostatic, possibly mediated by inhibition of cell cycle progression at G1. Additionally, in *in vitro* or *in vivo* models, mTOR inhibitors have also been demonstrated to decrease cell size, induce autophagy, promote senescence, inhibit angiogenesis, reduce motility, and selectively target stem cells (reviewed in [251-253]).

Recently developed small molecular ATP competitive mTOR kinase domain inhibitors inhibit both mTORC1 and mTORC2 (e.g. PP242 and PP30 [243], Ku-0063794 [244],Torin [245] and WAY-600, WYE-687, and WYE-354 [246]). These drugs block the phosphorylation of Akt at Ser473 and inhibit cell proliferation more completely than sirolimus in a manner that is at least partially dependent on suppression of sirolimus resistant functions of mTORC1 involved in cap-dependent translation and suppression of autophagy.

Chapter 4: Preclinical and clinical studies of mTOR inhibition in tuberous sclerosis and lymphangioleiomyomatosis

4.1 Studies in model systems

The potential efficacy of sirolimus in TSC1/2 deficient cells has been demonstrated in preclinical *in vitro* and *in vivo* models. In cell culture *Tsc1-/-* and *Tsc2-/-* MEFs show up regulation of mTORC1 activity and have a proliferative advantage over their wild type counterparts. Sirolimus has been shown to normalise deregulated mTOR signalling in cells that lack TSC1 or TSC2 [108, 137, 155, 184, 185, 254] and to reverse their proliferative advantage [137, 232].

The Eker rat carries a germline mutation in the *TSC2* gene [255]. Spontaneous renal cortical epithelial tumours and pituitary adenomas occur in these animal due to biallelic inactivation of *Tsc2* [256] and these tumours exhibit strong immunostaining for phospho-S6 [257]. Kenerson *et al.* administered sirolimus intraperitoneally to 12 month old Eker rats for 3 days. This was associated with reduced phosphorylation of S6, induction of apoptosis and decreased cellular proliferation in the renal tumours [257]. In a follow up study [258] Eker rats were treated with sirolimus for a longer time period (48 days) and most renal tumours underwent a >90% volume regression.

In transgenic mouse models of tuberous sclerosis sirolimus or rapalogs reduced renal tumour growth, associated with decreased cell proliferation and increased apoptosis. Similarly, in TSC2 deficient xenograft nude mouse models sirolimus or rapalogs reduced tumour growth [259-264].

Mouse models also suggest that aspects of cognitive function and seizures in tuberous sclerosis can be improved by sirolimus. mTOR signalling appears to be required for molecular and cellular aspects of learning and for the encoding and recall of spatial and auditory memory [265-269]. Administration of rapamycin to wild-type rodents impaired long-term spatial [267] and auditory recall memory [266]. In contrast, mTOR inhibition did not influence recognition memory, motor or sensory systems in wild-type rodents [268].

Studies in heterozygous *Tsc1* and *Tsc2* mouse models have shown the presence of cognitive deficits in the absence of structural brain lesions or seizures [270, 271]. Both *Tsc1*+/- and *Tsc2*+/- mice show deficits in hippocampal dependent learning and memory, including deficient spatial learning and abnormalities in contextual fear conditioning. Additionally, *Tsc1*+/- mice displayed deficits in social behaviour. These findings support a model in which haploinsufficiency for the TSC genes leads to aberrations in neuronal functioning resulting in impaired learning and social behaviour. Short term (3-5 days) treatment of $Tsc2^{+/-}$ mice leads to a reversal of hippocampal-dependent learning deficits.

Mice with conditional deletion of *Tsc1* in neurones have been generated, using either a Synapsin-I or a αCaMKII promoter to drive Cre recombinase. The Synapsin-I KO mice exhibited brain enlargement, largely due to neuronal hypertrophy, which was accompanied by astrogliosis, compromised survival and abnormal neurological findings and seizures. Sirolimus normalised brain weight and aspects of neuronal morphology and was associated with increased survival [272]. Sirolimus substantially increased

survival of Tsc1cc–aCaMKII-Cre mice, decreasing brain enlargement and improving aspects of the neurological phenotype [271].

A mouse model with conditional inactivation of the *Tsc1* gene in glial cells developed progressive epilepsy, encephalopathy and decreased survival associated with progressive astrogliosis, increased brain size, and abnormal neuronal organization. Early treatment with sirolimus, before the development of seizures prevented, the development of epilepsy and improved survival. Late treatment with sirolimus after the development of seizures suppressed seizures and prolonged survival [273].

4.2 Clinical studies

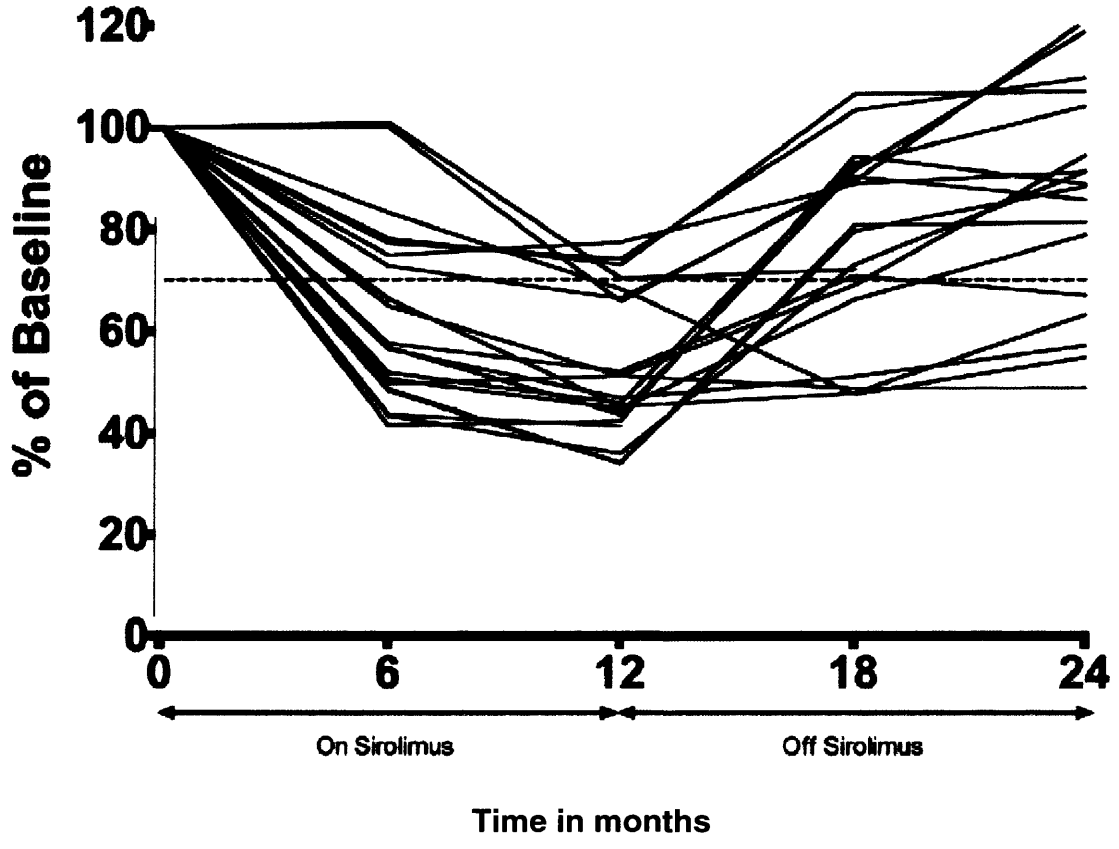
Case reports have suggested that sirolimus induced reduction in the size of AMLs [274, 275], astocytomas [276, 277] and facial angiofibroma [278] in tuberous sclerosis and in sporadic LAM.

Bissler *at al.* conducted a 24 month, nonrandomised trial to determine if sirolimus reduced angiomyolipoma volumes in patients with tuberous sclerosis or sporadic LAM [279] Patients received sirolimus for 1 year and were followed for an additional year after the therapy had stopped. The primary endpoint was angiomyolipoma volume at 1 year, as assessed by renal MRI. Secondary endpoints included AML volume at 2 years and lung function measurements.

Twenty five patients were enrolled (7 with TSC only, 6 with sporadic LAM and 12 with TSC associated LAM). The mean (±SD) angiomyolipoma volume at 12 months was 53.2±26.6% of the baseline value and at 24 months was 85.9±28.5% of the baseline value. After one year in patients with lymphangioleiomyomatosis, the mean FEV_1

increased by 118±330 ml, the mean FVC increased by 390±570 ml and the mean residual volume decreased by 439±493 ml, as compared with baseline but there was no change in DLCO. One year after sirolimus was discontinued, the mean FEV_1 was 62±411 ml above the baseline value, the FVC was 346±712 ml above the baseline value, and the residual volume was 333±570 ml below the baseline value.

Figure 3: Change in AML volume during the trial of Bissler *et al* [279] (taken from manuscript)



AMLs were assessed with abdominal magnetic resonance imaging, and volumetric analysis was performed at baseline and at 2,4, 6, 12, 18, and 24 months. The AML volume is expressed as a percentage of the baseline size. The dashed line represents 70% of the baseline value;

The use of sirolimus as an experimental treatment for brain tumours in tuberous sclerosis has been reported for four patients with SEGAs and one with pilocytic astrocytoma, ranging in age from 3 to 21 years [276]. Blood sirolimus levels were between 5–15 ng/ml and the duration of treatment was 2.5–20 months. Prior to entry into the study all lesions demonstrated growth and on treatment all exhibited regression and in one case necrosis (as assessed by MRI). In one patient interruption and then resumption of sirolimus therapy was mirrored by regrowth then further regression of the tumour.

Krueger et al performed a phase II trial of the rapalog everolimus as a treatment for SEGAs [280]. The trial enrolled 28 patients of age 3 and over. The primary efficacy end point was the change in volume of SEGAs between baseline and 6 months with everolimus given orally, at a dose to achieve a trough concentration of 5 to 15 ng per millilitre. A reduction of at least 30% was seen in 21 patients (75%) and of at least 50% in 9 patients (32%). Marked reductions were seen within 3 months and were sustained. Of the 16 patients for whom 24 hour video electroencephalography data were available, seizure frequency for the 6-month study period decreased in 9, did not change in 6, and increased in 1. Single cases of grade 3 treatment-related sinusitis, pneumonia, viral bronchitis, tooth infection, stomatitis, and leukopenia were reported.

Chapter 5: A phase II trial of sirolimus as a therapy for renal angiomyolipomas in patients with tuberous sclerosis or sporadic lymphangioleiomyomatosis

5.1 Study aims/objectives

The primary aim of this study was to determine if sirolimus induces regression of renal angiomyolipomas (AMLs) in persons with tuberous sclerosis complex (TSC), or sporadic lymphangioleiomyomatosis (LAM). Secondary aims were to assess its safety in these patients and determine whether sirolimus therapy affects respiratory function test results in LAM or the results of cognitive testing in TSC.

5.2 Study approval

The study protocol was approved by a multi-centre research ethics committee (Thames Valley MREC) and by institutional review boards of the participating institutions. The study was conducted according to the UK Human Use (Clinical Trials) Regulations 2004, the Directive 2001/20/EC of the European Parliament and the Declaration of Helsinki and its amendments. The trial was registered with European Clinical Trials database (EudraCT number 2004-00420-33), the Medicines and Healthcare products Regulatory Agency (CTA number 21323/0002/001-0001) and ClinicalTrials.gov (identifier:NCT00490789). The trial was sponsored by Cardiff University (sponsor number UWCM001).

5.3 Participating centres

The study was conducted at University Hospital of Wales, Cardiff; the City Hospital, Nottingham; The Royal Sussex Hospital, Brighton and Zurich University Hospital, Zurich.

5.4 Inclusion and exclusion criteria

Eligible patients had to:

- have a clinically definite diagnosis of tuberous sclerosis (modified Gomez criteria) or sporadic LAM (biopsy-proven or compatible high resolution chest CT scan and respiratory function tests.);
- be between 18 and 65 years old;
- have one or more renal angiomyolipomas of at least two centimetres or greater in largest diameter;
- have adequate renal function: glomerular filtration rate > 40 ml/min (*);
- be competent to give informed consent;
- if female, have documentation of negative pregnancy test prior to enrolment;
- use an effective form of contraception, whilst taking sirolimus and for twelve weeks after stopping the drug (**);

Patients were excluded from the study if they:

- had a significant haematological or hepatic abnormality (transaminase levels > 150 i.u./L serum albumin < 30 g/L, haematocrit< 30%, platelets < 100,000/

mm^3, adjusted absolute neutrophil count < 1,500/mm^3, total WBC < 3,000/ mm^3);

- had greater than 1 g proteinuria daily (***);
- had uncontrolled hyperlipidaemia (****);
- had embolisation for AML(s) within the preceding 6 months;
- had a renal haemorrhage within the preceding year or who had a renal haemorrhage at any time previously and had a known conservatively managed renal aneurysm(s) greater than 10mm.);
- were pregnant or breastfeeding;
- had multiple bilateral AMLs, where individual lesions could not be distinguished;
- were unable to walk 100 metres on the flat;
- continuously required supplemental oxygen;
- had or were being considered for organ transplant;
- had intercurrent infection at initiation of Sirolimus;
- had surgery within last 2 months;
- had used an investigational drug within the last 30 days;
- had changed anti epileptic drug medication within the last 3 months;
- were likely to need vaccination e.g. for travel during the course of the trial (except for influenza vaccine in patients with LAM);

- were using strong inhibitors of CYP3A4 (such as ketoconazole, voriconazole, itraconazole, tilithromycin or clarithromycin) or strong inducers (such as rifampicin or rifabutin);

* estimated from creatinine clearance calculated by the abbreviated MDRD study equation [281]:

GFR (mL per minute per 1.73 m^2) = 186 X (SCr)-1.154 X (Age)-0.203 X (0.742 if female) X (1.210 if black)

** oestrogen containing contraceptives are not recommended in LAM. Acceptable contaceptive measures included prior hysterectomy, ovarectomy or tubal ligation, vasectomy, complete abstinance, barrier methods which included both a cervical diaphragm and spermicidal jelly and progesterone based contraceptives.

*** as measured on 24 hour protein collection

**** hyperlipidaemia was defined and managed according to clinical guidelines [282]. In participants, with unacceptably high lipid levels, these levels were reduced to recommended levels prior to initiation of sirolimus.

5.5 Patient recruitment

Tuberous sclerosis patients were selected from individuals followed by the Medical Genetics Service for Wales, the Nephrology service at Zurich University Hospital and from participants in the UK National Registry of Renal Complications of Tuberous Sclerosis. Sporadic LAM patients were selected from the national cohort followed by The Respiratory Service at Nottingham University (The LAM Action Register). Patients followed at The Medical Genetics Service for Wales and the Zurich University Hospital were under the clinical care of one of the study doctors. The study doctor involved in their care selected potential participants and contacted them via mail. Participants in The National Registry of Renal Complications of TSC and The LAM Action Register already had given permission to be contacted about research studies. They were contacted by mail by one of the principal investigators. Potential participants received an information sheet (appendix 1) and a response sheet for return by prepaid post. Those that expressed a potential interest in participating were offered a face to face interview with one of the study doctors to provisionally assess eligibility, answer questions and obtain written consent prior to enrolment (appendix 2 –consent form).

5.6 Study design

After giving informed consent, patients had a baseline evaluation at a screening visit. This included a medical history and physical examination by one of the study doctors, cognitive function testing (see section 5.7), a renal MRI scan (see section 5.8), brain MRI scan (see section 5.8) , laboratory studies including renal profile, liver profile, bone profile, fasting lipids, fasing glucose, thyroid function, creatine phosphokinase (CPK), full blood count and differential, dipstick urine analysis, 24 hour urine protein clearance and spot urine protein-creatinine ratio. All female participants had a pregnancy test.

Baseline chest X-ray and spirometry (FEV1 and FVC) was performed in all patients and high-resolution chest CT scan (see section 5.8) was performed in female patients where chest CT films from within the preceding twelve months were not available. Individuals with evidence of LAM had more extensive baseline pulmonary function tests consisting of FEV1, FVC and DLCO in a lung function laboratory. Patients who had epilepsy were given an epilepsy diary and instruction in its use. The current frequency and nature of seizures was recorded and the diary reviewed at each follow-up assessment. Mutation analysis was performed in participants with tuberous sclerosis in whom the genotype was unknown.

Patients were given a sheet with information regarding the use of other drugs when on sirolimus (appendix 3) and a contact number for where advice was available 24 hours a day (this was provided by the renal on call service at the Royal Sussex Hospital). Doctors involved in their care were given an information sheet regarding the trial and the investigational drug (appendix 4).

Patients received sirolimus orally once daily. Within two weeks of the baseline evaluation sirolimus was initiated at a dosage of 0.5 mg/m^2/d. The start date of taking sirolimus was designated as day 1. Dosages were adjusted to achieve a trough level of 3-6 nanograms per milliliter (ng/ml). Dose adjustments were calculated using the formula:

New sirolimus dose = current dose × (target concentration/current concentration)

Sirolimus levels were measured 7 days after a dosage increase.

