CLINICAL AND PRE-CLINICAL STUDIES OF RENAL DISEASE IN TUBEROUS SCLEROSIS

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SUMMARY

Tuberous Sclerosis Complex (TSC) is a rare autosomal dominant disease characterized by benign hamartomatous growths in a variety of organs. Renal involvement with angiomyolipomas and/or cysts is common. A subset of patients develops severe polycystic kidney disease in association with contiguous deletion of the *TSC2* and *PKD1* genes. Little is known about the natural history of the renal disease in this group.

The present study had dual aims. The first was to further characterize the *TSC2/PKD1* contiguous gene deletion syndrome and to compare renal function and its change over time in these patients with those having mutations affecting *TSC2* alone. Clinical and renal function data were assimilated on 44 patients with *TSC2/PKD1* deletion and 69 patients with *TSC2* mutations. Additional genetic analyses were used to more fully characterize the genomic extent and mosaicism of *TSC2/PKD1* deletions. In comparison to patients with *TSC2* mutations, those with *TSC2/PKD1* deletion presented significantly earlier with significantly more advanced chronic kidney disease and lower estimated glomerular function rate and they were more likely to progress to end stage renal disease (P = 0.007).

The second aim was to investigate the efficacy of rapamycin, sunitinib, and nelfinavir in the treatment of renal lesions in a $Tsc1^{+/-}$ mouse model in which spontaneous renal cysts arise and progress to papillary cyst-adenomas and to solid adenomas and carcinomas. Compared to controls, rapamycin alone or in combination with sunitinib reduced significantly the number and size of papillary and solid lesions while sunitinib or nelfinivir treatment alone led to a lesser

reduction in numbers of solid lesions but no change in overall lesion numbers. Rapamycin, a highly specific inhibitor on mTORC1, appears to hold more promise than the other agents for the treatment of TSC-associated renal disease.

DECLARATION

This work has not previously been accepted in substance for any degree and is not concurrently submitted in candidature for any degree.

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ABBREVIATIONS

ACE- angiotensin converting enzyme

aCGH- array comparative genomic hybridization

ADHD- attention deficit hyperactivity disorder

ADPKD- autosomal dominant polycystic kidney disease

AKT- protein kinase B (also known as PKB)

AMPK- AMP-activated protein kinase

AML- angiomyolipoma

ARPKD- autosomal dominant polycystic kidney disease

CSF- cerebrospinal fluid

CT- X-ray computed tomography

DMSO- dimethyl sulfoxide

DNA- deoxyribonucleic acid

eGFR- estimated glomerular filtration rate

ERK- extracellular signal regulated kinase

ESRD- end-stage renal disease

FISH- fluorescence *in situ* hybridization

GFR- glomerular filtration rate

GP- general practitioner

HE- hematoxylin and eosin stain

HIV- human immunodeficiency virus

IP- Intraperitoneal (injection)

Kb- kilobases

LAM- lymphangioleiomyomatosis

LOH- loss of heterozygosity

MEFs- mouse embryonic fibroblasts

MLPA- multiplex ligation-dependent probe amplification

mTOR- mammalian target of rapamycin

MRI- magnetic resonance imaging

PC1- polycystin 1 (protein)

PC2- polycystin 2 (protein)

PEG- polyethylene glycol

PHA- phytohaemagglutinin

PI- protease inhibitor

PI3K- phosphatidylinositol-4,5-bisphosphate 3-kinase

PKD- polycystic kidney disease

PKD1- polycystic kidney disease 1 gene

PKD2- polycystic kidney disease 2 gene

RCC- renal cell carcinoma

RHEB- Ras homologue enriched in brain

RTK- receptor tyrosine kinases

SEGA- subependymal giant cell astrocytomas

TSC- Tuberous Sclerosis Complex

TSC1- tuberous sclerosis complex 1 gene

TSC1- tuberous sclerosis complex 1 protein

TSC2- tuberous sclerosis complex 2 gene

TSC2- tuberous sclerosis complex 2 protein

CHAPTER 1

GENERAL INTRODUCTION

1.1 Tuberous Sclerosis Complex

Tuberous Sclerosis Complex (TSC) is a rare autosomal dominant disorder characterised by benign growths (hamartomas) in a variety of organs. It typically affects the brain, kidneys, skin, and heart, although severity and presentation vary widely from patient to patient. Manifestations can range from minor skin involvement to severe mental retardation and renal failure. Table 1.1 shows a list of manifestations associated with the disease. TSC has an estimated prevalence of up to 1 in 6000 (Osborne, Fryer, & Webb, 1991). This classifies it as a rare disease (defined as affecting < 1 in 2000 in the EU), though it is relatively common compared to many other genetic disorders. Approximately one third of cases are inherited while the remaining two thirds carry *de novo* mutations. TSC occurs in all races and ethnic groups, and in both genders.

1.1.1 History of the disease

TSC was first reported by Désiré-Magloire Bourneville in 1880. He wrote of a young female patient with facial rash, retardation, and seizures. Upon her death, examination of the brain revealed small, dense masses with the firmness of a potato (tuber). He henceforth described her condition as tuberous sclerosis (Bourneville, 1880). As time progressed multiple physicians reported on the various manifestations of the disease but it was not until the mid-twentieth century that

the name “tuberous sclerosis complex” was agreed upon and used in diagnosis thereafter.

1.1.2 Manifestations

1.1.2.1 Dermatological manifestations

Dermatological abnormalities are one of the most common features of TSC. There are four major types of skin lesions: angiofibromas, hypomelanotic macules, shagreen patches, and ungual fibromas.

Facial angiofibromas are over growths skin and vascular cells that present as reddish bumps. They are especially seen on the nose and cheeks, though they can also be seen on the chin, forehead, and eyelids. Jozwiak et al. found 74% of patients have facial angiofibromas (2000).

Hypomelanotic macules are found in upwards of 97% of patients (Jozwiak et al, 2000), making them the most common skin manifestation of TSC. These macules present as a small area of skin (often ash-leaf shaped) lighter than the skin around it (“hypomelanotic” meaning less pigment). They can be found anywhere on the body, and are sometimes only visible under the ultraviolent light of a Wood’s lamp. Numbers of macules vary from patient to patient.

Ungual fibromas present as fibrous growths found around or underneath fingernails and toenails. They are relatively harmless but can bleed and disturb nail growth.

Shagreen patches are elevated, rough, pebbly areas of skin most often found on the lower back or buttocks. They are common in TSC patients, found in about 48% (Jozwiak et al, 2000). They rarely cause problems.

1.1.2.2 Neuropsychological Manifestations

Perhaps the most challenging manifestations of TSC are neuropsychological abnormalities. Traditionally, they have been believed to reflect structural brain abnormalities. Histopathological examination of brain specimens post-mortem reveal three main types of brain lesions: cortical tubers, subependymal nodules, and subependymal giant cell astrocytomas (SEGAs).

Cortical tubers are found in 80-100% of patients with TSC (Ridler et al, 2004). Their locations vary from patient to patient. Tubers appear as a firm, potato-like lesion and can form as early as twenty weeks gestation in utero (Park et al, 1997).

Subependymal nodules appear in 64-100% of patients (Ridler et al 2004). These lesions are usually small and found within the lining of the ventricles. Occasionally, in childhood or early adult life, subependymal nodules grow into larger subependymal giant cell astrocytomas (SEGAs). This occurs in about 10% of patients and can be much more damaging (Goh, Butler, & Thiele, 2004). SEGAs can grow to block cerebrospinal fluid from leaving the brain and lead to hydrocephalus and even death.

At least half of patients with TSC have some neuro-cognitive or neurodevelopmental difficulties (Gomez, Sampson, & Whittemore, 1999). Severity ranges from slight learning difficulties to severe mental retardation. In terms of IQ,

most individuals with TSC fall within a “normal distribution” or bell-curve. However, historical studies reveal a bimodal distribution and about 30% of patients have IQ scores that fall within the “profoundly impaired” range (<20) (Prather and de Vries, 2004). Half of patients also show some behavior difficulties (ranging from poor concentration to behaviors as significant as self- injury). Autism and Attention-Deficit Hyperactive Disorder (ADHD) are also much more common in the TSC population (approximately 50% of patients).

Epilepsy is the most common neurological problem in TSC. Approximately 60-90% of patients suffer from epilepsy and it usually first manifests during infancy (Holmes & Stafstrom, 2007). Infantile spasms are often the first reason for concern/diagnosis.

1.1.2.3 Renal Manifestations

Renal manifestations are common in patients with TSC, with a wide range of severity. Studies show that 48-80% of patients present with some sort of renal problem (Rakowski et al, 2006). There are three main types of kidney lesions in TSC: angiomyolipomas, renal cysts, and renal cell carcinoma, with the latter being the least common.

Angiomyolipomas (AMLs) are the most common kidney lesion occurring in 34-80% (Rakowski et al, 2006). They are tumours characterised by abnormal epithelial, fat containing, and smooth muscle cells in and varying proportions. The lesions have abnormal vasculature and are most often seen in the cortex of the kidney. AMLs are

most often slow growing tumours, but can become problematic with larger size. Risk of haemorrhage increases with size as do pain and discomfort.

Renal cysts occur in at least a quarter of patients with TSC. Most often they occur in small numbers in the cortex and remain asymptomatic. More rarely, patients suffer from polycystic kidney disease which proves to be much more problematic.

An overwhelming majority of renal lesions in TSC are benign. However, while rare, renal cell carcinoma (RCC) is said to occur slightly more often and earlier in this patient population (Bjornsson et al, 1996, Allison et al, 1999, & other anecdotal evidence).

1.1.2.4 Other manifestations

Lymphangioleiomyamatosis (LAM) characterised by proliferating smooth muscle cells and cystic destruction within the lungs affects almost exclusively females and can lead to respiratory failure.

Retinal astrocytic hamartomas are benign, white lesions of the retinal nerve fiber layer and occur in approximately half of the patient population (Martin, Rossi, Ferrucci, & Pian, 2010). They are rarely symptomatic.

Cardiac rhabdomyomas affect at least a half of patients with TSC and are benign tumours of cardiac muscle cells. These tumours are much more common in infants (and are often identified in utero) and frequently regress before adulthood (Nir et al, 1995). They may cause arrhythmia or obstruction and hence cardiac failure.

1.1.3 Diagnosis

Specific diagnostic criteria for TSC were slow to emerge, perhaps reflecting the extremely wide variation in manifestations seen from patient to patient. More recently progress made in the genetic study of the disease has led to genetic tests becoming available, though diagnosis remains chiefly clinically based. In 1998, a panel on international experts met to devise a set of specific criteria for diagnosis. Table 1.1 shows the clinical features of TSC, listed as major or minor symptoms (Northrup & Krueger, 2013). “Definite TSC” diagnosis requires either two major features or one major and two minor features. The criteria were revised again by an expert panel in 2012 and were published in 2013. The key change is the inclusion of genetic testing as a possible means of diagnosis. A “pathogenic mutation” is enough to make a definitive diagnosis, though it is important to note that a normal test result does not rule out TSC (Northrup and Krueger, 2013).

Genetic Diagnostic Criteria
Identification of either a TSC1 or TSC2 pathogenic mutation is sufficient for a definite TSC diagnosis.

Major Clinical Features
Brain
Cortical Dysplasias
Subependymal nodules
Subependymal giant-cell tumour (SEGA)
Eyes
Multiple Retinal hamartomas
Heart
Cardiac rhabdomyoma
Kidney
Angiomyolipoma (AML) (>2)
Lungs
Lymphangiomyomatosis (LAM)
Skin
Facial angiofibroma (>3) or fibrous cephalic plaque
Shagreen patch
Ungual fibromas
Hypomelanotic macules (>3)

Minor Clinical Features
Mouth
Multiple pits in dental enamel (>3)
Intraoral fibromas (>2)
Kidney
Multiple renal cysts
Skin
"Confetti" skin lesions
Eyes
Retinal achromatic patches
Other
Non-renal hamartoma

Table 1.1 Diagnostic criteria for Tuberous Sclerosis Complex. Adapted from Northrup, H., & Krueger, D.A. (2013). *Definite TSC diagnosis:* two major features or one major plus two or more minor features. *Possible TSC diagnosis:* Either one major feature or two or more minor features

1.2 Genetics of TSC

1.2.1 *TSC1* and *TSC2*

TSC results from mutation of one of two genes: *TSC1* or *TSC2*. *TSC1* is located on chromosome 9q34 and covers approximately 53 kb (Van Slegtenhorst et al, 1997). *TSC2* is located on chromosome 16p13.3 and is approximately 40 kb in length (European Chromosome 16 Tuberous Sclerosis Consortium, 1993). While mutations in both genes are represented in the patient population, cases with *TSC2* mutations tend to express a more severe phenotype. Due to the wide variety of clinical presentations and mutations, it has been very difficult to make any strong links between specific types of mutation and phenotypic outcome. Research in this area is becoming more feasible as larger numbers of patients are genotyped.

1.2.2 Cell signaling pathways

TSC1 and *TSC2* code for two proteins called hamartin and tuberin (or TSC1 and TSC2), respectively. These two gene products bind together to form a heterodimer which plays a role in the mammalian target of rapamycin (mTOR) signaling pathway.

mTOR is a protein kinase involved in a variety of cell processes via many downstream targets (see Figure 1.1). These include up-regulation of protein translation, growth and proliferation, as well as down-regulating autophagy. The tuberin/hamartin protein complex decreases the activity of Ras homologue enriched in brain (RHEB) which subsequently decreases the activity of mTOR. Thus, the tuberin/hamartin complex acts as a regulator of mTOR activity and links these

growth related cell processes to the availability of growth factors, nutrient and energy levels.