Follow-up evaluations were performed at 3 weeks, 2 months, 4 months, 6 months, 9 months, 12 months, 18 months, and 24 months after initiation of sirolimus. Follow-up blood biochemistry and haematology studies (as baseline), pregnancy tests, dipstick urine analysis, spot urine protein:creatinine ratios and trough sirolimus levels were performed at each follow-up visit. Renal MRI scans were repeated after 2, 6, 12 and 24 months on therapy.

Handheld spirometry (FEV1 and FVC) was carried out at each visit for patients with tuberous sclerosis alone. Patients with LAM (either sporadic or TSC associated) had handheld spirometry (FEV1 and FVC) at each visit apart from visits at baseline, 4 , 6 and 12 and 24 months when FEV1, FVC and DLCO were measured in a lung function laboratory.

High resolution chest CT (subjects with LAM only – sporadic or TSC related) was performed after 24 months on therapy to observe any effects on pulmonary disease. Brain MRI (TSC subjects only) was performed after 12 months on therapy to observe any effects on cortical tubers and subependymal nodules.

In between visits a study doctor telephoned participants to collect data regarding adverse events or changes in drug regimen. Calls occurred at 1 week, 1.5 months, 5 months, 7.5 months 10.5 months, 15 months, 21 months and 25 months.

After two months, unless there had been a reduction in diameter from baseline of at least 10% in all measured AMLs, the dose of sirolimus was altered to achieve a trough serum level of 6–10 ng/ml unless unacceptable toxicity occurred. The total duration of

treatment within the trial was 2 years unless the drug was stopped or temporarily withheld. The trough levels of sirolimus were selected in view of the trough level of 12-20 ng/ml recommended when sirolimus is used as a single agent immunosupressant in renal transplantation.

5.7 Cognitive function testing

Cognitive testing was carried out at baseline, 4 & 12 months by the same psychologist in a quiet and undisturbed testing environment. Only patients with English as their first language were evaluated for neurocognitive outcomes. IQ was determined for trial eligibility using the National Adult Reading Test [283]. Function was assessed in 3 neurocognitive domains:

1) Recall memory was assessed using the List Learning, Story and Complex Figure measures of immediate and delayed verbal and spatial recall memory from the Adult Memory and Information Processing Battery (AMIPB) [284];

2) Recognition memory was assessed using the spatial recognition memory and pattern recognition memory subtests from the Cambridge Neuropsychological Test Automated Batteries (CANTAB) [285];

3) Executive skills were assessed using the self-ordered Spatial Working Memory task (SWM), the Stockings of Cambridge (SoC) planning task and the Intra-Dimensional Extra-Dimensional shifting task (IDED) from the CANTAB [285].

Parallel versions of tests were used to reduce practice effects.

5.8 Imaging

Abdominal and brain imaging was performed by MRI using clinical 1.5-Tesla systems. Abdominal sequences comprised axial T2 Turbo Spin echo, T1 and T2 fat saturated with optional T1 coronal. Brain sequences comprised axial T2 weighted images, axial flair images and coronal 3D Spoiled Gradient Recalled. CT images of the lung were obtained during full inspiration, acquired with a low dose protocol, with a pitch of 1.375:1 at 5 mm thickness, reformatted on lung algorithm to 5 mm and 1.25 mm.

Figure 4: A flow chart of evaluations in the trial

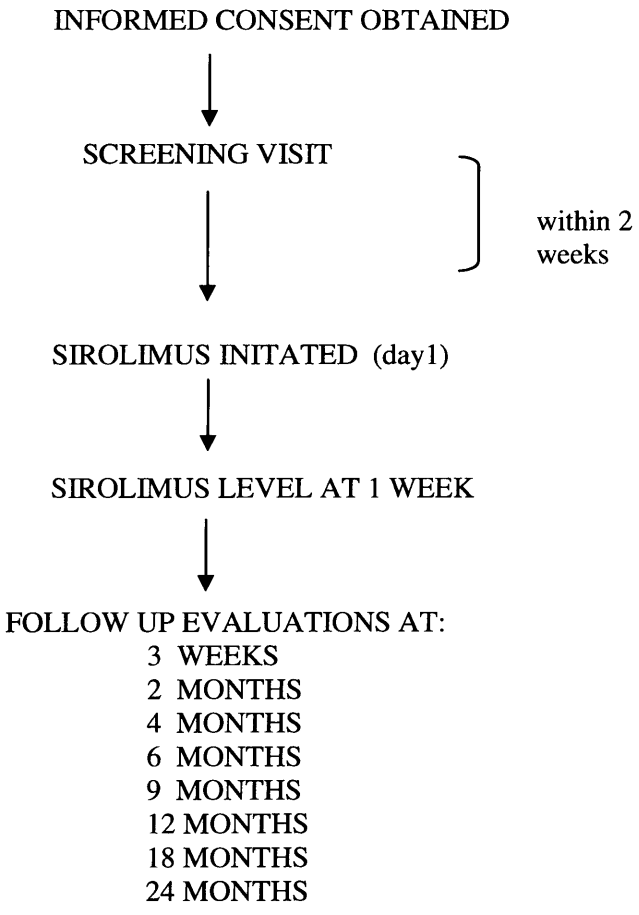


Table 3: Data capture summary

	Screening	1 wk	3 wk	2 mth	4 mth	6 mth	9 mth	12 mth	18 mth	24 mth
History and exam	✔		✔	✔	✔	✔	✔	✔	✔	✔
Blood Tests (1)	✔		✔	✔	✔	✔	✔	✔	✔	✔
Sirolimus levels		✔	✔	✔	✔	✔	✔	✔	✔	✔
Pregnancy test	✔		✔	✔	✔	✔	✔	✔	✔	✔
Spot urine Protein: creatinine ratio	✔		✔	✔	✔	✔	✔	✔	✔	✔
24 hour urine protein	✔									
Urine Analysis	✔		✔	✔	✔	✔	✔	✔	✔	✔
Genotype (2)	✔									
Chest X-ray	✔									
Spirometry	✔		✔	✔	✔	✔	✔	✔	✔	✔
Detailed lung function tests (3)	✔				✔	✔		✔		✔
Cognitive function tests	✔				✔			✔		
Renal MRI	✔			✔		✔		✔		✔
Brain MRI	✔							✔ (4)		
CT thorax	✔ (5)									✔ (6)
Review of epilepsy	✔		✔	✔	✔	✔	✔	✔	✔	✔

Telephone calls from a study doctor at 1 week, 1.5 months, 5 months, 7.5 months 10.5 months, 15 months, 21 months and 25 months.

Key to table

1	*Renal profile, liver profile, bone profile, fasting lipids, glucose, thyroid function, CPK, full blood count and differential.*
2	*For participants with tuberous sclerosis in whom genotype is not already known.*
3	*Only in patients with LAM.*
4	*Only in patients with tuberous sclerosis*
5	*At baseline evaluation a high-resolution chest CT scan was performed in female patients where chest CT films from within the preceding twelve months were not available.*
6	*Patients with LAM only.*

5.9 Evaluation of response of angiomyolipoma

Response was assessed according to Response Evaluation Criteria in Solid Tumors (RECIST) which are summarised in table 4 [286]. At baseline, AMLs were categorized by size as measurable (greater than 20mm in longest diameter) or non-measurable. Up to five measurable AMLs, greater than 20 mm in longest diameter, were selected in each kidney and identified as target lesions. The longest diameter (LD) of each target lesion was recorded and the sum of the LD for all target lesions calculated. Non-measurable AMLs were recorded as non-target lesions. A complete response represented the disappearance of all lesions. A partial response represented at least a 30% decrease in the sum of the LD of target lesions, using the baseline sum as the reference and stable non-target lesions. Progressive disease represented at least a 20% increase in the sum of the LD of target lesions (taking as a reference the smallest sum of the LDs recorded since the treatment started), progression of non-target lesions, or the appearance of 1 or more new lesions. Stable disease represented neither a partial response nor progressive disease. Patients with an overall complete or partial response were considered responders.

AML LDs were measured by a single radiologist (TD). Anonymized MRI scans were indentified by code numbers and the radiologist was not blind to the timing of the scans in relation to the study.

Table 4: Response Criteria

	Evaluation of target lesions
* Complete Response (CR):	Disappearance of all target lesions
* Partial Response (PR):	At least a 30% decrease in the sum of the LD of target lesions, taking as reference the baseline sum LD
* Progressive Disease (PD):	At least a 20% increase in the sum of the LD of target lesions, taking as reference the smallest sum LD recorded since the treatment started or the appearance of one or more new lesions
* Stable Disease (SD):	Neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD, taking as reference the smallest sum LD since the treatment started
	Evaluation of non-target lesions
* Complete Response (CR):	Disappearance of all non-target lesions
* Incomplete Response/ Stable Disease (SD):	Persistence of one or more non-target lesion(s)
* Progressive Disease (PD):	Appearance of one or more new lesions and/or unequivocal progression of existing non-target lesions

LD = largest diameter

Overall response

Target lesions	Non-Target lesions	New lesions	Overall response
CR	CR	No	CR
CR	Incomplete response/SD	No	PR
PR	Non-PD	No	PR
SD	Non-PD	No	SD
PD	Any	Yes or No	PD
Any	PD	Yes or No	PD
Any	Any	Yes	PD

5.10 Safety assessments

Safety assessments were based on reports of adverse events and the results of physical examination, laboratory determinations and other scheduled procedures. Adverse event reporting was done in accordance with the principles of Good Clinical Practice and the requirements of the Medicines for Human Use (Clinical Trials) Regulations 2004. Sample adverse event reporting cards are included in appendix 5.

An adverse event (AE) was defined as any untoward medical occurrence in a patient which does not necessarily have a causal relationship with this treatment. An adverse reaction to an investigational medicinal product (AR) was defined as all untoward and un-intended responses to an investigational medicinal product related to any dose administered. All adverse events judged by either the reporting investigator or the chief investigator as having a reasonable causal relationship to a medicinal product qualified as adverse reactions.

All adverse events were evaluated for seriousness, causality and expectedness. Adverse events were graded according to the National Cancer Institute's Common Terminology Criteria for Adverse Events v3.0 (CTCAE) [287]. A serious adverse event or serious adverse reaction was defined as any untoward medical occurrence or effect that at any dose fulfils at least one of the following criteria:

results in death;

is life-threatening;

requires inpatient hospitalisation or prolongation of existing hospitalisation;

results in persistent or significant disability/incapacity; or

is a congenital anomaly/birth defect

The relationship of adverse events to the product being studied was determined according to the five categories classification below. Of the five, "possibly", "probably" and "definitely" related to an investigational medicinal product qualify as adverse reactions; "unlikely" and "not related" did not qualify as a reasonable causal relationship.

Not related - temporal relationship of the onset of the event, relative to administration of the product, is not reasonable or another cause can by itself explain the occurrence of the event.

Unlikely – temporal relationship of the onset of the event, relative to administration of the product, is reasonable but the event is more likely explained by another cause than by the product.

Possibly related - temporal relationship of the onset of the event, relative to administration of the product, is reasonable but the event could have been due to another, equally likely cause.

Probably related - temporal relationship of the onset of the event, relative to administration of the product, is reasonable and the event is more likely explained by the product than by another cause.

Definitely related - temporal relationship of the onset of the event, relative to administration of the product, is reasonable and there is no other cause to explain the event.

An unexpected adverse reaction was defined as an adverse reaction, the nature or severity of which is not consistent with the applicable product information i.e. the summary of product characteristics (SPC) or is not expected based on the knowledge of TSC and LAM. Expected advents based on knowledge of the natural history of TSC and LAM were defined as follows:

for TSC

Seizures; or

Haemorrhage from AML.

for LAM:

Pneumothorax;

Chest infection;

Chylothorax; or

Haemoptysis;

Sirolimus-related treatment emergent adverse reactions were reported and were events not present at baseline or those present at baseline that worsened during treatment, and were definitely, probably or possibly related to sirolimus administration.

5.11 Toxicity and interruption or discontinuation of treatment

5.11.1 Hyperlipidaemia

Subjects developing significant hyperlipidaemia were given the option of having the sirolimus dose reduced or discontinued, or continuing on the protocol with concurrent therapy of their hyperlipidaemia. Management of hyperlipidaemia was based on clinical guidelines [288].These guidelines recommend that for secondary prevention treatment should be initiated in those with a risk threshold of ≥ 20% cardiovascular disease risk over 10 years, calculated using the Joint British Societies chart. If resolution of hyperlipidaemia did not occur after 2 months of dietary therapy a lipid lowering agent,

typically a statin, was initiated.

If cholesterol and/or triglyceride levels were not reduced to recommended levels with lipid lowering drugs, then the sirolimus dose would have been reduced by 50% until this has been achieved. Re-escalation of the sirolimus dose was permitted if the toxicity resolved.

5.11.2 Haematological toxicity

In the event of a significant hematological abnormality as outlined in the exclusion criteria (i.e. Haematocrit < 30%, Neutrophil count < 1,500 mm^3, total WBC < 3,000 mm^3, platelets <100,000 mm^3) sirolimus was discontinued until resolution of the abnormality. Sirolimus was then restarted at 50% of the previous dose. Re-escalation of the sirolimus dose was permitted.

5.11.3 Seizure control

Sirolimus would have been stopped if seizure control deteriorated.

5.11.4 Pulmonary toxicity

If minor but unambiguous pulmonary infiltrates were found on a PA chest film, the investigator would have either held the drug or reduced the dose of sirolimus by 50%. If the infiltrates did not steadily resolve, or if grade 3 or greater pulmonary toxicities occurred at any time, the drug would have be withheld until symptomatic and radiographic resolution occurred, and then the sirolimus dose restarted at 50% of the prior dose. Re-escalation of sirolimus was permitted if the toxicity resolved.

5.11.5 Infectious toxicities

If grade 2 infectious complications occurred, and antibiotics that interfere with the metabolism of sirolimus were prescribed, appropriate dose adjustments were made or sirolimus withheld for the duration of the antibiotic treatment. Sirolimus was withheld for all grade 3 or greater infectious complications until infection was resolved. Re-escalation of the sirolimus was permitted if the toxicity resolved.

5.11.6 Mucositis/stomatitis

If grade 2 symptoms occured, the sirolimus dose was decreased by 50% or withheld for at least 2 weeks. Sirolimus was withheld if grade 3 or greater toxicities occurred and restarted at 50% of the prior dose. Re-escalation of the sirolimus was permitted if the toxicity resolved

5.11.7 Other toxicities

For grade 1 or 2 sirolimus-associated toxicity the dose of sirolimus could be maintained, reduced, withheld or increased at the study doctors' discretion. If a subject experienced sirolimus associated grade 3 or 4 toxicity, or persistent grade 2 toxicity unresponsive to treatment, the drug could be held until toxicity regressed to grade ≤ 1 or to the baseline from screening. The study drug could then be resumed at the previous dose. A dose decrease of 20-50% was permitted at the discretion of the investigator. Subsequent re-escalation of dose was also permitted if toxicity did not recur. If a subject experienced recurrent sirolimus-related grade 3/4 toxicity or persistent grade 2 toxicity unresponsive to treatment, despite a 50% dose reduction the sirolimus would have been withheld for the rest of the trial.

5.11.8 Discontinuation depending on response

Consideration would have been given for participants with tuberous sclerosis to dose reduction or discontinuation of sirolimus should the LDs of all measured angiomyolipomas be reduced by at least 75% from their original size. Sirolimus would also have been discontinued if there had been no decrease in size from the baseline documented on serial MRI scans after 4 months of the highest sirolimus dose. However in sporadic LAM patients it was to be continued for the full two years.

5.11.9 Dosage reduction due to high serum levels

Trough serum levels above 10 ng/ml resulted in dosage reduction. Dose reduction was done according to trough levels as follows;

Trough level	
10.1-15 ng/ml-	withhold dose for 24 hours then resume at 50% of previous dose
15.1-24 ng/ml-	withhold dose for 48 hours then resume at 25% of previous dose
over 24.1 ng/ml-	withhold dose for 72 hours then resume at 10% of previous dose

5.12 Statistics

5.12.1 Sample size

The sample size calculation was based on a Fleming single stage design setting p1 at 0.1, p2 at 0.4, α at 0.05 and β at 0.1 (where p1 is the response rate below which the treatment would not be considered for further study, p2 the response rate above which the treatment would certainly be considered for further study, α the probability of concluding that the response rate is greater than p1 when that is false and β the

probability of concluding that the response rate is less than p2 when that is false). A Fleming single stage design was chosen in view of the restricted numbers of potentially eligible participants. We required 14 patients and recruited 16 to allow for drop out.