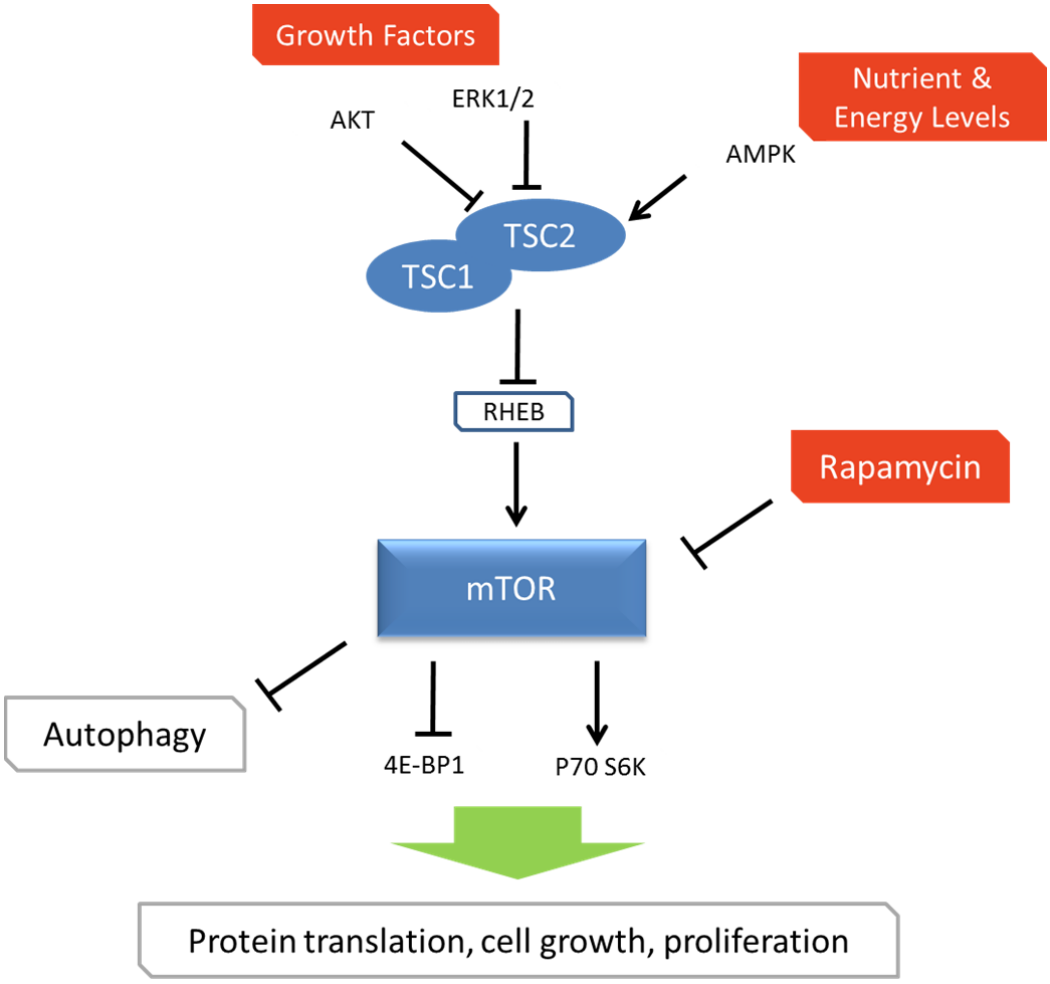


Figure 1.1 TSC/mTOR signaling cascade. The basic mTOR signaling cascade is shown. Normally, TSC1/TSC2 regulate Rheb and in turn, mTOR. When TSC1 or TSC2 is deficient, mTOR activity is uncontrolled and protein translation, cell growth and proliferation can persist with a lack of regulation.

1.2.3 Two-Hit Mechanism of Tumourigenesis in TSC

Molecular genetic studies have shown that the disease causing mechanism in TSC usually involves a second somatic event affecting the wild type allele, and often manifest as loss of heterozygosity, or LOH (Henske et al, 1995, Henske et al, 1996, Sepp, Yates, & Green, 1996, and Yu, Astrinidis, & Henske, 2001). TSC1 or TSC2 null cells are characterised by mTOR activation, up-regulated protein translation, growth, and proliferation and eventually a tumour forms. Interestingly, LOH has not been reported in many SEGAs (Henske et al, 1997 and Chan, Zhang, & Roberts, 2004) and has not been readily identifiable in cortical tubers (Qin et al, 2010). Somatic events triggering these lesions may be distinct from those in non-CNS lesions.

1.2.4 Mosaicism

Somatic mosaicism describes an organism with populations of cells containing different genetic makeups. A genetic error occurs early in development, giving rise to cell progeny with different genotypes. This could mean different amounts of DNA in different cells (e.g., one missing chromosome, as in mosaic Down Syndrome) or different copies of a gene in different cells (as in mosaic TSC). In mosaic TSC a person has populations of both “normal” cells and “mutant” cells. Patients with mosaic TSC tend to have a less severe phenotype than those with non-mosaic disease. Some patients initially characterised as sporadic may have resulted from mosaic parents who have no obvious signs or symptoms of TSC. The true frequency of mosaicism in TSC it not known as there could be many mosaics who do not know they are affected (Rose et al, 1999).

1.3 Surveillance and Treatment

1.3.1 Goals of treatment

Traditionally, treatment for patients with TSC has been in response to specific symptoms rather than preventative. Early and accurate diagnosis allowing prompt treatment appears to be key for a good outcome in most organ systems.

1.3.1.1 Renal management

Renal screening is important from the time of diagnosis as kidney involvement is often asymptomatic until significant renal damage has occurred. Renal ultrasound can readily give a view of number and nature of lesions (if present) but MRI has become the imaging modality of choice. Screening every 1-3 years is recommended to monitor lesion growth (Krueger & Northrup, 2013).

There are several treatment options when complications of AMLs arise. When haemorrhage occurs, arterial embolization is the preferred technique used to block blood vessels to the lesion. Traditionally, embolization was also sometimes used pre-emptively in large AMLs to block the blood/nutrient supplies to stop growth or cause shrinkage. In the last resort, if AMLs become large or numerous a partial or complete nephrectomy may be necessary. Very recent studies have shown that mTOR inhibitors can be used to shrink non-heamorrhaging AMLs and these have become first line treatment in this setting. (Krueger & Northrup, 2013).

Blood pressure and renal function monitoring is important in those with kidney involvement. The International Tuberous Sclerosis Complex Consensus Group currently recommends blood pressure and GFR be assessed at least annually

(Krueger & Northrup, 2013). Anti-hypertensive medication may be prescribed. If renal function is extremely poor, dialysis and/or transplant may be warranted. Patients with TSC and polycystic kidney disease represent a group at high risk for problems with renal function and are considered in more detail in Sections 1.5 and 1.6.

1.3.1.1.1 Rapamycin and AML

Rapamycin (an mTOR inhibitor) was first identified as a product of a bacterium on Easter Island and was developed as an anti-fungal agent. It was later found to have immunosuppressant properties and was subsequently used in clinical practice in transplant patients. Rapamycin and its analogues (e.g. everolimus) have been used in clinical trials for TSC in adults with AMLs (Bissler et al, 2008, Davies et al, 2011, and Bissler et al, 2013). Everolimus was recently approved for use in this indication in North America and Europe.

1.3.1.2 Neurological management

Tubers do not tend to increase in size but may be identified as epileptogenic foci and occasionally require surgical excision. SEGAs often increase in size and growing or symptomatic SEGAs have traditionally been resected. Treatment of SEGAs with everolimus has also been explored in clinical trials (Krueger et al, 2010 and Franz et al, 2013) and the drug was recently approved for use in this indication in North America and Europe.

Epilepsy is treated with anti-convulsant medications and sometimes surgeries. Vigabatrin has proved to be particularly effective for treatment of infantile spasms in TSC. Vagal nerve stimulators may also be used to treat epilepsy, but results have not been very promising in TSC. Some patients benefit from treatment with ketogenic diet, possibly reflecting effects of the diet on mTOR pathway activity.

Developmental and behavioural issues can be difficult to treat. Assessments must be made and subsequent special educational or psychiatric support may be required. Some medications can help problems such as ADHD, but effects on co-existing seizures may be problematic. It is generally accepted that early seizure control is of benefit in reducing risk of poor neurocognitive outcome, but randomised control trials have not yet been reported.

Pre-clinical trials in transgenic mice suggest that rapamycin and other mTOR inhibitors may help with some memory issues, autism, and/or learning difficulties in TSC (Ehninger et al, 2008, Zhou et al, 2009, and Ehninger & Silva, 2011), but future clinical research is necessary to confirm this.

1.3.1.3 Pulmonary management

Lymphangioleiomyomatosis (LAM) has been treated with hormone therapy and in its later stages by transplant. Use of rapamycin and its analogues for LAM treatment is also being explored in clinical trials and appears to at least slow progression of the disease (McCormack et al, 2011, Davies et al, 2011, and Bissler et al, 2008 & 2013).

1.3.1.4 Dermatological and ophthalmic management

Facial angiofibromas are most often treated with laser therapy. Ungual fibromas may be excised. Both may be left untreated. Retinal astrocytic hamartomas are rarely treated. Rapamycin and its analogues are being explored for treatment of skin lesions in clinical trials (Foster, Bint, & Halbert, 2012 and Salido et al, 2012.

1.3.1.5 Cardiologic management

Cardiac rhabdomyomas rarely require treatment. Occasionally, surgery may be warranted for extensive obstruction or drug treatment may be required for arrhythmia or heart failure.

1.3.2 Genetic Counseling

People with TSC have a 50% chance of passing on the disease to their offspring, but most cases are sporadic. When one family member is diagnosed, it is important for others to be offered assessment to see if they, too, have TSC. Early diagnosis is key to treatment of many of the manifestations. It is especially important for parents of diagnosed children to be evaluated to better understand the risk to subsequent offspring. For unaffected parents with an affected child, the risk is about 1-2% due to germline mosaicism (Rose et al, 1999).

1.3.3 Prognosis

Many of those with mild TSC lead a relatively normal lifestyle and with good care and disease management can expect a near-normal life expectancy. However, quality of life and life expectancy vary hugely from patient to patient. Patients with LAM, for example, may see a large reduction in life expectancy. One of the leading causes of death in patients with TSC is renal failure (Shepherd, Gomez, Lie, & Crowson, 1991). It is, therefore, especially important to explore new treatment options for renal disease in TSC. Fatal renal manifestations are most often numerous or large AMLs or extensive cystic disease (such as in the TSC/Polycystic Kidney Disease contiguous gene syndrome). Other leading causes of death are problems resulting from brain tumours (SEGAs) and sudden epilepsy associated death.

1.4 Pre-clinical trials

There have been numerous pre-clinical studies involving TSC covering a wide variety of disease facets. Many TSC2 and TSC1 null cell lines have been developed as have several rodent models. Both are used for studies of cell signaling (mTOR, AKT, etc.) as well as pre-clinical drug assessments.

1.4.1 The Eker Rat

The Eker rat model has been studied extensively. It has a naturally occurring heterozygous mutation in *Tsc2*. It spontaneously develops tumours in a variety of organs. It has been widely used for disease exploration. Its limitations include lack

of obvious neurological abnormalities and kidney lesions that are renal cysts and cancers rather than AMLs which are the usual lesions found in humans.

1.4.2 Traditional transgenic mouse models

Wilson et al (2005) developed a *Tsc1*$^{+/-}$ mouse model (Balb/c background) with deletion of part of exon 6 through exon 8. This model showed a more severe phenotype than previous *Tsc1* models. The mice develop renal cystadenomas ranging from purely cystic lesions to papillary lesions and solid adenomas and cancers. This and other TSC mouse models are summarized in Table 1.2, adapted from Kwiatkowski, (2010). Several models exist for mutations in either *Tsc1* or *Tsc2,* many of which also show cystadenomas of the kidney often accompanied by liver hemangiomas.

Species	Gene	Allele Name	Exon targeted/ mutation	Major features of heterozygote animals	Major Reference(s)
Rat	*Tsc2*	Eker	Intracisternal A-particle (IAP) element inserted into codon 1272	Cystadenomas-carcinomas of kidney Splenic hemangiomas Uterine leiomyomas Pituitary adenomas Subependymal and Subcortical hamartomas	Hino et al, 1993, Yeung et al, 1994, Eker et al, 1981, and Yeung et al, 1997
Mouse	*Tsc2*	-, Kwiatkowski	Neomycin cassette insertion into exon 2	Cystadenomas of kidney Liver hemangiomas Extremity angiosarcomas	Onda et al, 1999
Mouse	*Tsc2*	-, Hino	Neomycin cassette insertion into exon 2, deletion of exons 2-5	Cystadenomas of kidney Liver hemangiomas	Wilson et al, 2006
Mouse	*Tsc1*	-, Hino	Neomycin cassette insertion and deletion of exons 6-8	Cystadenomas of kidney Liver hemangiomas	Kobayashi et al, 2001
Mouse	*Tsc1*	-, Kwiatkowski	Deletion of exons 17 and 18	Cystadenomas of kidney Liver hemangiomas	Kwiatkowski et al, 2002
Mouse	*Tsc1*	-, Cheadle	Deletion of exons 6-8 with insertion of neomycin cassette	Cystadenomas of kidney Liver hemangiomas Reduced survival of $Tsc1^{+/-}$ when in C57BL/6 strain $Tsc1^{+/-}$ kidney cancer in BALB/c strain	Wilson et al, 2005
Mouse	*Tsc2*	*neo,* Gambello	Neomycin cassette insertion into exon 1	Hypomorphic allele: Renal cysts only at 20 months of age $Tsc2^{neo/neo}$ embryos survive to E17 in some cases	Hernandez et al, 2007
Mouse	*Tsc2*	*del3*, Kwiatkowski	Deletion of exon 3	Hypomorphic allele: Reduced severity of renal tumours 1-2 day longer survival of $Tsc2^{del3/del3}$ embryos	Pollizzi et al, 2009
Mouse	*Tsc2*	*KO,* Gambello	Deletion of exons 2-4	Cystadenomas of kidney Little published data	Hernandez et al, 2007
Mouse	*Tsc2*	-, Kobayashi	Deletion of exons 3-4	Little published data	Shigeyama et al, 2008

Table 1.2 Rodent models of Tuberous Sclerosis Complex (adapted from Kwiatkowski, 2010)

1.4.3 Conditional transgenic mouse models

Conditional mouse models also exist for *Tsc1* and *Tsc2* allowing for tissue-specific knock-out of these genes. This can be extremely helpful in pin-pointing specific roles of TSC1/TSC2 in certain organs. Unfortunately, though, none provide reproduction of the AML kidney manifestations of TSC (Kwiatkowski, 2010). Nevertheless, conditional models have been developed to successfully represent other aspects of the disease. Several conditional *Tsc1* brain models exist. Mice with astrocyte specific conditional knockout of *Tsc1* developed epilepsy and showed increased astrocyte proliferation (Uhlmann et al, 2002). Mice with cortical neuron specific knockout of *Tsc1* developed epilepsy and showed abnormal neuron myelination and location (Meikle et al, 2007). Finally, Ehninger et al (2008) compared a *Tsc1* conditional knockout in the postnatal forebrain to traditional transgenic mice heterozygous for *Tsc2*. The *Tsc1* conditional knockout showed a much more severe brain phenotype, with extreme macroencephaly and early death within the first week of life (Ehninger et al, 2008).

1.4.4 Xenograft mouse model

Xenograft mouse models for TSC allow for much more rapid tumour development than transgenic models. Lee et al (2005) generated a nude mouse model with tumour induction by injection of $Tsc^{-/-}$ mouse embryonic fibroblasts (MEFs). All of these mice developed tumours by 3-4 weeks, most of which tended to be large in size (Lee et al, 2005).

1.4.5 Drugs in Preclinical Trials

In addition to mTOR inhibitors, several other drugs have been used in pre-clinical trials to attempt to treat or prevent renal tumour growth in TSC including combined mTOR/PI3K inhibitors, statins, and proteasome inhibitors (Table 1.3). A few of these drugs have shown promise in the pre-clinical setting in tumour frequency reduction, volume reduction, or decreases in cell proliferation.

Drug	Setting	Outcome	References
Metformin	*Tsc2*$^{+/-}$ A/J mouse model	No effect on tumour size or frequency	Auricchio et al, 2012
Proteasome inhibitors			
Bortezomib	*Tsc2*$^{+/-}$ A/J mouse model	No effect on tumour size or frequency	Auricchio et al, 2012
MG-132	*TSC2*$^{-/-}$ human renal angiomyolipoma cells	Increased cell death, decreased proliferation	Siroky et al, 2012
Atorvastatin	TSC2-/- MEFs	Decreased proliferation	Finlay et al, 2007
	Tsc2$^{+/-}$ 129/SvJae mouse model	No effect on tumour size or frequency	Finlay et al, 2009
NVP-BEZ235 (dual mTOR/PI3K inhibitor)	*Tsc2*$^{+/-}$ C57BL/6J: 129S1/SvImJ mouse model	Decreased tumour frequency and cellular content	Pollizzi et al, 2009
MLN0128 (mTOR ATP competitive inhibitor)	*Tsc2*$^{+/-}$ A/J mouse model	Suppression of tumour development and size	Guo & Kwiatkowski, 2013
5-aminoimidazole-4-carboxamide ribonuclease (AICAR)	TSC2-/- MEFs	Cell cycle arrest at G1/S	Gwinn et al, 2008

Table 1.3 Drugs in pre-clinical investigations for treatment of TSC.

1.4.5.1 Nelfinavir

Two more recently explored drugs are nelfinavir and sunitinib, both of which have been used in cancer studies and are now being investigated for use in treatment of TSC. Nelfinavir is chiefly an antiretroviral drug used in treatment of human immunodeficiency virus disease (HIV). It belongs to the class of drug called protease inhibitors (PIs), which have been shown to decrease proliferation, induce growth arrest and induce apoptosis in a variety of cancer cells (Ikezoe et al, 2000, Yang et al, 2006, and Pajonk, Himmelsbach, Riess, Sommer, & McBride, 2002, and others). Furthermore, PIs such as nelfinavir have been shown to inhibit AKT/protein kinase B activity. AKT lies upstream in the TSC1/2 and mTOR pathway (see Figure 1.1). It also plays a role in other signaling pathways involved in cell growth and proliferation. Nelfinavir may therefore be useful in treating non-cancerous tumours such as hamartomas in TSC.