5.12.2 Statistical analysis of primary endpoint

The primary efficacy end point of this study was objective renal angiomyolipomas response rate (the percentage of patients with CR or PR). The primary efficacy analysis was based on the intention-to-treat population. The per protocol population was defined as all those participants in the study excluding those who had not complied with the protocol sufficiently that they were likely to show the effects of the treatment. Those who had failed to comply sufficiently were defined as those who did not receive at least four months at 6-10 ng/ml unless they achieved a response.

We also determined the number and percentage of those participants who were considered responders at the time of the 24 month MRI scan and those in whom the drug was discontinued because of a 75% reduction from the baseline LDs of all measured angiomyolipomas combined.

5.12.3 Statistical analysis of lung function

The lung function data is expressed as percentage of predicted values to allow for change over time. The annual rate of change in FEV1, FVC and DLC0 was determined by linear regression.

5.12.4 Statistical analysis of cognitive function

Test scores were converted to percentile band scores (below 5^{th}, 5^{th}-9^{th}, 10^{th}-24^{th}, 25^{th}-49^{th}, 50^{th}-74^{th}, 74^{th}-89^{th} or 90^{th} and above) according to published UK population norms

for age, and sex for each test version. This enabled comparison across parallel forms of tasks. We calculated summary scores across tasks[30] by allocating an integer score to each centile band (below 5^{th} = 1, 5^{th}-9^{th} = 2, 10^{th}-24^{th} = 3, 25^{th}-49^{th} = 4, 50^{th}-74^{th} = 5, 75^{th}-89^{th} = 6, 90^{th} and above = 7) and adding the integer scores across the tests in each domain of neurocognitive function. The total immediate recall memory score was the sum of the integer scores for list learning, story recall and figure recall; the total immediate recognition memory score was the sum of the pattern recognition and spatial recognition integer scores and the total executive score was the sum of the SWM, SoC and IDED integer scores.

5.13 Results

5.13.1 Characteristics of the patients

A total of 16 patients, 13 female and 3 male, were enrolled from October 2005 to September 2007. Six patients had sporadic LAM, 10 had tuberous sclerosis; of whom 3 also had tuberous sclerosis related LAM. Three patients had undergone an unilateral nephrectomy. Of the 13 patients with both kidneys remaining the target lesions were unilateral in 7 patients (6 with LAM and 1 with tuberous sclerosis) and bilateral in 6 patient (all with tuberous sclerosis). The median number of designated target lesions was 3 (range1-9). Four patients had epilepsy which was well controlled with antiepileptic medication.

During the trial one patient with sporadic LAM died from a respiratory infection. One patient withdrew for lung transplantation, two withdrew for personal reasons unrelated to the study, one withdrew because of sirolimus related peripheral oedema and one patient for elective surgery for AML. Additionally, sirolimus was permanently

discontinued in one patient due to fatigue and in another due persistent proteinuria.

The genotypes of the patients with tuberous sclerosis are shown in table 5.

Table 5: The genotypes of the patients with tuberous sclerosis

Patient	**Result**	
TSC1(L)	no mutation found	
TSC2(L)	*TSC2*	c.5227_5244 del 18bp
TSC3	no mutation found (*)	
TSC4	no mutation found	
TSC5	*TSC2*	c.1111C>T
TSC6	*TSC2*	c.4947C>G
TSC7	*TSC2*	deletion exon 7-19 (somatic mosaic)
TSC8(L)	no mutation found	
TSC9	mutation analysis not done	
TSC10	mutation analysis not done	

TSC1 and TSC2 mutation screening was performed using denaturing high-performance liquid chromatography. PCR products were on an ABI 3100 Genetic Analyser. Multiplex ligation-dependent probe amplification (MLPA) was performed on TSC2 using a standard kit, P046-B2 (MRC Holland, Amsterdam, the Netherlands).

(MLPA not performed to date)*

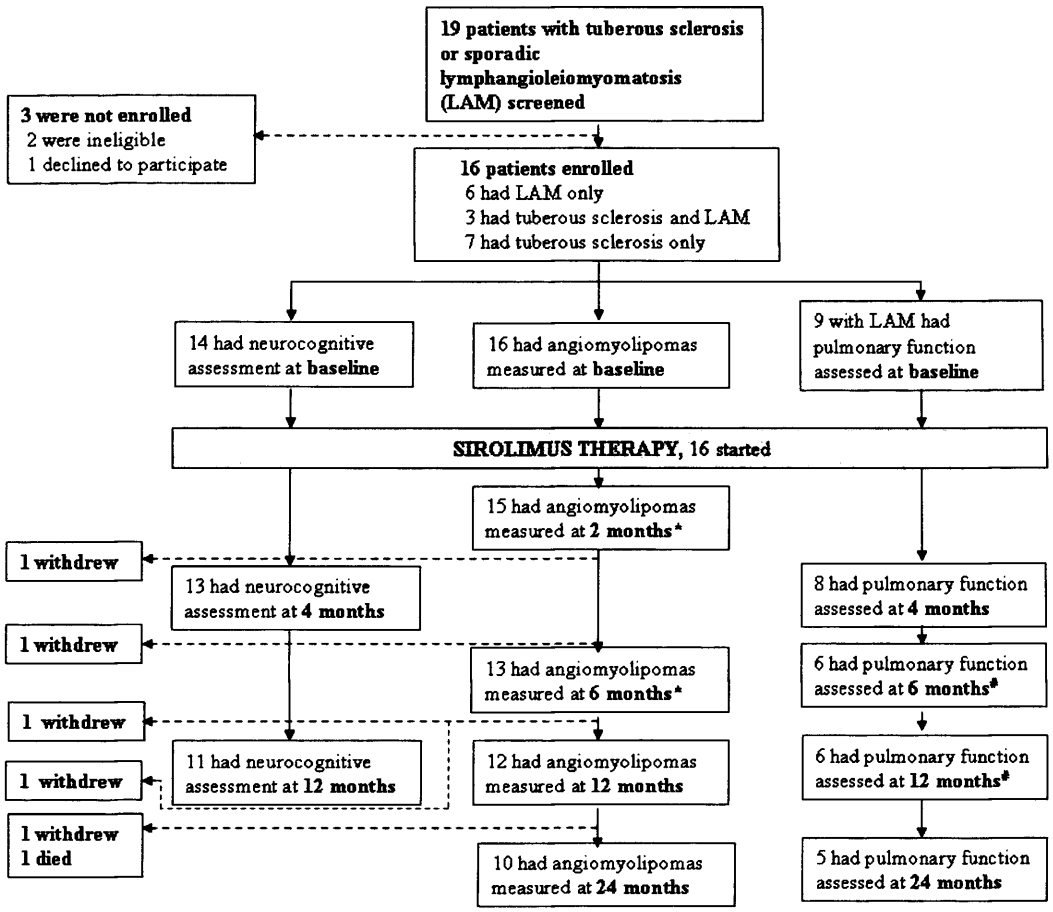
Ten patients completed the 24 month trial (Figure 5). At 2 months 15 patients underwent renal MRI evaluation, one patient fractured her tibia and fibular shortly prior to the 2 month assessment. Thirteen patients underwent renal MRI evaluation at 6 months, 2 patients had withdrawn from the study at this point and one had a respiratory infection and was unable to attend for the scan. At 12 months, 12 patients underwent renal MRI evaluations four patients having withdrawn. At 24 months 10 underwent renal MRI evaluation as 5 had withdrawn and one patient had died.

Pulmonary function data for patients with LAM (tuberous sclerosis related or sporadic) was available for 9 patients at baseline, for 8 at 4 months, for 6 at 6 months, for 7 patients at 12 months and for 5 patients at 24 months.

Cognitive function data was available for 14 patients at baseline and at four months and for 11 patients at 12 months, including 7 with tuberous sclerosis.

At 2 months 7 patients achieved a decrease greater than 10% in all measured AMLs, six were maintained at the 3-6 ng/ml range and one (LAM2) increased to 6-10ng/ml (originally this was done in error but was well tolerated and continued at this level following discussion with the patient). A decrease greater than 10% in all measured AMLs at 2 months was not achieved in 8 patients. Of these 5 were maintained at the 3-6 ng/ml range, 3 because of toxicity and 2 because of patient preference and 3 were escalated to the 6-10 ng range. In the patient who did not have a 2 month renal MRI because of a fractured tibia and fibula the sirolimus was discontinued until after the fracture healed and was not escalated above 3-6 ng/ml. Sirolimus was not discontinued on the basis of responses in any patients as no reduction of 75% or greater was seen from the baseline LDs of measured AMLs in any patients.

Figure 5. Flow diagram summarising patients and assessments.



** One patient who was still in the study was unable to undergo magnetic resonance imaging of the kidneys at the 2-month assessment and another at the 6-month assessment because of inter-current illness. # One patient was unable to undergo pulmonary function tests at 6 and at 12 months because of inter-current illness.*

5.13.2 Response of AMLs

The data on treatment response is illustrated in table 6 and figure 6. Figure 7 shows MRI findings in patient TSC5 at baseline and at 24 months. A response at any time during the study period was seen in 8 of 16 patients (50%). No patients achieved a complete response and 8 (50%) achieved a partial response, 4 of 10 with tuberous sclerosis and 4 of 6 with sporadic lymphangioleiomyomatosis. One new non-target lesion was seen in one patient with sporadic lymphangioleiomyomatosis at the 12 month renal MRI but this was no longer seen at the 24 months. In all other participants at all other time points the responses of non-target lesions were evaluated as incomplete responses/ stable disease.

In the per protocol group of 10 patients the response rate was 80%. All the responses that occurred were seen in the per protocol group.

Of the 10 participants who were evaluated at 24 months a response was seen in 4 (40%), at that time point, i.e. 25% of the original 16 participants. Of the other 6 evaluated at 24 months, one patient achieved a response at 2 months but there was a subsequent increase in the sum LD sufficient to be designated progressive disease although the sum LD remained below baseline and the other 5 had stable disease. Of the 6 participants who were not evaluated at 24 months 1(17%) had achieved a response during their participation in the study. Of the 11 participants who were evaluated by 24 month MRI scan or were deceased by this time point, 4 (36.3%) were classified as being responders at that time point.

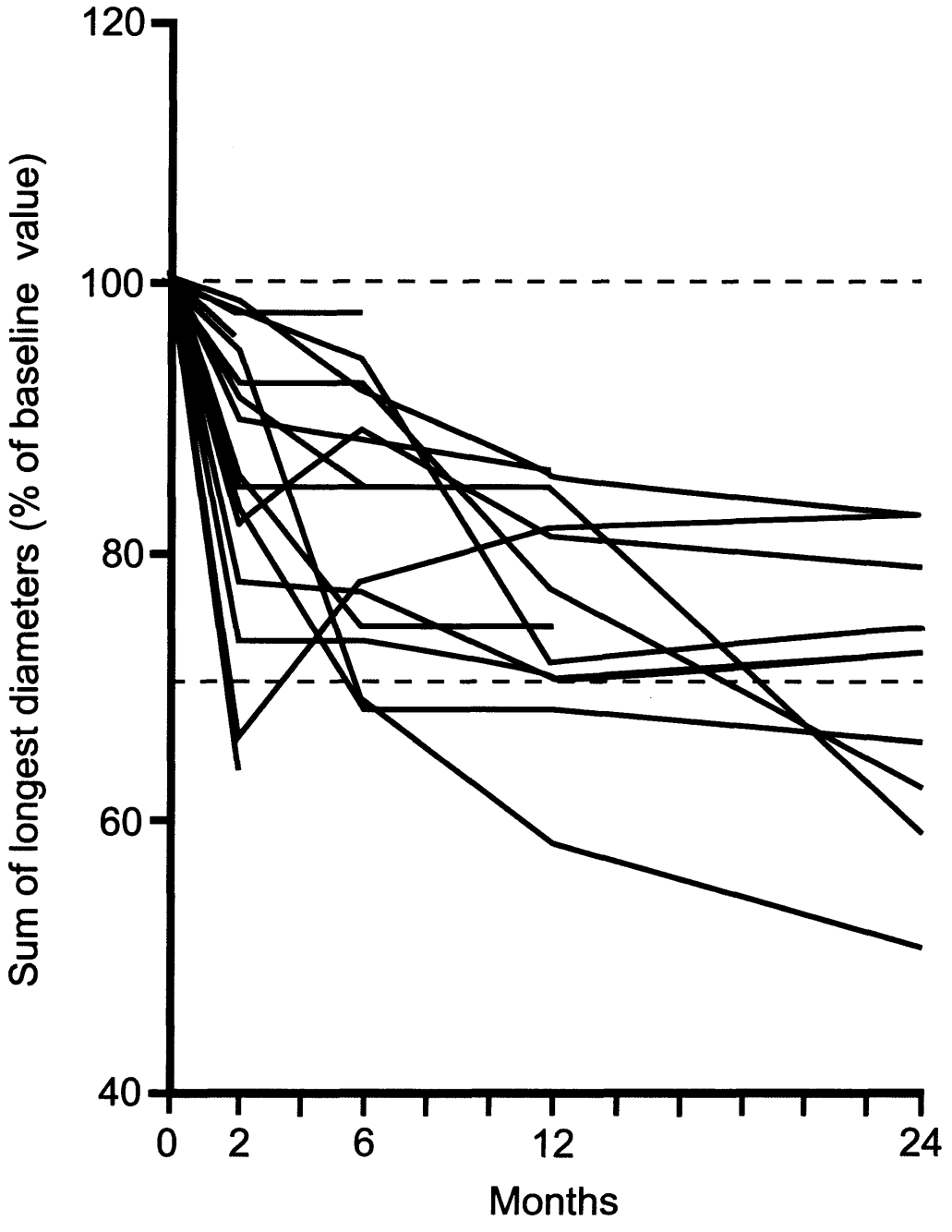
Of the 12 participants maintained at 3-6ng/ml at 2 months 5 (42%) achieved a response

at any time during the study period. Of the 6 maintained at that level because of a 10% or greater decrease in size of measured AMLs at 2 months, 4 (67%) achieved a response at any time during the study period. Of the 5 maintained at 3-6ng/ml or less at 2 months because of toxicity or patient preference one (20%) achieved a response at any time during the study period although only 3 of these patients were evaluable at 24 months. Of the 7 patients who were maintained on 3-6 ng/ml or lower who were evaluated at 24 months one (14.3%) was considered a responder at the time of the 24 month evaluation which is 6.3% of the initial 16 participants.

Of the 4 patients escalated to 6-10 ng/ml at 2 months, 3 (75%) achieved a response and one withdrew. Three of these patients had not achieved a 10% or greater decrease in size of measured AMLs at 2 month but of these 3 patients 2 achieved a response during the trial.

Of the 48 individual target lesions evaluated, 41 (85%) were smaller at the last measurement made than at baseline, 2 were the same size and 5 were larger, including 4 from one patient (table 7). A decrease in LD at at least one time point was seen in 44 (92%) lesions. At least one target lesion exhibited a decrease in LD from baseline in each patient. Three target lesions (all in the same patient) showed no decrease in LD but rather a continued increase in size over the duration of the study. Nine target lesions showed a decrease in sum LD followed by an increase back towards or above baseline LD at the last evaluation available. Of 23 target lesions evaluated at 24 months, 21 showed a decrease in LD compared with baseline, 2 lesions had the same LD as baseline.

Figure 6: Changes in angiomyolipoma burden during sirolimus therapy.



Renal angiomyolipomas were measured at baseline and at 2,6,12 and 24 months by magnetic resonance imaging. The sum of the longest diameters of all target angiomyolipomas in each patient was calculated at each time point and the percentage reduction calculated by comparison to the baseline value. Each solid line shows change

in angiomyolipoma burden in one patient. The upper dashed line represents the baseline value and the lower dashed line 70% of the baseline value. Patients in whom the sum of the longest diameters of target angiomolipomas fell below 70% of the baseline value are defined as partial responders by the RECIST criteria.

Table 6: Response of angiomyolipomas to sirolimus.

	Percent Reduction in Sum of Longest Diameters of Angiomyolipomas			
	2 months	**6 months**	**12 months**	**24 months**
LAM1	10	ND	13	Deceased
LAM2	6	**31**	**42**	**50**
LAM3	14	14	14	**41**
LAM4	8	8	23	**38**
LAM5	4	Withdrew		
LAM6	**37**	Withdrew		
TSC1 (L)	28	28	**30**	28
TSC2 (L)	13	27	27	Withdrew
TSC3	9	14	Withdrew	
TSC4	2	2	Withdrew	
TSC5	16	**32**	**32**	**33**
TSC6	ND	5	29	26
TSC7	22	23	**30**	28
TSC8 (L)	34	24	18	17
TSC9	16	11	18	21
TSC10	2	6	14	17

Response is expressed as percentage reduction in the sum of the longest diameters of target angiomyolipomas in each patient measured by magnetic resonance imaging (MRI) compared to baseline. Reductions of 30% of more are categorised as partial responses by the RECIST criteria and are highlighted in red . 8 of 16 patients (50%) achieved a partial response. (ND = MRI not done due to inter-currrent illness).

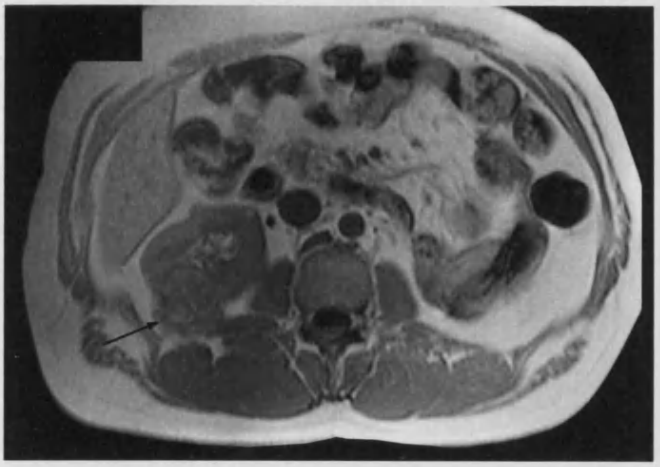
Table 7: Change in longest diameters of individual target angiomyolipomas.

Patient	Angiomyolipoma	baseline	2 Months	6 Months	12 Months	24 Months
LAM1	R1	2.2	1.4	ND	2.0	Deceased
	R2	2.6	2.7	ND	2.3	Deceased
	R3	2.3	2.3	ND	1.9	Deceased
LAM2	R1	2.8	2.5	1.4	1.2	1
	R2	2.0	2.0	1.9	1.6	1.4
LAM3	R1	2.2	1.9	1.9	1.9	1.3
LAM4	L1	2.8	2.5	2.8	2.4	2.2
	L2	2.0	1.9	1.6	1.3	0.8
LAM5	R1	2.3	2.2	Withdrew		
LAM6	L1	5.6	3.4	Withdrew		
	L2	2.7	1.7	Withdrew		
	L3	2.6	1.8	Withdrew		
TSC1(L)	R1	2.4	1.9	1.9	1.9	2.0
	R2	2.2	1.4	1.4	1.3	1.3
TSC2(L)	R1	3.2	2.6	2.1	2.3	Withdrew
	R2	2.2	1.8	1.4	1.6	Withdrew
	R3	2.2	2.1	2.0	2.0	Withdrew
	L1	3.8	3.4	2.8	2.4	Withdrew
TSC3	R1	2.4	2.2	2.1	Withdrew	
	R2	2.4	1.9	1.9	Withdrew	
	L1	5.4	4.6	4.3	Withdrew	
	L2	2.2	2.4	1.9	Withdrew	
	L3	2.7	2.6	2.8	Withdrew	
TSC4	R1	14.1	12.8	13.3	Withdrew	
	R2	2.7	2.7	2.5	Withdrew	
	R3	3.4	3.3	3.7	Withdrew	
	R4	3.2	4.2	4.2	Withdrew	
	R5	5.4	5.4	6.4	Withdrew	
	L1	10.6	10.2	8.3	Withdrew	
	L2	4.4	4.5	4.8	Withdrew	
	L3	3.6	3.5	3.4	Withdrew	
	L4	3.4	3.3	3.2	Withdrew	
TSC5	R1	2.3	1.9	1.8	1.8	1.6
	R2	4.5	3.8	2.8	2.8	2.9
TSC6	R1	2.4	ND	2.3	1.8	1.6
	L1	2.8	ND	2.3	1.7	2.1
	L2	2.4	ND	2.6	1.9	1.9
TSC7	R1	2.5	2.4	2.2	1.8	1.8
	R2	3.8	2.8	2.7	2.8	3.2
	R3	3.3	2.4	2.3	2.1	2.1
	R4	2.9	2.2	2.4	2.1	1.9

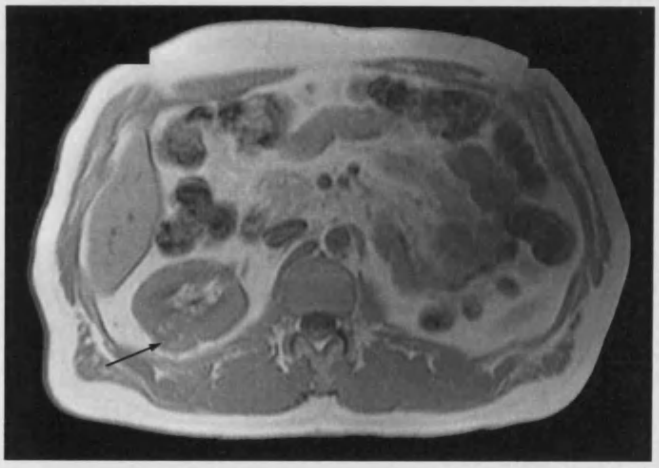
TSC8(L)	R1	3.9	2.3	2.6	2.5	2.5
	L1	4.3	3.1	3.6	4.2	4.3
TSC9	R1	2.0	1.7	1.7	1.9	1.7
	R2	4.2	3.5	3.8	3.2	3.2
TSC10	R1	2.9	2.8	2.8	2.2	2.0
	L1	3.2	2.9	2.7	2.5	2.5
	L2	3.3	3.5	3.3	3.4	3.3

Target angiomyolipomas in the right (R) and/or left (L) kidney in each patient were visualised by magnetic resonance imaging (MRI) at baseline and at 2,6,12 and 24 months and the longest diameter of each angiomyolipoma was measured. Values are in cm. ND = MRI not done due to inter-current illness.

Figure 7: MRI scans showing AMLs in the abdomen of a patient (TSC5) with the tuberous sclerosis.



Baseline



24 months

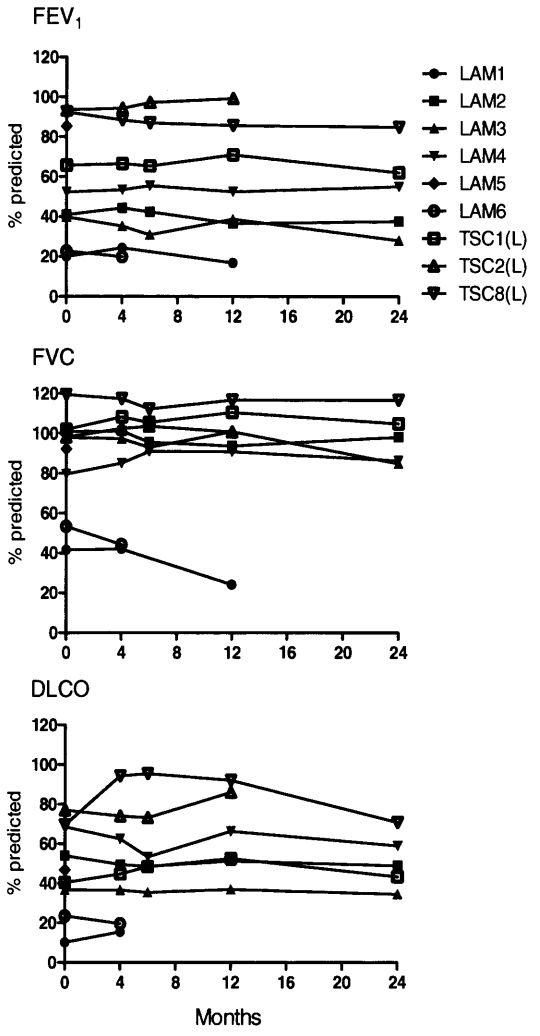
Unilateral AMLs in a patient with a single kidney are shown at baseline and after 24 months of sirolimus therapy. A lesion in the right kidney is identified by an arrow.

5.13.3 Pulmonary function

Baseline lung function in patients with lymphangioleiomyomatosis showed wide variation in airflow obstruction and gas transfer (fig. 8 and table 8). FEV1 and FVC fell in most patients during the trial. The mean +/-SD decline in FEV_1 and FVC were 76 +/-52 and 55 +/-94 ml/yr respectively for the 5 patients with measurements over 2 years and for the 7 patients with at least one year of measurements 49 +/-93 and 100 +/-181 ml/yr respectively. Mean changes in DLCO for the 5 patients completing 2 years and for the 6 completing at least one year were -0.49 +/- 0.55 and -0.04 +/-1.23 ml/min/mmHg/yr.

Four patients (LAM1, LAM2, LAM4 and TSC1) had serial measurements of FEV1 prior to enrollment, for 16, 132, 42 and 106 months respectively. Mean annual change in FEV1 for these patients before and during the trial were -172 and -94, -122 and -89, -90 and +10 and -49 and -69 ml/yr respectively. There were no clinically relevant changes in lung CT appearances during the trial.

Figure 8: Pulmonary function studies in patients with sporadic or tuberous sclerosis-associated lymphangioleiomyomatosis



Forced expiratory volume at 1 second (FEV1), forced vital capacity (FVC) and diffusing capacity of the lung for carbon monoxide (DL_{CO}) are shown as percentages of the predicted value for women of equivalent age and height.

Table 8: Pulmonary function tests in patients with tuberous sclerosis-associated or sporadic lymphangioleiomyomatosis.

FEV_1							
	% pred.	**Litres**					**Annual rate of change, ml/yr**
Patient	**Baseline**	**Baseline**	**4 Mo**	**6 Mo**	**12 Mo**	**24 Mo**	
LAM1	20	0.41	0.50	not done	0.34	died	-94
LAM2	41	1.11	1.20	1.15	0.98	1.00	-89
LAM3	40	1.03	0.91	0.80	0.99	0.71	-120
LAM4	52	1.50	1.53	1.59	1.49	1.55	+9
LAM5	85	2.55	withdrew				
LAM6	23	0.63	0.55	withdrew			-240
TSC1(L)	66	2.29	2.32	2.28	2.46	2.13	-69
TSC2(L)	93	2.43	2.45	2.53	2.56	withdrew	+138
TSC8(L)	92	2.69	2.57	2.53	2.47	2.43	-114
FVC							
	% pred	**Litres**					**Annual rate of change, ml/yr**
LAM1	42	1.01	1.02	not done	0.58	died	-463
LAM2	101	3.19	3.20	3.02	2.94	3.05	-78
LAM3	98	2.96	2.94	2.81	3.02	2.52	-198
LAM4	80	2.65	2.83	3.03	3.00	2.83	+52
LAM5	92	3.18	withdrew				
LAM6	53	1.71	1.42	withdrew			-870
TSC1(L)	102	4.10	4.35	4.24	4.41	4.16	0
TSC2(L)	98	3.01	3.14	3.17	3.06	withdrew	+34
TSC8(L)	119	4.01	3.94	3.77	3.89	3.86	-49
DLco							
	% pred	**ml/min/mmHg**					**Annual rate of change ml/min/mmHg/yr**
LAM1	10	2.12	3.25	not done	not done	died	+3.39
LAM2	54	13.43	12.33	12.1	12.70	12.03	-0.44
LAM3	37	8.87	8.81	8.54	8.84	8.21	-0.29
LAM4	69	17.70	16.12	13.77	17.02	15.05	-0.70
LAM5	47	12.42	withdrew				
LAM6	24	5.94	4.96	withdrew			-2.94
TSC1(L)	40	11.97	13.25	14.32	15.52	12.68	+0.22
TSC2(L)	77	18.75	17.98	17.8	20.83	withdrew	+2.27
TSC8(L)	70	18.10	24.55	24.84	23.83	18.22	-1.28

5.13.4 Neurocognitive function

The mean (+/-SD) IQ of patients with tuberous sclerosis was 107 (+/-12) and of sporadic lymphangioleiomyomatosis patients 105 (+/-15). Patients with tuberous sclerosis showed neurocognitive deficits (performance <5th percentile) in 9 of 88 tests (10.2%) at baseline, in 6 of 88 (6.8%) at 4 months and in 7 of 77 (9.1%) at 12 months (table 9). By comparison, patients with sporadic lymphangioleiomyomatosis showed deficits in 2 of 63 tests (3.2%) at baseline, 1 of 45 (2.2%) at 4 months and none of 44 at 12 months. Most deficits were in executive function. All patients showed changes in test performance during the trial. Six patients (TSC1, TSC 5, TSC7, TSC8, LAM1 and LAM4) had greater than one percentile band decrease at 12 months compared to baseline in at least one test.

Seven patients with tuberous sclerosis completed neurocognitive assessment at 12 months and 1 (TSC4) withdrew after the 4 month assessment. Seven of the 8 showed an increase in recall memory scores from baseline. In contrast, recognition memory scores fell in 5 of 8 and none showed an increase. Executive scores increased in 5 of 8 patients (figure 9).

Of the 5 patients with sporadic lymphangioleiomyomatosis, 4 completed 12 month assessments and one (LAM5) withdrew after the 4 month assessment. Three showed increases in recall memory scores, two increased recognition memory scores and 4 increased executive scores (Figure 9).

One patient with tuberous sclerosis and 2 with sporadic lymphangioleiomyomatosis showed decreased recall memory scores. These patients (TSC3; LAM3; LAM6) had

the highest baseline scores. No patient showed an increase across all three domains and none a decrease across all three domains (figure 9).

Table 9: Neurocognitive Tests Percentile Scores

TESTS		TSC 1	TSC 2	TSC 3	TSC 4	TSC 5	TSC 6	TSC 7	TSC 8	LA M1	LA M2	LA M3	LA M4	LA M5	LA M6
AMIPB															
List learning															
Immediate recall	B	90	<5	50	25	50	75	25	75	75	25	25	75	75	75
	4	90	<5	50	90	25	90	50	75	90	25	50	75	---	75
	12	90	<5	50	---	75	90	50	75	90	50	50	90	---	---
List learning															
Delayed one list Recall	B	90	6	75	25	50	90	25	50	---	10	50	90	50	75
	4	75	6	50	90	50	90	75	50	90	10	50	75	---	75
	12	90	10	50	---	50	90	50	75	90	50	50	90	---	---
Complex figure															
Immediate recall	B	50	10	90	25	50	75	6	90	50	10	50	50	25	75
	4	90	50	75	50	75	50	50	75	90	25	75	25	---	90
	12	90	50	90	---	90	50	50	50	50	10	50	25	---	---
Complex figure															
Delayed recall	B	75	25	90	25	75	75	10	90	---	10	50	50	50	75
	4	90	25	90	50	75	25	50	90	90	25	75	25	---	90
	12	90	50	90	---	90	50	25	75	25	25	50	25	---	---
Story															
Immediate recall	B	50	10	50	50	75	50	50	90	90	50	10	90	90	25
	4	90	25	50	90	25	50	90	50	90	50	25	25	---	90
	12	75	25	75	---	25	75	75	90	90	90	50	25	---	---
Story															
Delayed recall	B	75	25	50	90	75	25	50	90	90	50	6	90	90	25
	4	90	10	75	90	10	75	90	75	90	75	25	25	---	90
	12	50	25	75	---	25	90	50	90	90	90	75	25	---	---
CANTAB															
SWM															
≥4 betwn errors	B	10	<5	75	10	25	10	<5	50	50	10	10	75	10	50
	4	10	<5	90	10	25	10	6	50	90	25	25	50	---	50
	12	50	<5	90	---	10	10	<5	25	10	50	90	90	---	---
SOC															
Trials in min moves	B	75	<5	25	50	<5	25	25	25	25	50	25	25	10	<5
	4	90	<5	75	25	50	50	<5	50	50	90	25	75	---	25
	12	50	10	90	---	<5	25	<5	75	25	90	25	75	---	---
IDED															
Errors over	B	6	50	50	10	25	<5	<5	<5	25	25	<5	<5	50	50

TESTS		TSC 1	TSC 2	TSC 3	TSC 4	TSC 5	TSC 6	TSC 7	TSC 8	LA M1	LA M2	LA M3	LA M4	LA M5	LA M6
stages 1-7	4	10	75	50	25	50	<5	10	75	25	25	6	75	---	<5
	12	10	50	50	---	10	25	25	75	25	75	6	25	---	---
PRM															
Percent correct	B	90	10	50	50	75	75	25	50	90	10	25	90	50	90
	4	90	10	25	25	90	75	25	10	90	10	25	25	---	90
	12	90	10	10	---	75	75	25	25	90	6	50	50	---	---
SRM															
Percent correct	B	25	<5	25	75	25	75	6	50	25	25	10	50	---	25
	4	25	<5	25	25	50	25	6	25	10	6	10	25	---	25
	12	25	<5	25	---	6	50	6	<5	50	10	10	50	---	---

Numbers refer to the following percentile bands:

<5 = "Below the 5th percentile

6 = "Above 5th but below the 10th percentile"

10 = "Between 10th and 24th percentiles"

25 = "Between 25th and 49th percentiles"