1.4.5.2 Sunitinib

Sunitinib is considered to be a receptor tyrosine kinase (RTK) inhibitor. RTKs are involved in many normal cell processes and appear to play a critical role in the progression of some cancers. Sunitinib is currently in trials for many cancers and approved for treatment of renal cell cancer and imatinib-refractory gastrointestinal stromal tumours (Chow & Eckhardt, 2007). Like nelfinavir, it also can inhibit AKT signaling, induce apoptosis, and induce growth arrest in cancer cells (Yang et al, 2010). Due to its anti-tumour activity and actions in relevant signaling pathways, sunitinib may have potential in the treatment of TSC.

1.5 Polycystic Kidney Disease

Autosomal Dominant Polycystic Kidney Disease (ADPKD) is an inherited disorder associated with mutations of *PKD1* or *PKD2* affecting predominantly the kidneys. It is characterised by renal cyst development and is typically diagnosed in adults. By contrast, the significantly less common Autosomal Recessive Polycystic Kidney Disease (ARPKD) is typically a disease of childhood or prenatal onset.

ADPKD has a prevalence of about 1 in 1000 live births. While a majority of cases are inherited, a notable portion are thought to be sporadic (about 10%). Severity varies from case to case, but in general the disease involves extensive, bilateral renal cystic growth. Often, the number and size of cysts impairs renal function so much that end stage renal disease (ESRD) is reached and dialysis and/or transplant is warranted (Torres, Harris, & Pirson, 2007). ADPKD can also lead to cyst development in the liver and pancreas.

1.5.1 Diagnosis

Diagnosis of ADPKD relies chiefly on imaging. Ultrasound, CT, or MRI may be used to see presence of multiple cysts as well as to monitor their progression. Genetic analysis can also be useful in diagnosis, and enables predictive diagnosis prior to onset of manifestations.

Criteria for diagnosis via imaging are as follows: at least three renal cysts (distributed in one or both kidneys) in patients aged 15-39, at least two renal cysts in each kidney in those aged 40-59, and four renal cysts in each kidney in those over 60 (Ravine et al, 1994). This system of diagnosis is mainly aimed at those at risk for

the disease (i.e., family members of those with ADPKD). These criteria have been questioned as they may lack sensitivity in less severe cases with *PKD2* mutations (Pei & Watnick, 2010).

1.5.2 Symptoms and findings

Pain is common, often over one or both kidneys or in the abdomen. Blood and protein in the urine is also common. Infections of the kidney or urinary tract may occur as well as kidney stones. High blood pressure is frequent. End stage renal disease (ESRD) is usually reached in mid to late adult life.

1.5.3 Genetics and Signaling

1.5.3.1 PKD genes

ADPKD results from mutations in one of two genes: *PKD1* or *PKD2*. The former is located on chromosome 16p13.3 and the locus spans approximately 50 kb (European Polycystic Kidney Disease Consortium, 1994). *PKD2* is located on chromosome 4q21 and spans approximately 68 kb (Mochizuki, 1996). Mutations in *PKD1* tend to present with a more severe phenotype. It is estimated that 85% of cases have a mutation in *PKD1* while the remaining 15% have mutations in *PKD2* (Rossetti et al, 2007). There is a wide variety of clinical presentations and mutations in ADPKD and significant variation in disease severity even within families. Mutation type and location has been shown to affect severity and progression of ADPKD. Mutations at the 5' region of *PKD1* cause significantly more severe disease than those in the 3' region (Rossetti et al, 2002). More information on correlations

between mutation and severity of disease might aid development of more targeted or individualised surveillance and treatment.

1.5.3.2 Cell signaling

PKD1 and *PKD2* code for proteins called Polycystin-1 (PC1) and Polycystin-2 (PC2), respectively. PC2 is a membrane protein that may be involved in cell-cell interactions and renal development. PC1 is also an integral membrane protein. Both have been shown to interact with each other and they co-localize in several cellular locations including the primary cilium.

PC1 appears to play a role in several signaling pathways, including the mTOR pathway (see Figure 1.2, & Shillingford et al, 2006). This proposed role is supported by observation of elevated mTOR activity in ADPKD cyst epithelia (both in human samples and PC1 inactivated mice).

PC1 may regulate tuberin via extracellular-signal-regulated kinases (ERK). PC1 appears to block ERK-mediated phosphorylation of tuberin (Distefano et al, 2009). This model suggests that PC1 and tuberin may work together to regulate mTOR and that where either protein is lacking, increased cell proliferation, growth, and protein translation follow. PC1 may be involved in keeping tuberin at the cell membrane “protecting” it from ERK deactivation and facilitating mTOR regulation.

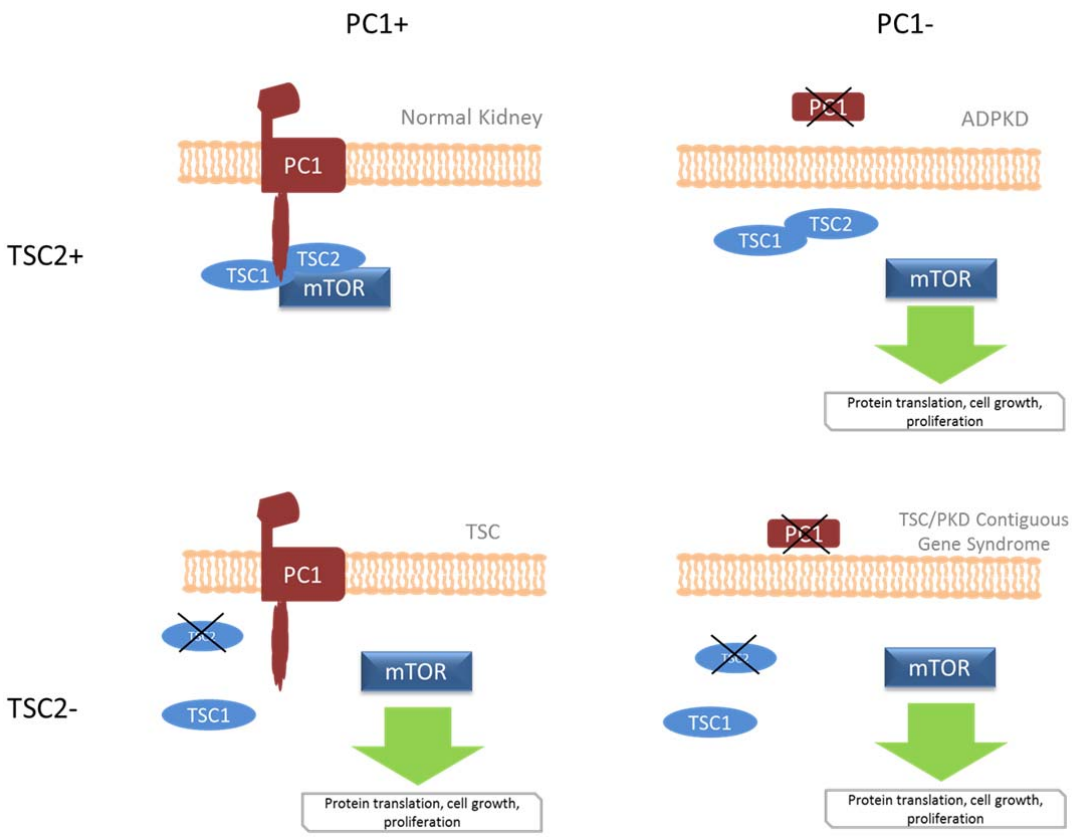


Figure 1.2 PC1 involvement in mTOR signaling. This model suggests that PC1 and TSC2 may act together to modulate mTOR. Where either is lacking, increased cell proliferation, cell growth and protein translation can occur.