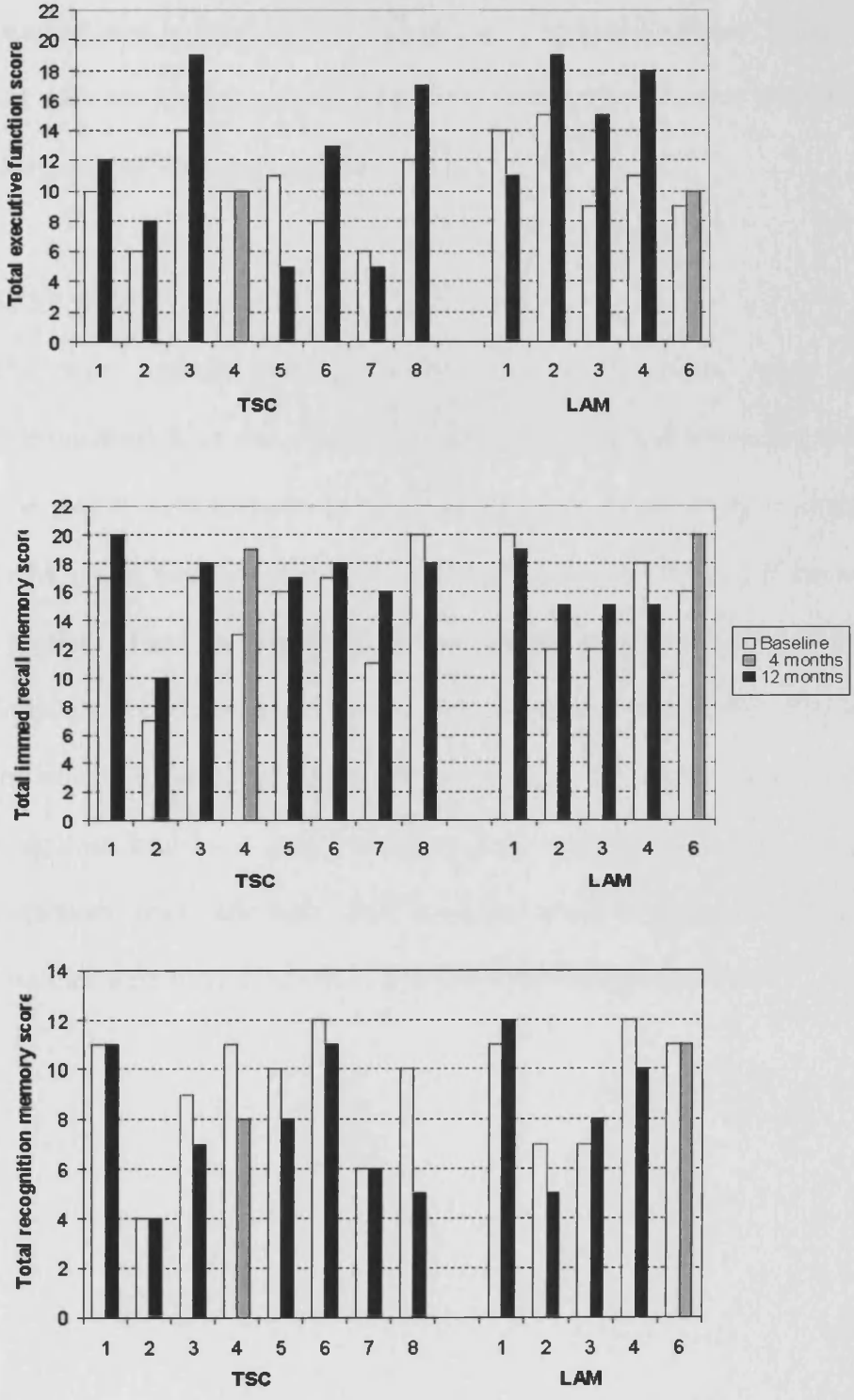
50 = "Between 50th and 74th percentiles"

75 = "Between 75th and 89th percentiles"

90 = "In or above the 90th percentile"

Key: AMIPB = Adult Memory and Information Processing Battery; CANTAB = Cambridge Neuropsychological Testing Automated Battery; SWM = Spatial Working Memory subtest; ≥4 betwn errors = between errors on 4 or more boxes; SOC = Stockings of Cambridge subtest; Trials in min moves = Trials in minimum moves; IDED = Intra-Dimensional Extra-Dimensional subtest; PRM = Pattern Recognition Memory subtest; SRM = Spatial Recognition Memory subtest. B = Baseline testing session; 4 = 4 months testing session; 12 = 12 months testing session.

Figure 9: Neurocognitive test percentile scores.



Test performance was measured at baseline and at 12 months (at 4 months in patients TSC4 and LAM6 who withdrew prior to the 12 month assessment) using parallel test versions.

5.13.5 Neuroimaging

Baseline cranial MRI scans in patients with tuberous sclerosis showed cortical tubers and sub ependymal nodules but no subependymal giant cell astrocytomas and no changes were seen at 12 months.

5.13.6 Safety

The most common adverse events were mouth ulcers, upper respiratory tract infection/bronchitis and proteinuria (table 10). Seven serious adverse events occurred. One patient with sporadic LAM died following a respiratory infection. Another two LAM patient were admitted to hospital, one with pharyngitis and the other with a chest infection. The four remaining serious adverse events were thought to be unlikely or definitely not related to sirolimus: a fracture of the tibula/fibula after a fall, chest pain for which no cause was found, urinary obstruction secondary to an ovarian cyst and musculoskeletal back pain. No patient had a seizure during the study period. Upper respiratory tract infections were more common in patients with LAM, metabolic toxicities were more common in patients with tuberous sclerosis.

Table 10: Sirolimus-related treatment -emergent adverse events

Category	Diagnosis	No. of events	No. of patients	Grade 1-2	Grade 3-4	Grade 5
Gastrointestinal						
Oral mucositis	All	9	9	9	-	-
	SLAM	4	3	4	-	-
	TLAM	3	2	3	-	-
	TSC	2	1	2	-	-
Diarrhoea	All	1	1	1	-	-
	SLAM	1	1	1	-	-
	TLAM	-	-	-	-	-
	TSC	-	-	-	-	-
Nausea	All	1	1	1	-	-
	SLAM	1	1	1	-	-
	TLAM	-	-	-	-	-
	TSC	-	-	-	-	
Infection						
Upper respiratory tract or bronchitis	All	16	5	15	-	1
	SLAM	16	5	5	-	1
	TLAM	-	-	-	-	-
	TSC	-	-	-	-	-
Pharyngitis	All	1	1	-	1	-
	SLAM	1	1	-	1	-
	TLAM	-	-	-	-	-
	TSC	-	-	-	-	-
Urinary tract infection	All	3	3	3	-	-
	SLAM	2	2	2	-	-
	TLAM	1	1	1	-	-
	TSC	-	-	-	-	-
Cellulitis	All	2	2	2		
	SLAM	-	-	-	-	-
	TLAM	1	1	1	-	-
	TSC	1	1	1	-	-
Oral cavity	All	1	1	1	-	-
	SLAM	1	1	1	-	-
	TLAM	-	-	-	-	-
	TSC	-	-	-	-	-
Metabolic						
Proteinuria	All	5	5	5	-	-
	SLAM				-	-
	TLAM	1	1	1	-	-
	TSC	4	4	4	-	-
Raised creatinine kinase	All	3	3	3	-	-
	SLAM				-	-
	TLAM	2	2	2	-	-
	TSC	1	1	1	-	-
Hypertriglyceridaemia	All	2	2	2	-	-
	SLAM	-	-	-	-	-
	TLAM	1	1	1	-	-
	TSC	1	1	1	-	-
Raised ALP	All	1	1	1	-	-
	SLAM	-	-	-	-	-
	TLAM	-	-	-	-	-
	TSC	1	1	1	-	-
Hypokalaemia	All	1	1	1	-	-
	SLAM	1	1	1	-	-
	TLAM	-	-	-	-	-
	TSC	-	-	-	-	-
Soft tissues						
Peripheral oedema	All	3	3	3	-	-
	SLAM	-	-	-	-	-
	TLAM	1	1	1	-	-
	TSC	2	2	2	-	-
					-	-
					-	-
					-	-
					-	-

Category	Diagnosis	No. of events	No. of patients	Grade 1-2	Grade 3-4	Grade 5
Constitutional symptoms						
General malaise	All	1	1	1	-	-
	SLAM	1	1	1	-	-
	TLAM	-	-	-	-	-
	TSC	-	-	-	-	-
Fatigue	All	3	3	3	-	-
	SLAM	-	-	-	-	-
	TLAM	2	2	2	-	-
	TSC	1	1	1	-	-
Dermatolgy						
Acneform rash	All	2	2	2	-	-
	SLAM	-	-	-	-	-
	TLAM	-	-	-	-	-
	TSC	2	2	2	-	-
Exacerbation of eczema	All	1	1	1	-	-
	SLAM	-	-	-	-	-
	TLAM	1	1	1	-	-
	TSC	-	-	-	-	-
Cardiac						
Palpitations	All	2	2	2	-	-
	SLAM	2	2	2	-	-
	TLAM	-	-	-	-	-
	TSC	-	-	-	-	-
Neurology						
Depression	All	1	1	1	-	-
	SLAM	-	-	-	-	-
	TLAM	-	-	-	-	-
	TSC	1	1	1	-	-
Endocrine						
Hypothyroidism	All	1	1	1	-	-
	SLAM	-	-	-	-	-
	TLAM	-	-	-	-	-
	TSC	1	1	1	-	-
Ocular						
Retinal tear	All	1	1	1	-	-
	SLAM	-	-	-	-	-
	TLAM	1	1	1	-	-
	TSC	-	-	-	-	-
Blood						
Anaemia	All	1	1	1	-	-
	SLAM	-	-	-	-	-
	TLAM	-	-	-	-	-
	TSC	1	1	1	-	-

5.14 Discussion

5.14.1 Summary

This trial determined the response of renal angiomyolipomas in patients with tuberous sclerosis or sporadic lymphangioleiomyomatosis to 2 years of sirolimus treatment. A response (as defined by the RECIST criteria) was achieved in 8 out of 16 (50%) patients overall and in 8 of 10 (80%) in the per protocol group. The reduction in sum LD was sustained over the 2 year duration of sirolimus treatment for the majority of patients. A response was seen in 42% of patients maintained at a sirolimus dose of 3-6 mg/ml. Of 23 angiomyolipomas evaluated at 24 months, 21 were smaller and 2 were unchanged.

One previous trial reported by Bissler *et al.* investigated sirolimus treatment for angiomyolipoma in patients with tuberous sclerosis or sporadic lymphangioleiomyomatosis and found a mean reduction in angiomyolipoma volume of 47% at 12 months [279]. In the current trial angiomyolipomas measured at 12 months showed a mean reduction in their longest diameters of 25% compared to baseline, equivalent to a volume reduction of 60% if a spherical shape is assumed.

While 12 of 16 patients in the current trial were maintained at trough blood levels of 3-6 ng/ml and the other 4 at 6-10 ng/ml all but one of the patients in the trial by Bissler *et al.* were escalated to trough blood levels of 10-15 ng/ml. The current trial shows that sirolimus levels at the lower end of the immunosuppressive range are effective in reducing angiomyolipoma size in tuberous sclerosis or sporadic lymphangioleiomyomatosis.

In the trial of Bissler *et al.* angiomyolipoma volumes increased after sirolimus therapy was withdrawn at 12 months, returning to 85.9% of the baseline value by 24 months. Our study suggests that AML regression is maintained or increased during the second

year of sirolimus therapy. Longer term therapy might need to be considered in patients with tuberous sclerosis or lymphangioleiomyomatosis.

In the 10 patients with LAM there was no sustained improvement in lung function, nor was there any apparent change on chest imaging. In recent studies of lymphangioleiomyomatosis, the mean rate of decline in FEV_1 has ranged from 75-118 ml/yr [32, 41]while in this trial it was 76 ml/yr in 5 patients who had measurements over 2 years and 49 ml/yr in 7 patients with measurements over one year or more. Our findings in four patients with serial measurements prior to as well as during treatment were compatible with sirolimus causing some slowing in the rate of decline in FEV1. We did not observe the improvement in FEV1 and FVC seen by Bissler *et al.* although in both studies there was no sustained improvement in DLCO. This difference between studies may reflect differences in sirolimus levels achieved or in the baseline lung function of the patients or may be a reflection of the small patient numbers in both studies.

The reversal of spatial learning deficits by rapamycin (sirolimus) treatment in heterozygous *Tsc2* mice [271] has suggested that mTOR inhibitors might also improve specific neurocognitive problems associated with tuberous sclerosis. In most of the patients with tuberous sclerosis scores for recall memory, that has been associated with mTOR activity[15], increased while those for recognition memory, a domain not consistently associated with mTOR activity, did not. We included tests of executive skills because of the high rates of such deficits in tuberous sclerosis. More executive scores increased than decreased in patients with tuberous sclerosis and in those with sporadic lymphangioleiomyomatosis; observations that might reflect drug or practice effects.

Adverse events were common and consistent with the known toxicity profile of sirolimus. The majority of patients experienced some mild/moderate adverse events and sirolimus had to be withdrawn due to toxicity in two patients. There were six serious adverse events including one patient with LAM who died from a respiratory infection. The toxicities seen in our study and that of Bissler *et al.* are broadly comparable. The pattern of toxicities appears to differ between those patients with sporadic LAM and those with tuberous sclerosis, with the LAM patients having more respiratory toxicities and those with tuberous sclerosis more metabolic toxicities. This may reflect underlying differences in organ involvement but the effects of haploinsufficincy in the *TSC* genes may influence toxicity in patients with tuberous sclerosis. Sirolimus is a relatively toxic drug with frequently occurring side effects of mild to moderate severity but also rarely occurring toxicities that are potentially life threatening such as interstitial peumonitis. This raises important questions with regard to the risk/benefit analysis of sirolimus use in the treatment of tuberous sclerosis and LAM.

This study has important limitations including the small sample size, the inclusion of patients with two conditions (tuberous sclerosis and sporadic LAM) with linked but separate aetiologies, the lack of a control group and that 6 of the 16 patients who were recruited did not undergo the 24 month evaluation. The primary endpoint was the response of AMLs as defined by RECIST criteria and the clinical significance of a reduction in AMLs size needs to be established. The small sample size particularly restricts the conclusions that can be drawn from the secondary outcome measures. No biopsies were taken that would have allowed assessment of mTORC1 modulation by sirolimus and of possible mechanisms of reduction in AML. The reduction in AML size may reflect an induction of apoptosis, a decrease in cell size, an inhibition in proliferation, interference in angiogenesis or other mechanisms.

Model systems suggest that *TSC1/2* deficient cells are 'addicted' to the mTORC1 pathway [289, 290]. However, this clinical study and others highlight the limitations of sirolimus in terms of AMLs response and effects on lung function. Not all AMLs respond, the degree of response is variable, incomplete and largely reversible on cessation of drug treatment.

Against many cell types, sirolimus is mainly a cytostatic agent and its efficacy may be compromised by effects on feed back loops and crosstalk between intracellular signalling pathways. Also, sirolimus would not influence mTOR independent consequences of TSC1/2 deficiency nor sirolimus resistant mTOR functions.

Alternative strategies to target TSC1/2 deficient cells that potentially overcome these problems include the use of:

- drug combinations;
- alternative mTORC1 inhibitors; and
- agents that target processes downsteam of mTORC1.

Inhibition of mTOR can relieve inhibitory feedback to other signalling molecules such as ERK or AKT and up regulation of compensatory pathways may allow 'addiction bypass' and attenuate the efficacy of mTOR inhibitors. This might be overcome by the use of multiple agents to target differences in a pathway [291] or by the use of drugs that interact with multiple targets [264].

ATP competitive mTORC1 and mTORC2 inhibitors have greater effects on cell proliferation and induction of apoptosis than sirolimus in a variety of cell types, both *in*

vitro and *in vivo*. This increased efficacy has been attributed to increased inhibition of mTORC1 and mTORC2 as well as suppression of sirolimus-resistant functions [292]. However, use of these agents in animal models or patients has not been reported.

TSC1/2 deficient cells appear to be highly sensitive to some forms of stress. The cells have an altered metabolic phenotype and exhibit a metabolic inflexibility that results in glucose addiction [111, 122]. The cells are also highly sensitive to other forms of stress which may reflect an inability to down regulate mTORC1 activity in response to stress. TSC1/2 deficient MEFs exhibit increased basal levels of ER stress compared to wild type cells and the use of drugs which increase ER stress is associated with a selective increase in apoptosis in the TSC1/2 deficient cells in culture [194, 195]. However, experimental use of nelfinavir, a HIV protease inhibitor that is known to induce ER stress [293], did not appear to shrink or prevent renal tumours in transgenic mouse models (Dr Ming Shen, personal communication)

TSC1/2 deficiency may be a biomarker of responsiveness to mTOR inhibition, not only in the context of tuberous sclerosis and LAM but also in sporadic cancers. Small mutations in *TSC1* have been demonstrated in bladder cancer (16% c [294] and in *TSC2* in pancreatic neuroendocrine tumours (8.8% of tumours) [295]. Decreased expression of either *TSC1* or *TSC2* mRNA has been noted in acute myeloid leukaemia [296], endometrial cancer [297] and breast cancer [298] and this has been associated with promoter hypermethylation. Loss of heterozygosity for either *TSC1* or *TSC2* has been demonstrated in bladder cancer [294] and lung cancer [299] and the possibility of a role of haploinsufficncy in *TSC1* or *TSC2* has been suggested. Phosphorylation of TSC2,

leading to probable inhibition of function, mediated either by ERK or Akt has been demonstrated in breast, colon, endometrial and papillary renal cancer [72].

5.14.2 Conclusion

Our trial represents an early example of therapeutic targeting of a signalling pathway in Mendelian and sporadic disorders that share a common molecular pathology. The high response rate seen in the trial underscores the potential for effective targeted treatment when the setting is one of relative molecular homogeneity. Although our findings are based upon a small number of patients in a non-controlled open label trial, they suggest that mTOR inhibition may ameliorate some aspects of tuberous sclerosis and LAM and that larger controlled trials are warranted.

Very recently, the mTOR inhibitor, everolimus, has been approved by the American Food and Drug Administration for patients with SEGAs associated with tuberous sclerosis who require therapy but are not candidates for surgical resection on the basis of the single-arm open label trial of Krueger *et al* [280]. However, how mTOR inhibition will effect the long term outcome in this patient group remains unclear. The clinical trails database http://www.clinicaltrials.gov lists several completed, ongoing or approved trials of rapalogs for AMLS, brain lesions, epilepsy, lung disease and skin disease in tuberous sclerosis or lymphangioleiomyomatosis (table 11).

Table 11: Clinical trials of rapalogs in tuberous sclerosis or lymphangioleiomyomatosis

Identifiers	Patient group	Recruitment target	Drug	Trial deign	Primary outcome measure
NCT00790400 Exist-2	Tuberous Sclerosis or Sporadic LAM	99	Everolimus (RAD001)	Randomized, Double Blind, Placebo Control, Crossover Assignment, phase III	AML response rate
NCT00457964	Tuberous Sclerosis or Sporadic LAM	30	Everolimus (RAD001)	Non-Randomized, Open Label, Uncontrolled, Single Group Assignment, phase II	AML burden
NCT00126672	Tuberous Sclerosis or Sporadic LAM	36	Sirolimus	Non-Randomized, Open Label, Uncontrolled, Single Group Assignment, phase II	AML response rate
NCT01059318	LAM	20	Everolimus (RAD001)	Non-randomized, Open Label, Within-patient Multiple Dose-escalation, phase II	Safety, PK and PD endpoints
NCT00414648	LAM	120	Everolimus (RAD001)	Randomized, Double Blind (Subject, Outcomes Assessor), Placebo Control, Parallel Assignment, phase III	Lung function, adverse events
NCT01031901	Tuberous Sclerosis or NF1	60	sirolimus	Randomized, Double Blind, Placebo Control, Parallel Assignment, phase 1	Sirolimus levels
NCT00411619	Tuberous Sclerosis	20	Everolimus (RAD001)	Non-Randomized, Open Label, Uncontrolled, Single Group Assignment, phase I/11	SEGA response rate
NCT00789828	Tuberous Sclerosis	99	Everolimus (RAD001)	Randomized, Double Blind, Placebo Control, Crossover Assignment, phase III	SEGA response rate
NCT01070316	Tuberous Sclerosis	20	Everolimus (RAD001)	Non-Randomized, Open Label, Uncontrolled, Single Group Assignment, phase I/II	Reduction in seizure frequency

Identifiers	Patient group	Recruitment target	drug	Trial design	Primary outcome measure
NCT00411619	Tuberous Sclerosis	20	Everolimus (RAD001)	Non-Randomized, Open Label, Uncontrolled, Single Group Assignment, phase I/11	SEGA response rate
NCT00789828	Tuberous Sclerosis	99	Everolimus (RAD001)	Randomized, Double Blind, Placebo Control, Crossover Assignment, phase III	SEGA response rate
NCT01070316	Tuberous Sclerosis	20	Everolimus (RAD001)	Non-Randomized, Open Label, Uncontrolled, Single Group Assignment, phase I/II	Reduction in seizure frequency

Clinical trials of rapalogs in tuberous sclerosis or lymphangioleiomyomatosis currently listed on http://www.clinicaltrials.gov

Chapter 6: Small-molecule signal-transduction inhibitors as targeted therapeutic agents for single-gene disorders

6.1 Introduction

Mutations affecting over 2,000 of the 20,000 or so genes in the human genome have been linked so far to specific inherited diseases, most of which are rare. Many of the genes involved encode components of intracellular signalling pathways that regulate processes such as the growth, proliferation, differentiation, and survival or programmed death of cells during development and the maintenance of tissues and organs. Mutations that change the function of genes encoding signalling proteins thereby cause disorders ranging from birth defects to cancer. For Mendelian disorders, the essentially causal relationship between mutation and disease may present direct opportunities to therapeutically manipulate intracellular signalling.

6.2 Targeting tumours

Mutations in oncogenes and tumour suppressor genes result in the deregulation of intracellular signalling pathways that control apoptosis, proliferation, angiogenesis, metabolism, stress responses and other cellular functions. In recent years, many agents that manipulate intracellular signalling have been developed as potential cancer treatments. The successes of signal transduction inhibitors such as imatinib, gefitinib and erlotinib show this approach can yield valuable clinical benefit, but experience of their use has highlighted the importance of patient selection [300]. When signal-transduction inhibitors have been used in patient groups defined by tumour histology, the results have been generally disappointing, reflecting underlying heterogeneity in molecular pathology. In contrast, successes have occurred when these drugs have been used against tumours that share a specific molecular defect. Examples include gastrointestinal stromal tumours, most of which carry mutations in the c-kit oncogene,

the target for imatinib, and non-small cell lung cancer in which specific mutations in epidermal growth factor receptor (EGFR) predict response to gefitinib or erlotinib [301, 302]. Such tumours exhibit a dependence upon a specific aberrant signalling pathway or gene product, and this phenomenon has been termed "oncogene addiction" or "tumour suppressor hypersensitivity", depending on the nature of the responsible gene [303]. In these settings, suppressing oncogenes activity or restoring tumour suppressor activity has been shown to be potentially deleterious to a cancer cell.

A related approach utilises the concept of synthetic lethality. Two genes have a synthetic relationship when inhibition or mutation of either gene alone does not cause loss of viability, but simultaneous inhibition or mutation of both genes results in reduced cell viability [304]. Synthetic lethality underlies the rationale for the use of Poly(ADP-Ribose) Polymerase 1 (PARP) inhibitors in BRCA 1 or 2 deficient tumours [305]. PARP is required to repair single stranded DNA breaks. If PARP is inhibited single stranded DNA breaks persist and during DNA replication are converted to double stranded breaks. These can normally be repaired by BRCA1/2 mediated homologous recombination which is a relatively error free process. In the absence of functional BRCA1/2 the double stranded DNA breaks persist or are repaired by error prone non-homologous end joining, leading to cell death. Many synthetic lethal relationships represent examples of functional buffering where when one molecular function, gene or protein is lost, another can readily be used to mitigate any effects that could limit either cell survival [306]. Exploiting oncogene or tumour suppressor gene induced lack of flexibility in metabolic or stress responses can be thought of as a synthetic lethal approach where multiple targets involved in a cellular process may be synthetically lethal with the mutated oncogene or tumour suppressor gene.

Tumours arising in specific Mendelian tumour predisposition syndromes share at least one key molecular abnormality, for example, mutations in the tumour suppressors *TSC1/2* in tuberous sclerosis, *VHL* in Von Hippel-Lindau disease or *NF1* in neurofibromatosis type 1. This relative molecular homogeneity may facilitate selection of therapeutic agents to exploit oncogene addiction/tumour suppressor hypersensitivity or synthetic lethality.

Trials of mTOR inhibitors in sporadic cancer have generally been disappointing. The rapalog temsirolimus has been licensed in the UK and USA as a treatment for advanced clear cell renal carcinoma following a phase III study that showed that temsirolimus improved overall survival compared to treatment with interferon. Median overall survival times in the interferon group and the temsirolimus group were 7.3 and 10.9 months respectively. The objective response rate was 8.6%, among patients receiving temsirolimus, the proportion of patients with stable disease for at least 6 months or an objective response was 32.1% in this group [307]. VHL is mutated in 55% [308] and methylated in approximately 30% [309] of cases of clear renal cell cancer. The efficacy of mTOR inhibition in the clear cell renal cancer may, at least in part, be due to a reduction in the abnormally high HIF activity caused by VHL deficiency [310]. However, the objective response rate to temsirolimus in renal cancer is far below that seen in our trial. This may reflect that the tumours we treated were benign and do not exhibit the genomic instability of more malignant tumours, with the accompanying ability to develop drug resistance. However, it may also reflect the underlying homogeneity of addiction in the lesions we treated.

6.3 Targeting the brain

Many Mendelian disorders are associated with cognitive deficits. Recently, interest has become focused on whether some of these deficits might be amenable to the therapeutic targeting of intracellular signalling pathways, even if this is started in adulthood.

de Vries and Howe hypothesized that neurocognitive deficits in tuberous sclerosis are a direct result of aberrant intracellular signalling and that tubers and seizures were therefore neither necessary nor sufficient to explain the observed neurocognitive phenotypes [8]. A corollary of this is that molecular modulation may improve or reverse such neurocognitive deficits. The beneficial effects of mTOR inhibition on cognitive function seen in tuberous sclerosis animal models are consistent with the results of our trial. Larger placebo controlled trials of mTOR inhibition in tuberous sclerosis, where cognition function is the primary outcome, are currently being planned in the USA and UK.

A number of neurocognitive deficits occur in Fragile X syndrome [311]. This condition results from a trinucleotide repeat expansion in the promoter of the *FMR1* (fragile X mental retardation–1) gene [312] which leads to transcriptional silencing of *FMR1* and reduced expression of the *FMR1* protein (FMRP) [313]. FMRP is an RNA binding protein which modulates protein synthesis through mechanisms including inhibition of group 1 metabotropic glutamate receptor (mGluR1 and mGluR5) mediated mRNA translation [314]. mGluR antagonists ameliorated memory impairments in a *Drosophila* model of Fragile X [315] and a number of Fragile X phenotypes in mice [316, 317]. Very preliminary studies in humans using lithium [318] or fenobam [319], mGluR5 antagonists, are encouraging but properly powered trials are required.

The difficulties of extrapolating from mouse models to humans and of conducting trials in the area of cognitive function have been illustrated in a trial of statins in neurofibromatosis type 1 (NF1) [320]. This condition is characterised by a number of neurocutaneous manifestations, including tumour growth and the frequent occurrence of intellectual disability and specific neuropsychological deficits. Neurofibromatosis type 1 is caused by mutations in the NF1 gene, which encodes a GTPase activating protein whose normal role is to regulate RAS signalling. [321] Increased RAS signalling has been implicated in the neuronal plasticity defects and spatial learning and attention problems seen in neurofibromatosis type 1 mouse models [322, 323]. RAS signalling can be inhibited by farnesyl transferase inhibitors and 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, which block the isoprenylation required for RAS activity. Treatment of neurofibromatosis type 1 mouse models with these agents for just a few days was found to lead to a reversal of cognitive function deficits [324]. On the basis of these findings, Krab et al [320] conducted a clinical trial to assess the effect on cognitive function of the HMG-CoA reductase inhibitor simvastatin in children with NF1. Sixty-two children were randomised to receive either simvastatin or placebo for 12 weeks. However, no significant differences were observed in the primary outcome measures of cognitive function. The authors reflected that this study had a number of limitations. The lack of effect may reflect an inadequate treatment duration or failure to achieve adequate drug concentrations in the brain. The placebo group performed better in a number of cognitive function tests and there was a relatively high amount of missing data.

Cognitive function is inherently complex and multifaceted. In trials assessing cognitive function careful thought must be given to the selection of the cognitive tests performed, to maximise the chance of capturing changes. Equally, consideration must be given as to whether changes in cognitive function will result in clinically meaningful benefits to the patient and that the potential risks of taking an investigational drug are justified.

Inhibition of mTORC1 has also been proposed as a therapeutic strategy for neurodegenerative conditions such as Huntington disease where the formation of abnormal protein aggregates may contribute to pathogenesis [325]. Inhibition of mTORC1 with sirolimus in mouse models of Huntington disease enhanced the clearance of these proteins, possibly by up regulating autophagy, and ameliorated behavioural abnormalities [326].

6.4 Targeting tissue integrity

The maintenance of cell and tissue integrity requires a balance between cell death and division, control of cell differentiation and regulated interplay between cells and the extracellular matrix.

Marfan syndrome is characterised by abnormalities of the skeletal, cardiovascular and ocular systems and affects approximately 1 in 20 000 individuals [327]. It is caused by mutations in the *FBN1* gene, which encodes fibrillin-1. This microfibril protein contributes to the mechanical integrity of organs and the vasculature. Aortic root dissection is a major cause of mortality in patients with Marfan syndrome. Recent studies have shown that aortic aneurysm in Marfan syndrome may arise not simply as a result of inherent weakness of connective tissues due to deficiency of fibrillin-1 but also

because of defective interaction between fibrillin-1 and transforming growth factor beta (TGFβ), leading to impaired tissue maintenance [328]. The TGFβ family of growth factors regulate many cellular processes such as proliferation, differentiation and survival. TGFβ is normally sequestered by fibrillin-1 and is released in a tightly controlled manner. Deficiencies of fibrillin-1 may thereby lead to excessive TGFβ activity and promote processes such as matrix degradation, which may contribute to vascular pathology [329].

Analysis of *Fbn1* knockout mouse models that develop fatal aortic aneurysms showed up regulation of TGFβ signalling in affected tissue, and treatment of these mice with TGFβ neutralising antibodies reduced aortic root dilatation [330]. TGFβ-neutralising antibodies are not currently available for clinical use in humans. However, another way of reducing TGFβ signalling is to target the angiotensin pathway with a small-molecule angiotensin II type 1 receptor blocker such as losartan, a widely used antihypertensive agent. AT1-receptor blockade decreases TGFβ-mediated intracellular signalling by incompletely understood mechanisms that may involve a direct effect on TGFβ synthesis and modulation of cross-talk between signalling pathways [331, 332]. Habashi *et al.* treated *Fbn1* knockout mice with losartan, the β-blocker propranolol or placebo [333]. The doses of losartan and propranolol were titrated to obtain comparable changes in blood pressure. Propranolol reduced the aortic root dilatation compared with placebo, but losartan completely inhibited aortic root dilatation, and losartan-treated animals were indistinguishable from wild-type controls. In a small-cohort study of 18 patients with Marfan syndrome, treatment with an angiotensin II type 1 receptor blocker was associated with a dramatic slowing in the rate of aortic root dilatation [334]. In light of

these promising results, a number of clinical trials of losartan for Marfan syndrome are being undertaken [335-339].

TGFβ inhibition may have a therapeutic role in a wider range of inherited conditions. Increased TGFβ activity has been implicated in the pathogenesis of the X-linked Duchenne and Becker muscular dystrophies, caused by mutations in the *DMD* gene. These conditions are characterised by progressive skeletal muscle weakness and wasting. The more severe Duchenne form is usually fatal by early adult life. Early in the disease both muscle cell death and regeneration are seen, but regeneration slowly fails and fibrogenesis occurs [340]. This process has been linked to increased TGFβ activity [341, 342]. Treatment of *DMD* knockout mice with losartan resulted in improvement of muscle architecture and function by maintaining muscle regeneration [342]. These findings have led to proposals for clinical trials of losartan for the human disease.

6.5 Treatment in utero

Many Mendelian disorders are characterised by congenital anomalies, which reflect abnormal development in utero. Recent studies in mouse models have suggested that molecularly targeted therapies might be used in utero to treat malformation syndromes. Apert syndrome is characterised by craniosynostosis and syndactyly of the hands and feet. It is caused by activating mutations in fibroblast growth factor receptor 2 (FGFR2) gene, most commonly S252W [343]. Such mutations up regulate a number of downstream signalling pathways including the mitogen activated protein kinase (MAPK) pathway [344]. Shukla *et al.* [345] developed a mouse model of Apert syndrome in which embryonic expression of a mutant *FGFR2* allele led to craniosynostosis. Introduction of a short hairpin RNA (shRNA) complementary to the

mutant allele prevented its expression, normalised signalling in the MAPK pathway, and corrected the phenotype. The therapeutic use of RNA interference to knock down the expression of target genes is an exciting prospect, but issues of delivery and safety will have to be overcome [346]. However, Shukla *et al.* [345] also treated pregnant transgenic mice with U0126, a small-molecule MEK1/2 inhibitor, and this led to complete repression of craniosynostosis in affected embryos. Cranial abnormalities emerged in the postnatal period, but were ameliorated by reinitiating treatment with U0126. MAPK pathway inhibitors may have a role in other inherited disorders that exhibit increased MAPK signalling such as the phenotypically related conditions cardiofaciocutaneous, Costello and Noonan syndromes, all caused by mutations in genes encoding components of the MAPK pathway [347]. However, significant difficulties exist for the translation of *in utero* therapy to the clinical setting, including the need for sufficiently early prenatal diagnosis and the risk of drug-related teratogenicity.