1.5.3.3 Two-hit mechanism in PKD

The disease mechanism in ADPKD may be a 2-hit process, analogous to that in TSC. The "first hit" is the inherited mutated allele of *PKD1*or *PKD2*, and the "second hit" occurs in the second (wild type) allele during life as cells proliferate. This leaves cells with no functional PC1 or PC2. LOH at the PKD1 locus has been documented in cysts found in patients with ADPKD (Brasier and Henske, 1997, Koptides et al, 1998, and others).

1.5.3.4 Mosaicism

Mosaicism may occur in ADPKD. As with TSC, mosaic cases of ADPKD appear to present with a less severe form of the disease. There are several documented cases of mosaic ADPKD (Consuger et al, 2008 and Reiterova et al, 2013). Mosaicism is important to consider in taking family histories of those with the disease and especially in selecting related donors for those in need of transplants (Connor et al, 2008).

1.5.4 Surveillance and treatment

1.5.4.1 Management of ADPKD

Management of ADPKD is aimed at prevention of symptoms and slowing progression of disease. Pain may be managed with medication. Infections of either the kidney or urinary tract may be treated with antibiotics. Urinary tract infections should be treated as soon as possible to avoid the infection spreading to the kidney

(and cysts). Kidney infections are often difficult to treat if they are located within cysts, and can require long term antibiotic treatment. Kidney stones can be removed or passed.

Blood pressure control is very important as hypertension can quicken progression of kidney disease. It may be treated with anti-hypertensive medications (ACE inhibitors, angiotensin receptor blockers, etc.) Diet and lifestyle changes are also recommended (low-salt diet, weight loss, exercise).

Cysts normally increase in number and size with age. Regular imaging is performed along with kidney function tests and blood pressure screening. If ESRD is reached, dialysis and/or transplant are necessary. Tolvaptan, a vasopressin receptor 2 inhibitor, is being used in clinical trials and has been shown to slow cyst growth and kidney function decline in patients with ADPKD (Torres et al, 2012).

1.5.4.2 Genetic counseling

Patients with ADPKD have a 50% chance of passing the disease on to their offspring. If a family member is diagnosed, it is important that others at risk are evaluated. Early diagnosis, usually by imaging, is key to the treatment and management of ADPKD. At present, genetic testing for ADPKD is expensive but as DNA based testing becomes more widespread and inexpensive, it will better aid in early diagnosis of those at risk for the disease.

1.5.4.3 Prognosis

Patients with ADPKD can expect to live many decades. Often renal function is relatively preserved until mid to later adult life. It tends to decrease predictably until it reaches a critical point. A rapid decline then occurs (Franz & Reubi, 1983). Research shows that cyst development occurs significantly prior to a major decline in renal function (Chapman et al, 2003) and normotensive patients tend to have less cyst development than those who are hypertensive.

1.6 The TSC2/PKD1 Contiguous Gene Syndrome

The *TSC2/PKD1* Contiguous Gene Syndrome is a rare disease in which both genes are involved in a deletion mutation. The prevalence is unknown but it is unlikely to account for more than a few percent of all TSC cases.

1.6.1 Genetics

The *TSC2* and *PKD1* genes lie adjacent to each other on chromosome 16p13.3 (European Polycystic Kidney Disease Consortium, 1994). This makes it possible for one deletion event to occur and affect both genes. *TSC2* and *PKD1* lie only 60 bp apart in a tail-to-tail orientation (European Chromosome 16 Tuberous Sclerosis Consortium, 1993 and European Polycystic Kidney Disease Consortium, 1994).

Mosaicism has been reported in a significant proportion of patients with the contiguous gene syndrome and was often associated with preserved renal function, Sampson et al. (1997).

1.6.2 Manifestations/diagnosis

The TSC2/PKD1 contiguous gene syndrome is characterised by the presence of polycystic kidneys within the context of TSC and its accompanying manifestations. This severe cystic phenotype should be distinguished from the scattered, often cortical cysts that are seen frequently in TSC patients. In the contiguous gene syndrome, severe polycystic kidney disease is usually present, with enlarged kidneys in which renal tissue appears to be replaced with confluent cysts much earlier in life than in either ADPKD or TSC alone. There have been few studies of the contiguous gene syndrome.

In the contiguous gene syndrome, cysts can often be diagnosed prenatally or early in life but this is not always the case (Laass et al, 2004, Sampson et al, 1997, Brook-Carter et all, 1994, Kacerovska et al, 2009, and Smulders, Eussen, Verhoef & Wouters, 2003). Diagnosis is usually clinical in the first instance, based on the presence of grossly polycystic kidneys via renal imaging (ultrasound, CT, MRI) and the age of the patient, but several genetic approaches to confirm the diagnosis are available including multiplex ligation-dependent probe amplification (MLPA), array comparative genomic hybridization (aCGH), and fluorescence *in situ* hybridization FISH.

Bisceglia, Galliani, Carosi, Simeone, and Ben-Dor (2008) determined the contiguous gene syndrome diagnosis of one patient based on ADPKD with “round, polyhedral cysts with flat to cuboidal epithelium”, classic TSC cysts of smaller size with “tall, eosinophilic, and granular epithelium”, and AMLs. The coexistence of all these lesions made this diagnosis extremely likely even without genetic analysis. Another

study characterised cysts of TSC as small with plump epithelium, and those of PKD are large with flattened epithelium with both present in the contiguous gene syndrome (Martignoni et al, 2002).

1.6.2.1 Genetic testing

Infants and small children presenting with polycystic kidneys and TSC with no family history of ADPKD should be suspected of having a contiguous deletion of *TSC2* and *PKD1* (Torra et al, 1998). Likewise, presence of AMLs in the context of polycystic kidneys should suggest a contiguous deletion (Martigononi et al, 2002). Genetic testing in those patients who are suspected cases may be helpful as contiguous gene deletion predicts faster renal disease progression than in TSC or PKD alone (Sampson et al. 1997). More insight into the contiguous deletion syndrome would be beneficial in order to clarify the prognosis for renal function. Several studies have characterised a variety of mutations affecting both *TSC2* and *PKD*, but the mechanisms determining severity of disease have not been identified at this time (Oyazato et al, 2011, Boehm, Bacher, & Neumann, 2006, Consugar et al, 2008, Sampson et al, 1997, Longa et al, 1997, Torra et al, 1998, and Brook-Carter et al, 1994).

1.6.3 Possible Mechanisms in TSC2/PKD1 Deletion Syndrome

The two-hit model may apply, but there is currently no evidence supporting this theory. Indeed, one study found PC1 expression in multiple cysts of two *TSC2/PKD1* deletion cases while rarely observing absence of expression (Ong et al, 1999) although antibody specificity could be an issue.

1.6.3.1 Signaling

Signaling studies specific to the contiguous gene syndrome are virtually non-existent. Figure 1.2 shows the relationship between PC1 and TSC2 in the mTOR pathway. This signaling pathway may well play a role in disease pathogenesis, but this has not been investigated experimentally.

1.6.4 Surveillance and treatment

1.6.4.1 Management

Treatment of the *TSC2/PKD1* contiguous gene syndrome largely follows that of TSC and PKD. It remains based on management of symptoms with few preventative measures or options for slowing the disease progression. Cyst progression is monitored closely, along with kidney function tests. Hypertension is treated aggressively. Dialysis and transplant may be warranted if ESRD is reached and partial or full nephrectomy is occasionally needed.

1.6.4.2 Genetic counseling

Genetic counseling is of great importance in the *TSC2/PKD1*deletion syndrome. Patients have a 50% chance of passing on their mutation to offspring. The risk may be lower where a parent in mosaic, but offspring, if affected, will not be mosaic and are at high risk of early ESRD.

1.6.4.3 Prognosis

It is generally accepted that the *TSC2/PKD1* contiguous gene syndrome carries a more severe renal prognosis than TSC or ADPKD alone. However, there is little data

on progression of disease and how consistent this is from patient to patient. Most evidence comes from cross sectional studies and case reports (Llamas Velasco et al, 2013, Longa et al, 1997, Culty et al, 2006, others). Data published by Sampson et al (1997) suggested a relatively rapid decline in kidney function in patients with a contiguous deletion with many, perhaps most non-mosaic cases, predicted to reach ESRD by early adult life. One limit of this study, though, was the young age of most patients. Long-term follow up is needed to more clearly define the course of the disease.