6.6 Limitations to molecularly targeted therapy

Simply inhibiting one component of a perturbed signalling pathway may fall far short of restoring normal signalling, with its nuanced responses to multiple stimuli, and in practice it is difficult to titrate dose against molecular response resulting in limited efficacy and in toxicity. The cellular pharmacodynamics may be very different for heterozygous as opposed to wild type cells resulting in a different toxicity profile between patients with a single gene disorder and the general population. The multi-organ involvement seen in many tumour predisposition disorders may further contribute to an altered toxicity profile. Many signal transduction inhibitors are "promiscuous" drugs, inhibiting multiple signalling molecules, and these off-target effects may also

contribute to a wide side-effect profile. Feedback mechanisms and cross-talk characterise many signalling networks and may lead to unintentional effects when a pathway is targeted. Functional redundancy between pathways may also limit drug efficacy. Combination therapy or the use of "promiscuous" drugs to target multiple signalling pathways or multiple components of the same pathway may overcome some of these difficulties. As not all types of signalling molecules are currently "drugable", new classes of drugs that target protein/protein interactions or protein/DNA binding are likely to be required to expand the range of genetic diseases amenable to a molecularly targeted approach.

Chapter 7: Challenges to the clinical development of molecularly targeted therapy for single gene disorders

7.1 Introduction

Individual inherited single gene disorders are often rare, but collectively they affect a significant proportion of the population. Effective treatments for these diseases represent an area of unmet medical need and, whilst an increased understanding of the molecular pathology underpinning these disorders has presented new therapeutic opportunities, there are significant challenges in developing drugs to treat these conditions. The patient population is usually small and geographically dispersed and may include vulnerable groups such as children or people with intellectual disability. The diseases are often multi-system with variable expression and the molecular biology underlying the disease and response to treatments is complex.

7.2 Clinical trials in rare genetic diseases

The Committee for Medicinal Products for Human Use (CHMP) has produced guidelines for clinical trials in small populations which recognise that the conduct, analysis, and interpretation of studies in rare conditions may be constrained to varying degrees [348].

Hierarchies of evidence have been described which usually place in order:

- meta-analyses of randomised controlled clinical trials;
- individual randomised controlled trials;
- meta-analyses of observational studies;

- individual observational studies;
- published case-reports;
- anecdotal case-reports; and
- opinion of experts in the field.

All such forms of evidence provide some information. For rare disorders it may not be feasible to perform randomised control trials. Potentially, the combined evaluation of single case studies may be the only way to accumulate evidence. In such studies, standardised treatment conditions and data collection would facilitate systematic review.

A number of trial designs especially lend themselves to studies with small numbers of participants, including single subject (*n*-of-1) designs, sequential designs and response adaptive designs. The small number of potential participants in rare disease trials may be insufficient for a classical (frequentist) trial based on hypothesis testing at accepted statistical levels of confidence. One option is to relax the type I and type II error boundaries. An alternative is to use Bayesian methods [349, 350]. Here, a power calculation is not required, the data can be analysed as it accumulates and a probability range would be obtained even with a small number of participants, albeit not at conventionally accepted levels of certainty. But a lower degree of certainty in a result is better than no result at all.

The credibility of study results may be increased if a clear chain of events can be established (for example, drug exposure to molecular target occupancy, evidence of target modulation, biomarkers of response and then clinical outcome). Bradford-Hill's

criteria for determining causality in observational studies may be helpful [351]. These include:

- consistency of association;
- biological gradient: Is there a dose response effect?
- specificity of association;
- biological plausibility;
- strength of association.

Surrogate markers of outcome may have to be used as alternatives to clinical endpoints to allow a study to be carried out. Use of a biomarker as a valid surrogate endpoint requires it to be reasonably likely – based on epidemiologic, pathological, or other evidence – to predict benefit. However, validation of surrogate endpoints is often difficult, particularly in rare diseases.

In trials in rare disorders it is particularly important that every patient participating in a study contributes as much information as possible and that opportunities to perform related translational studies are exploited fully. The restricted patient pool may preclude multiple studies. As single gene disorders are often multisystem disorders thought needs to be given to including a range of endpoints to maximise the information gained from each person entered into a trial. To reflect this multisystem involvement the team involved in the design and conduct of a trial may need to be multi-disciplinary.

Slow participant accrual is likely to be a problem in trials in rare conditions. In the UK, the care of people, particularly adults, with genetic multisystem disorders is often

fragmentary and poorly co-ordinated and this is reflected in a lack of infrastructure to carry out clinical trials in these conditions. Patient registers will have an important role by facilitating recruitment and providing information on the natural history of a disease. Regional clinical genetic services have already developed registers for some disorders and recently the families of patients affected by Duchenne muscular dystrophy were instrumental in setting up a national register of patients and their genotypes specifically to facilitate clinical trials [352].

The likely small sample sizes in rare disease trials means minimising loss to follow up is important. Bio-noise, the sum of avoidable and unavoidable non-systematic errors in the design and conduct of a trial, is a particular problem. Therefore, minimisation of avoidable errors such as those that occur through loss to follow up is of great importance. Loss to follow up may be reduced by scheduling visits at reasonable intervals and at times convenient to patients, providing transport where necessary making adequate provision for parents and/or carers. Involving patient groups at an early stage in the trial design may help to ensure the trial is acceptable to potential participants.

Clinical trials in rare diseases will often require international co-operation and co-ordination and this would benefit from further simplification and standardisation of regulatory and ethical approval processes. Given the restricted numbers of potential participants, it is important that careful thought is given as to which drugs are to be tested and that the research community communicates to prevent unnecessary duplication of research.

Cost is a major challenge in the development of novel agents. Pharmaceutical companies may elect to explore drug development in some rare but biologically informative conditions to open niche markets or to gain insights into disease processes relevant to more common conditions. World wide a number of programmes have been introduced to promote the development of 'orphan' medicinal products for rare diseases. The European Union defines an 'orphan' medicinal product as a product intended for the diagnosis, prevention or treatment of life-threatening or chronically debilitating conditions that affect no more than 5 in 10,000 people in the European Union (EU), or one which, for economic reasons, would be unlikely to be developed without incentives. The EU 'orphan' medicinal products programme provided community wide economic incentives to pharmaceutical companies such as market exclusivity provision,. regulatory fees waivers and protocol assistance.

Most drugs currently in trials for genetic disorders have already been used to treat other sporadic conditions. It is likely that repositioning of such agents will remain the most realistic strategy for the future treatment of many of the disorders that constitute the workload of clinical genetics services. This repositioning may be viewed by pharmaceutical companies as a relatively low risk strategy to optimise product pipelines but conflict may arise between the interests of these companies and those of healthcare purchasers with regards the benefits of repositioning older, well established drugs, nearing the end of their patent period compared with newer, more expensive derivatives.

Despite programmes to support orphan medicinal product development, economic considerations mean that for many single genes disorders translational research will

have to be driven from outside the pharmaceutical industry. Clinical and laboratory genetics services have been quick to translate knowledge of the human genome into diagnostic advances. They should now accept the challenge of delivering the therapeutic advances in inherited disorders that were anticipated by the Human Genome Project.

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Appendices

Appendix 1: Sample patient information sheet for participants

version 18 (01/09/05)

A trial of the efficacy and safety of sirolimus (rapamycin) in tuberous sclerosis (TSC) and lymphangioleiomyomatosis (LAM (TESSTAL).

INFORMATION SHEET FOR POTENTIAL PARTICIPANTS WHO HAVE TUBEROUS SCLEROSIS

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. Thank you for reading this.

1. What is the purpose of the study?

This study will examine the safety and efficacy (beneficial effects) of the drug sirolimus in people who have benign (not cancerous) growths in the kidneys called angiomyolipomas (AMLs) and either or both of the related conditions tuberous sclerosis (TSC) and lymphangioleiomyomatosis (LAM)).

Sirolimus is also known as rapamycin. Sirolimus has been used for over 4 years in patients having organ transplants but, until now, has not been used to treat AMLs or TSC. Research has suggested that sirolimus may be helpful in treating AMLs and the related conditions TSC and lymphangioleiomyomatosis of the lungs (LAM).

2. Why have I been chosen?

You have been invited to participate in this study because you have tuberous sclerosis (TSC) and in the past have had scans that have shown a benign fatty lump or lumps in your kidney(s) called angiomyolipomas. Your doctor has considered that you are eligible for the study as you are mildly affected by the tuberous sclerosis (and do not have a learning disability as some patients do) but the lumps in your kidney(s) are big enough to detect a response to the treatment, if one occurs.

3. Do I have to take part?

No, it is entirely up to you to decide whether or not to take part. If you do decide to take part you should keep this information sheet and you will be asked to sign a consent form. If you decide to take part you are still free to withdraw at any

time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect your future medical care in any way.

4. What will happen to me if I take part?

Before you start the drug one of the study doctors will examine you and you will have blood and urine tests. If the alteration in the gene responsible for TSC in yourself is not known this will be looked for. If we need to look for a genetic alteration we will give you a separate information sheet and ask you to sign a separate consent form. You will have a MRI scan of your kidneys to accurately measure the angiomyolipomas. You will have a chest X-ray. Females will also have a CT scan of the lungs, as these organs are sometimes affected by TSC in women (unless you have already had a recent CT scan of the lungs for which the result is available). You will also have a MRI of your brain and be asked to do some tests to assess your concentration, learning and memory. If you have epilepsy we will give you a diary to record any seizures. These are 'baseline' tests which will help us to look for positive effects or side effects of the sirolimus as the trial progresses. As a result of these baseline tests we may find a reason why you cannot take part in the study and this will be shared with you if this is the case.

You will need to see the study doctor after three weeks, two months, four, six, nine, twelve, eighteen and twenty four months during the study. We can reimburse you reasonable travel expenses for all visits. At each of these visits you will have further blood and urine tests. To follow the effect of the drug on your kidneys you will have further kidney MRI scans after two, six, twelve, and twenty four months (four more times) on sirolimus treatment. After 12 months in the study you will have another MRI scan of your brain. After four months and after twelve months you will be asked to do further tests of concentration, learning and memory. All participants will have basic lung function (breathing) tests at the start of the study, after three weeks and then at two, four, six, nine, twelve, eighteen and twenty four months (eight more times). If you are a female with affected lungs you will have more detailed basic lung function (breathing) tests at the first study visit and at 4 months, 6 months, 12 months and 24 months and you will also have a further CT scan of the lungs at the end of the study. In between visits a study doctor will telephone you to ask about any changes in medication, side effects or other medical problems. We will call after one week, one and a half months, five months, seven and a half months, ten and a half months, fifteen months, twenty one months and 25 months.

Sirolimus comes as a liquid which you take by mouth. You will receive a low dose of sirolimus for the first two months. If your kidney scan does not improve by the end of these two months you will receive a higher dose for the rest of the trial. During the trial sirolimus will be stopped if your scans get worse, if the AMLs haven't shrunk at all after 4 months on the higher dose or if you have significant side effects from the sirolimus (section 8 of this information sheet lists some of the potential side effects of sirolimus). We will discuss with you reducing the dose or stopping the sirolimus if your angiomyolipomas shrink to less than a quarter of their original size. Using a blood test at each visit, we will

monitor the level of sirolimus in your blood and measure the levels of red and white blood cells, kidney function, liver function and fats in the blood. This will require us to take 30 mls of blood (four small tubes, equal to about 2 tablespoons) at each visit.

The whole study will last two years although you may not need to take sirolimus for the whole of this time.

5. What do I have to do?

Sirolimus comes as a liquid which you take by mouth. When you take sirolimus you can draw up the dose using a plastic syringe which we will provide and then mix it with a glass of water or orange juice. After you have drunk this you should drink another glass of water or orange juice straight away.

When you are taking sirolimus we will ask you to inform us before taking any new medication. You should avoid grapefruit juice because this increases the levels of sirolimus in the blood.

You should not give blood, become pregnant or breast feed while you are on the sirolimus. Women will be asked to have a pregnancy test before taking part to exclude the possibility of unexpected pregnancy and will also have a pregnancy test at each visit. If you are male or female you will be asked to use a reliable form of contraception for the duration of the study and for 12 weeks after you stop taking sirolimus. We will discuss with you and help you decide what will be the most suitable form of contraception given the options available.

It will be important to take your medication regularly and at the same time in relation to food. We will ask you to take the sirolimus straight after breakfast. You will have monitoring blood tests at the appropriate times. In order to measure the fat levels in the blood accurately we need fasting samples. This means we will ask that you do not eat from midnight of the night before a study visit until you have had your blood tests done the next morning. We will ensure that your blood tests are done immediately when you arrive for a study visit so that you can then have some breakfast.

If you have private health insurance you will need to inform your insurers that you are in a trial.

6. What is the drug or procedure that is being tested?

Sirolimus was first used in 1999 and has been given to over 4000 patients. It is currently used in patients who have received a kidney transplant to help prevent rejection. Studies in the laboratory predict that it will have a beneficial effect in patients with TSC who have angiomyolipomas. This study is designed to assess the safety and hopefully beneficial effects in patients with TSC and angiomyolipomas.

7. What are the alternatives for diagnosis or treatment?

There are currently no drug treatments for angiomyolipomas. Lesions that grow very large or bleed are normally treated by having their blood supply blocked during a minor operation or they are removed surgically with a bigger operation. These treatments can still be performed during the study should they become necessary. Your normal treatment for other complications of TSC should not be affected.

8. What are the side effects of any treatment received when taking part?

For any drug there are possible side effects. Occasionally, sirolimus may cause a reduction in blood cells leading to anaemia, increased risk of infection or delayed wound healing. Some people receiving the drug develop raised cholesterol or other blood fats which may need a low fat diet or extra tablets. More rarely, patients have developed a pneumonia-like reaction. Other side effects that have been seen include abdominal discomfort, diarrhoea, a fast heart rate, a decrease in the level of elements like potassium in the blood, joint pain, acne and mouth ulcers. All of the above effects should be detected by the monitoring procedures and usually go back to normal if the drug is stopped.

Sirolimus can also cause blood clotting problems that can result in either an increased risk of bleeding or the formation of abnormal blood clots (e.g. in the legs or lungs).

As sirolimus has not been used to treat many people with TSC, there may be other side effects not previously seen. For example, there is a theoretical risk that shrinking angiomyolipomas may make them more likely to bleed. Similarly, if you have epilepsy the drug could make this worse or it could trigger a seizure in someone who has been previously seizure free. If this happens your driving licence could be withdrawn. However, to date neither increased bleeding nor worsening epilepsy have been seen in TSC patients who have already received sirolimus. However, the numbers treated so far are too small to discount these risks. There is no evidence that sirolimus has any effect on concentration, learning and memory but this will be carefully monitored as part of the study. You will be given a card with emergency contact numbers and the study details to carry while you are in the study.

As sirolimus can affect wound healing we would recommend that the drug is stopped at least two weeks before an operation and not re-started until at least two weeks after an operation. Therefore, you should contact a study doctor if you have had or are about to have an operation. Similarly, if you have an accident or any treatment in A&E that involves a cut to the skin or stitches then please contact a study doctor for advice about sirolimus.

If you develop signs such as fever (a temperature greater than 38 degrees Celsius which is the same as 100.4 degrees Fahrenheit), sore throat, abdominal pain, bleeding, bruising or mouth ulcers you should seek immediate medical advice. You could contact your GP, attend at your local A&E department or contact a study doctor.

You can contact a study doctor between 9 am and 5 pm Monday to Friday on:
029-20-744672

For emergencies outside these hours you may contact a study doctor via the switchboard at Brighton hospital on:

01273-696955

9. What are the possible disadvantages and risks of taking part?

In women who become pregnant, while on treatment with sirolimus, the drug may harm the unborn child. Pregnant women cannot take part in this study; neither can women who plan to become pregnant during the 2 year study period. Any woman who finds that she has become pregnant while taking part in the study should immediately contact the study doctor. We will ask for your consent to follow up any children who are born to women taking sirolimus in this study. This is not for research purposes but to allow any health problems in these children to be detected as early as possible and appropriate medical care arranged. Women who are breast feeding should not take part in the study as the drug can enter breast milk.

There is a possible risk that by suppressing the immune system sirolimus could increase the risk of cancers, such as of the lymphoid system and skin. Because of the potentially increased risk for skin cancer exposure to sunlight and UV light should be limited by wearing protective clothing and using a sunscreen with a high protection factor.

There is mild discomfort associated with the taking of blood samples as required in this study.

The MRI scan involves lying still for about 30 minutes in the scanner. Some patients experience a feeling of claustrophobia during MRI scans, though the scans themselves cannot be felt and are not harmful.

Having a chest X-ray and a CT scan does involve exposure to radiation. The dose of radiation associated with a chest X-ray is equivalent to about 3 day's worth of background radiation i.e. the normal environmental radiation we are all exposed to. The dose of radiation associated with a CT scan is equivalent to about 12-24 months worth of background radiation. The total risk of harm from radiation from one chest X-ray and one CT scan is about 1 in 2800.

In females it is possible that the lung CT scan will show evidence of lung involvement by TSC which was not already known to be present. If we discover that you are affected in this way, but have not been aware of it, we will give you full advice on what to do about this, how the lung changes will be monitored throughout the study and what other steps you should take after the study.

During treatment with sirolimus vaccination may be less effective and some types of vaccine (live vaccines such as BCG for tuberculosis, varicella-zoster [the virus that causes chickenpox and shingles] vaccine, yellow fever vaccine, measles, mumps and rubella [MMR] vaccine and the live type of poliomyelitis [polio] vaccine) cannot be given. Because of this, if you are likely to need vaccination e.g. for travel during the course of the study you will not be able to participate. People with LAM can have influenza (flu) vaccination and still participate in the study but because of taking sirolimus it is possible that this vaccine will be less effective.

10. What are the possible benefits of taking part?

We hope that sirolimus will shrink the angiomyolipoma(s) in your kidney(s). The information we get from this study may help us to improve treatment for other people with TSC or LAM.

11. What if new information becomes available?

Sometimes during the course of a research project new information becomes available about the drug that is being studied. If this happens, one of the doctors working on the study will tell you about it and discuss with you whether you can and want to continue in the study. If you decide to withdraw from the study, arrangements will be made for your standard care to continue. If you decide to continue in the study you will be asked to sign an updated consent form.

12. What happens when the research study stops?

If suitable, you may be able to continue sirolimus treatment after the study has ended, but we cannot guarantee this at the present time.

13. What if something goes wrong?

If taking part in this research project harms you, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanism should be available to you.

14. **Will my taking part in this study be kept confidential?**

If you consent to take part in the research the investigators may look at your medical records to aid with analysing the results. Your records may also be looked at by regulatory authorities to check that the study is being carried out correctly. Your name, however, will not be disclosed outside the participating hospitals, except to inform your GP and hospital consultant (if you have one) that you are participating in the study.

15. **What will happen to the results of the research study?**

The results of this research will be published in a medical journal. You will not be identified in any publications.

16. **Who is organising and funding the research?**

The study is being organised by doctors with special interests in tuberous sclerosis and lymphangioleiomyomatosis. It is being funded by the Tuberous Sclerosis Association and LAM Action. No doctor will receive extra payment for including you in the trial.

17. **Who has reviewed the study?**

Independent experts in TSC, kidney and lung disease have reviewed the study. The Thames Valley Multi-Centre Research Ethics Committee has also reviewed the study and has no objections on ethical grounds.

18. **Contacts for further information**

Further information about the study can be obtained from;

Thank you for considering this study.

Appendix 2: Consent form

version 18
(01/09/05)

A trial of the efficacy and safety of sirolimus (rapamycin) in tuberous sclerosis (TSC) and lymphangioleiomyomatosis (LAM). (TESSTAL)

Participant Number :

CONSENT FORM

Please initial box

1. I confirm that I have read and understand the information sheet dated 01.09.05 (version 18) for the above study and have had the opportunity to ask questions.

☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.

☐

Participant Number :

3. I understand that sections of any of my medical notes may be looked at by the health professionals undertaking this study, or by regulatory authorities where it is relevant to my taking part in the research. I give permission for these individuals to have access to my records.

☐

4. I agree to take part in this study. ☐

5. I agree to my GP being notified of my participation in this study ☐

6. I agree to my hospital consultant(s) being notified of my participation in this study ☐

for females only

7. I agree that if I become pregnant whilst taking sirolimus any child born can be followed up in a medical clinic.. ☐

_____	_____	
Name of Patient	Date	Signature
_____	_____	
Name of Person taking consent (if different from researcher)	Date	Signature

_____	_____	_____
Researcher	Date	Signature

1 copy for patient; 1 for researcher; 1 to be kept with hospital notes

Appendix 3: Drug interactions card

TESSTAL version 14 (12/01/05)

Sirolimus and other drugs

Sirolimus can interact with other drugs. There are some drugs that shouldn't be used in combination with sirolimus and there are other drugs that should be avoided if possible or used with caution. Both types of drug are listed overleaf.

All drugs have a generic name; most drugs also have a trade name. For example, sirolimus is a generic name and Rapamune is its trade name. Both the generic name and the trade name (if the drug has one) will be displayed on the medication's packaging. On the list overleaf the generic name and the trade name (where applicable) are given.

We would be grateful if you could contact a study doctor if you start any new medication (this also includes over the counter medication and herbal remedies). Please also contact us if either you or one of your doctors would like advice about the use of other drugs in combination with sirolimus.

Contact details for study doctors

Between 9am and 5 pm telephone

029-20-744672

for emergencies outside these hours a study doctor can be contacted via the switchboard at Brighton hospital on:

012-73-696955

Drugs which should not be used with sirolimus

Generic name	Trade name(s)
Clarithromycin	Klaricid, Klaracid XL
Itraconazole	Sporanox
Ketoconazole	Nizoral
Rifabutin	Mycobutin
Rifampicin	Rifadin, Rimactane, Rifater, Rifinah 150, Rifinah 300. Rimactazid 150, Rimactazid 300
Telithromycin	Ketek
Voriconazole	Vfend

NB Grapefruit juice interacts with sirolimus and should be not drunk by someone taking sirolimus.

Drugs which should be avoided or used with caution in combination with sirolimus

Generic name	Trade name(s)
Amiodarone	CordaroneX
Barbiturates	Amytal, Sodium Amytal, Soneryl, Seconal Sodium, Tuinal
Bromocriptine	Parlodel
Carbamazepine	Tegretol, Tegretol Retard, Teril Retard Timonil retard
Ciclosporin	Neoral, Sandimmun
Cimetidine	Dyspamet, Tagamet
Ciprofloxacin	Ciproxin
Clotrimazole	Canesten
Danazol	Danol
Diltiazem	Tildiem, Adizem-SR, Adizem-XL. Angitil SR, Angitil XL, Calciacard CR, Dilcardia SR, Dilzem SR, Dilzem XL, Slozem, Tildiem LA, Tildiem Retard, Viazem XL, Zemtard
Erythromycin	Erymax, Erythrocin, Erythroped, Erythroped A
Fluconazole	Diflucan
Fluvoxamine	Faverin
Gestodene	Femodette
Glucocorticoids (steroids)	
Prednisolone	not applicable
Betamethasone	Betnelan, Betnesol
Cortisone	not applicable
Deflazacort	Calcort

Dexamthasone	Decadron
Hydrocortisone	Efcortesol, Hydrocortone, Solu-Cortef
Methylprednisolone	Medrone, Solu-Medrone, Depo-Medrone
Triamcinolone	Kenalog
HIV antivirals	
Atazanavir	Reyataz
Amprenavir	Agenerase
Efavirenz	Sustiva
Indinavir	Crixivan
Lopinavir	Kaletra
Nelfinavir	Viracept
Nevirapine	Viramune
Ritonavir	Norvir
Saquinavir	Fortovase, Invirase
Ketoconazole	Nizoral
Mifepristone	Mifegyne
Modafinil	Provigil
Nicardipine	Cardene, Cardene SR
Norfloxacin	Utinor
Phenobarbital	not applicable
Phenytoin	Epanutin
Pioglitazone	Actos
St. John's wort	
Verapamil	Cordilox, Securon, Half Securon SR, Securon SR, Univer, Verapress MR, Vertab SR 240.

Appendix 4: Doctor's information card

A study of the efficacy and safety of sirolimus (rapamycin) in tuberous sclerosis complex (TSC) and lymphangioleiomyomatosis (LAM). (TESSTAL)

Dear (doctors name).

Re: (patient details)

Your patient (patient name) has agreed to participate in a study of the efficacy and safety of sirolimus therapy in patients with tuberous sclerosis and/ or lymphangioleiomyomatosis (LAM). (Sirolimus is known in the USA as rapamycin). Below is a summary of the study and information regarding sirolimus. Also attached is a copy of the patient information sheet.

The study

The primary aim of this study is to determine if sirolimus is effective in inducing regression of renal angiomyolipomas (AMLs) in persons with tuberous sclerosis complex (TSC), or sporadic lymphangioleiomyomatosis (LAM). A secondary aim is to determine whether sirolimus therapy affects respiratory function test results in LAM or the results of cognitive testing in TSC.

What patients are eligible for the study?

Patients with either tuberous sclerosis or sporadic lymphangioleiomyomatosis and one or more renal angiomyolipomas are potentially eligible.

What does the study involve?

After giving informed consent, patients will have a thorough baseline evaluation including a review of their medical history, physical examination, baseline blood tests, urine analysis, cognitive function tests, a chest X-ray and lung function tests. A high-resolution chest CT scan will also be performed in female patients where chest CT films from within the preceding twelve months are not available. All patients will have a baseline MRI of the brain and kidneys. Patients with TSC who have epilepsy will be given an epilepsy diary and instruction in its use. For participants with TSC whose genotype is not already known, blood will be drawn for genetic analysis.

As sirolimus is thought to be teratogenic women should not become pregnant whilst taking the drug nor should the partners of male participants. Female participants will have a pregnancy test on entry and at every study visit. Participants will be required to use an effective form of contraception during the study. Use of a contraceptive should continue for twelve weeks after stopping sirolimus.

The patients will undergo regular follow up evaluations involving a combination of clinical review, blood tests, urine tests, lung function test and radiological examinations.

Unless they are withdrawn patients will be treated, as part of the study, for 2 years.

What are the potential benefits?

This study may result in the identification of a medical treatment for renal angiomyolipomas in tuberous sclerosis. Untreated these lesions usually increase in size over time and lead to complications including haemorrhages and renal failure that are the second most common cause of death in TSC (after neurological causes). This may in turn lead to identification of sirolimus as an effective treatment for other progressive TSC lesions such as lymphangioleiomyomatosis or giant cell astrocytoma. This study may also result in a greater understanding of the development of hamartomatous malformations in TSC and other more malignant tumours in patients with TSC and the general population. This study may identify a useful treatment for sporadic LAM. This is a progressive condition and the only available treatments are supportive.

What are the potential risks?

Sirolimus is an immunosuppressant medication and has been associated with a number of potential risks. Sirolimus has been commercially available in the United States and UK since 1999. It is indicated for the prevention of renal transplant rejection in adult patients. As of July 2004, over 4000 patients have been treated with the drug. It has also been used to prevent rejection of other transplanted organs. In these patients, it is typically administered with other immunosuppressive agents such as tacrolimus, cyclosporin, or steroids. In addition to its immunosuppressive action, sirolimus may produce dose-related anaemia and thrombocytopenia, and a reversible idiosyncratic leucopoenia. Other individuals may experience dose related delay in wound healing and hyperlipidemia. This lipid alteration may require treatment with cholesterol lowering medications. An interstitial pneumonitis has been reported in 35 renal transplant patients receiving sirolimus (Wyeth database.) These individuals were also receiving immunosuppressants and lipid lowering drugs, and the interstitial pneumonitis resolved after withdrawal of sirolimus therapy. Other side effects seen are tachycardia, abdominal pain, diarrhoea, hypokalaemia, joint pain, acne and mouth ulcers. For the purposes of this protocol, sirolimus will be initiated at a dose lower than the recommended starting dose for immunosuppression, and will not exceed the recommended immunosuppressive starting dose. It is hoped that these measures will minimize the risk for side effects.

Since May 2003, patients with TSC have been treated with sirolimus in the USA in a parallel study. Side effects as of September 2004 have included oral

ulceration in about one third of patients, and elevated serum lipids in about one quarter of patients. There have been a number of brief hospitalisations one for mouth ulcers causing inability to eat and drink, one for community acquired pneumonia which cleared while on sirolimus and two for diarrhoea attributed to Giardia, statins and possibly sirolimus. Although there have been several instances of bronchitis and upper respiratory infections, many of which prompted holding the drug, there have been no opportunistic infections or unexplained pulmonary infiltrates consistent with sirolimus pneumonitis.

Periodic laboratory tests will be performed to evaluate potential side effects of sirolimus. If patients develop significant side effects the dose of sirolimus may have to be reduced or the drug withheld. In the event of significant hyperlipidemia therapy with dietary modification and/or a HMG CoA reductase inhibitor (statin) +/- a fibrate will be initiated.

Sirolimus should be withheld for at least two weeks prior to and two weeks following elective surgery requiring more than three stitches, entry into a body cavity, or optimal healing (e.g.-laser dermatologic surgery on the face). Sirolimus should be withheld for at least two weeks following accidents or emergency surgeries that meet the criteria above.

If you would like any advice about the management of medical problems in patients taking sirolimus or dose reduction in response to side effects then please contact a study doctor (contact details are at the end of this letter). Sirolimus may be discontinued at the discretion of any of the patient's doctors.

Patients in this study will be exposed to a maximum of two high-resolution chest CT scans and one chest X-ray as part of the protocol.

An independent Trial safety monitoring committee will regularly review the study. They will be empowered to stop the study, at any time, (or withdraw an individual) if they perceive that the risks outweigh the potential benefits.

Drug interations

Sirolimus is extensively metabolised by the CYP3A4 isoenzyme. Drugs which are inhibitors or inducers of this enzyme should be avoided if possible or used with caution (see table 1). Inhibitors of CYP3A4 may decrease the metabolism of sirolimus and increase sirolimus levels, while inducers of CYP3A4 may increase the metabolism of sirolimus and decrease sirolimus levels. Sirolimus may require dose adjustment or interruption during therapy with the following agents, and, conversely, dose adjustments or interruption of the following agents may be required during sirolimus therapy. Co-administration with strong inhibitors of CYP3 (such as ketoconazole, voriconazole, itraconazole, telithromycin or clarithromycin) or strong inducers (such as rifampicin or rifabutin) is not recommended. Grapefruit juice is an inhibiter and should be avoided when taking sirolimus. If you would like any advice about the administration of any medication with sirolimus then please contact a study doctor (contact details are at the end of this letter).

Table1

Cytochrome P450 inhibitors and inducers

Inhibitors	Inducers
Amiodarone	**Barbiturates**
Bromocriptine	**Carbamazepine**
Cimetidine	**Glucocorticoids**
Ciclosporin	**HIV antivirals**
(efavirenz,	
Ciprofloxacin	**nevirapine)**
Clarithromycin	**Modafinil**
Clotrimazole	**Phenobarbitone**
Danazol	**Phenytoin**
Diltiazem	**Pioglitazone**
Erythromycin	**Rifabutin**
Fluconazole	**Rifampicin**
Fluvoxamine	**St. John's wort**
Gestodene	
HIV antivirals (atazanavir, amprenavir, indinavir,	
lopinavir, nelfinavir, ritonavir, saquinavir)	
Itraconazole	
Ketoconazole	
Mifepristone	
Nicardipine	
Norfloxacin	
Telithromycin	
Verapamil	
Voriconazole	

Grapefruit juice interacts with sirolimus and should be avoided in someone taking sirolimus.

During treatment with sirolimus vaccination may be less effective. The use of live vaccines should be avoided during treatment with sirolimus.

Ethics.

We have obtained ethical approval for this study. The ethics approval is from the Thames Valley Multi-Centre Research Ethics Committee. Informed consent from all participants will be obtained prior to their entry into this study. All data will be kept strictly confidential and suitably anonymised prior to publication.

How we are asking you to help?

Patients will remain under the care of their principal physician but with regular review in the study centres. We would be grateful if any adverse effects were reported immediately to the study co-ordinator. We would also be grateful if you would inform any deputising services you use about this study.

If you have any specific concerns about your patient in relation to this study or any general enquiry about the study, please contact:

You can contact a study doctor between 9 am to 5 pm Monday to Friday on:

029-20-744672

For emergencies outside these hours you may contact the study doctor via the switchboard at Brighton hospital on:

01273-696955

Thank you.

Appendix 5: Sample case report forms

N.B. Adverse event (1) was for local investigators, Adverse event (2) for the chief investigator or his delegate.

Adverse events (1)

Patient number :

Nature of event:

Date of event
Start time
Stop time

Action taken re sirolimus

Treatment/ medication given
(include date started and
stopped/changed)

Event outcome

Seriousness non-serious serious

CTCAE description and grade (e.g. cardiac general, hypertension, grade 2)

Causality	Not related	
	Unlikely	
	Possibly related	
	Probably related	
	Definitely related	
expectedness	expected	unexpected

Time and date of notification of adverse event

Time and date study co-ordinator informed of adverse event

Name of PI _____

Signed _____

Adverse event form II

Patient number :

Nature of event:

Date of event
Start time
Stop time

Seriousness non-serious serious

Causality Not related

Unlikely

Possibly related

Probably related

Definitely related

expectedness expected unexpected

CTCAE description and grade (e.g. cardiac general, hypertension, grade 2)

Does this event need to be reported Yes No

If yes

Time and date MHRA notified

Time and date ethics committee notified

Time and date Data and Safety committee notified