1.7 Project aims

The present study had dual aims to improve knowledge of renal disease in TSC. The first aim was to better define the natural history the *TSC2/PKD1* contiguous gene syndrome. Clinical histories of patients with either the contiguous gene deletion syndrome or with mutations affecting only *TSC2* were collected, including details of kidney function (via creatinine measurements and GFR estimation, see Appendix) and, where ESRD was reached, recording the age at which this occurred. We compared decline in kidney function in those with *TSC2/PKD1* deletions to those with mutations of *TSC2* alone.

The second aim was to explore nelfinivir, sunitinib, and rapamycin as candidate drug treatments for renal manifestations in a *Tsc1+/-* mouse model. Their effects on number, size, and cellular content of renal cysts and solid lesions were investigated.

CHAPTER 2

General Materials and Methods

2.1 TSC2/PKD1 Contiguous Gene Syndrome Clinical Investigation

2.1.1 Ethical Approvals

The study protocol was developed by Professor Sampson and Dr. Madeleine Tooley. It was approved by the Wales Research Ethics Committee (10/MRE09/2) and the sponsor was Cardiff University.

2.1.2 Identification and Recruitment of Cases and Controls.

2.1.2.1 Cases

Patients with contiguous deletions involving TSC2 and PKD1 were ascertained from the records of the genetic diagnostic laboratory at the Institute of Medical Genetics, Cardiff. This is one of two laboratories in the UK that provides a genetic diagnostic service for *TSC1* and *TSC2* to the NHS and also to health care providers internationally. Until 2007 the laboratory identified TSC2/PKD1 contiguous gene deletions by fluorescence in situ hybridization (FISH) using the probe JH2A (European Polycystic Kidney Disease Consortium, 1994 and Figure 2.2). The analysis was performed in cases suspected as likely to have the contiguous gene syndrome on clinical and radiographic grounds. From 2007 onwards, multiplex probe ligation analysis (MLPA) became the laboratory's primary method for identification of deletions (and other copy number changes) at the TSC loci and was performed for

the *TSC1* and *TSC2/PKD1* loci on all patients referred for molecular genetic testing for TSC.

2.1.2.2 Controls

Controls with *TSC2* mutations (mostly point mutations) that did not involve the PKD1 gene were identified from the records of the genetic diagnostic laboratory at the Institute of Medical Genetics, Cardiff and from the records of the tuberous sclerosis clinic at Cincinnati Children's Hospital where genotyping had been undertaken through Athena Diagnostics (a commercial US-based genetic diagnostics laboratory).

The study was also advertised on the web site of the Tuberous Sclerosis Association (UK) enabling interested patients to contact the study and volunteer for inclusion, if eligible.

2.1.2.3 Patient Contact

Initial contact with patients was made through the physicians who had referred their samples for genetic testing, following the procedure illustrated in Figure 2.1.

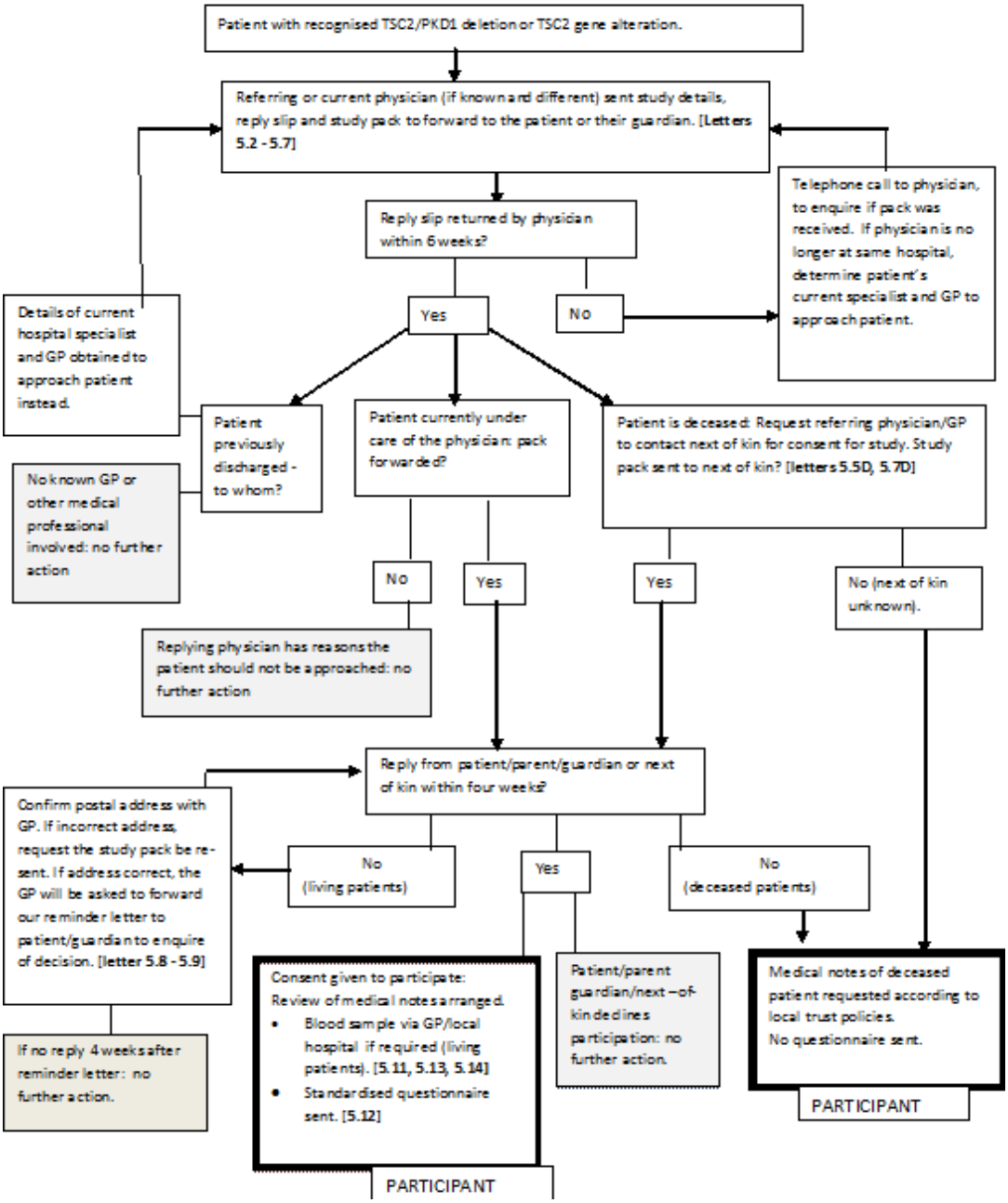


Figure 2.1 Communication flow for recruitment and consent of study participants. Chart obtained from study protocol for "A Study of the Natural History of Renal Disease in TSC2/PKD1 Contiguous Gene Deletion Syndrome." Chief Investigator: Professor Julian Sampson through the Institute of Medical Genetics, Cardiff University. After a patient with deletion was identified, their referring or current physician was sent study materials to be forwarded. We attempted to reach potential participants a limited amount of times through appropriate channels (shown in chart above). Patients were only included once informed consent was obtained from the patient him/herself, or from a parent/guardian/next of kin.

2.1.3 Clinical Data

Once written informed consent had been obtained from patients (or their relative/guardian where appropriate) and a questionnaire had been completed by the patient or relative/guardian, additional medical records were requested from relevant hospitals. They were reviewed and, if required, arrangements for further blood samples were made. Relevant data were extracted from the records and entered into a simple database in Excel format. Dr. Madeleine Tooley (trainee in clinical genetics), Miss Maia Walsh (medical student) and I undertook this work with clerical assistance from Miss Hannah Lister.

Information collected from medical records included presentation of TSC, age at diagnosis of TSC and method of diagnosis, age and method of diagnosis of renal disease, details of results of renal imaging (MRI, CT, and ultrasound scanning) and records of kidney function tests enabling estimation of glomerular filtration rate (eGFR) from serum creatinine values. In children under 18, height measurements were included in order to calculate eGFR by the Bedside Schwarz Equation method (Schwartz & Work, 2009 & Schwartz et al, 2009). Adult eGFR values were calculated using MDRD method (Levey et al, 1999). Any history of hypertension and its treatment were recorded. If ESRD was reached, age at ESRD and treatment was noted as were dates and causes of death. Any other likely relevant information found in patient notes (i.e., renal insults, urine analysis, etc.) was also recorded.

When critical information was not available in hospital notes, GP surgeries, the patient, and/or hospital clinicians and hospital laboratories were contacted. Where possible, parents of apparently sporadic cases with the TSC2/PKD1 contiguous gene

deletion syndrome were also consented and records of their clinical, radiographic and genetic investigation were reviewed in order to identify any who were mosaic for deletions. Where this was confirmed, they were also recruited.

2.1.3.1 Confidentiality

Each patient has been assigned a number for identification purposes and any identifying information has been removed from data presented here to maintain confidentiality.

2.1.4 Statistical Analysis

All statistical analysis was performed by John Gallacher using STATA 12 software (StataCorp. 2011. *Stata Statistical Software: Release 12*. College Station, TX: StataCorp LP). $p< 0.05$ was considered statistically significant. Two-sample t-tests were performed to compare eGFR data while Fisher's exact test was used to compare Chronic Kidney Disease (CKD) stages between groups.

2.1.5 Laboratory Analyses

2.1.5.1 Characterisation of Mutations

Patients with the contiguous gene syndrome reported previously in the study of Sampson et al (1997) had been investigated by a combination of FISH and conventional and pulse field gel electrophoresis (PFGE). Samples from patients identified since then had been studied by FISH, MLPA or both approaches. In order to better define the extents of the deletions array comparative genomic hybridization (aCGH) was undertaken on all cases where adequate DNA samples

could be obtained. In order to identify mosaic cases, where this had not already been done, all cases except those who had inherited their deletion from an affected parent were studied by FISH if adequate samples could be obtained.

Intragenic TSC2 mutations in control cases were taken from diagnostic laboratory records. Only confirmed pathogenic mutations were included (using the TSC2 mutation database www.chromium.liacs.nl/LOVD2/TSC/home.php?select_db=TSC2) and no further genetic studies were undertaken in these cases.

2.1.5.2 MLPA

An MLPA assay kit (MRC Holland, SALSA P081/082 and SALSA P122) that contained probes covering the entire TSC2 gene and in exon 46 of the PKD1 gene, surrounding regions and control probes was employed to screen for evidence of genomic deletions or insertions across the TSC2 gene and a portion of the PKD1 gene using the protocol outlined by the manufacturers (MRC Holland). 250ng of test DNA in addition to a positive control (characterised whole gene deletion) and a negative control (from a non-TSC2 patient sample) were required for each assay. Briefly, the DNA was heated to 98°C for 5minutes and then cooled to 25°C. All incubations were completed on a thermal cycler. The relevant probes were mixed with the buffers and 2µl was added to the DNA and incubated for 1 minute at 95°C followed by 16hours at 60°C. Following the 16 hour incubation, the samples were incubated at 54°C and 32µl of a mix containing a ligase, ligase buffer and dH2O was added to each sample and mixed. This was incubated for 15 minutes at 54°C, for 5 minutes at 98°C and finally briefly at 60°C just prior to the plate being removed from the

thermal cycler. 10µl of this reaction was added to a new plate and 30µl of a mix containing a PCR buffer and dH2O was added to each well in the new plate. Finally the plate was put back into the thermal cycler, 10µl of a mix containing PCR primers and polymerase was added and the PCR reaction was started using the following cycle conditions: 35x (95°C 30 seconds, 60°C 30 seconds, 72°C 60 seconds), 72°C 20 minutes. After the PCR reaction was complete, 7.7µl of HiDi and 0.3µl of ROX was added to each reaction. The plate was then analysed on an ABI 3100 analyser (Applied Biosystems, Warrington, Cheshire, UK).

2.1.5.3 FISH

FISH was performed using standard 72 hour PHA stimulated lymphocyte cultures established from peripheral blood samples and the JH2A probe (see Figure 2.2) was indirectly labeled with biotin using a nick translation kit (Qiagen). The signal was detected using fluorescein avidin and anti-avidin antibodies.

Slides were denatured at 72˚ C in a 70% formamide solution, placed in ethanol, and allowed to dry. Probe was denatured at 72˚ C for ten minutes followed by 37˚C for one hour. Probe was applied to the slide and allowed to hybridise overnight at 37˚C. Slides were then washed, blocking solution (Boehringer blocking reagent) applied and left at 37˚C for ten minutes. Fluroescein avidin was applied and slides were left at 37˚C for fifteen minutes followed by multiple washes. Biotinylated anti-avidin was then added, washed, and more fluorescein avidin applied. Finally, DAPI/PI was added to the slides and signal detected.

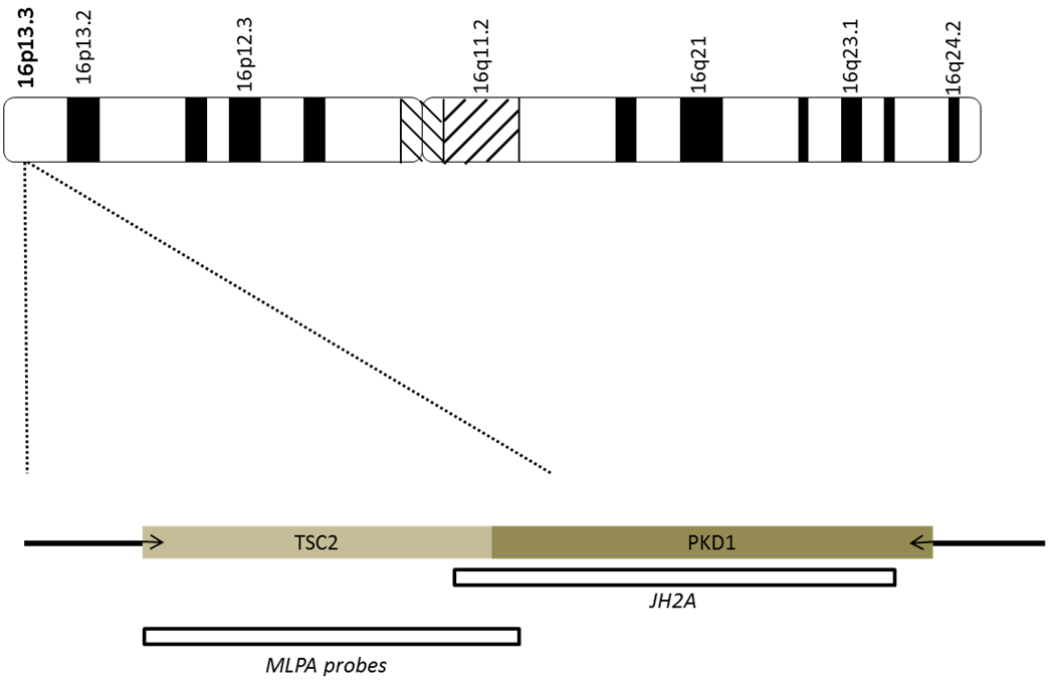


Figure 2.2 The TSC2 and PKD1 region of chromosome 16. (Adapted from Sampson et al, 1997 & The European Polycystic Kidney Disease Consortium, 1994.) *TSC2* and *PKD1* are shown with directions of transcription depicted by arrows. The JH2A probe and is shown below the map as well as the region covered by MLPA probes. The MLPA probes cover all of *TSC2* and exon 46 of *PKD1*.

2.1.5.4 aCGH

Array-CGH was performed on the BlueGnome CytoChip ISCA (v2.0) 8x60k oligonucleotide platform according to manufacturers' instructions. Commercial male or female control DNA provided by Promega was used in sex matched experiments.

DNA samples and random primers were denatured at 95˚C for ten minutes and cooled to 4˚C. A mix of buffers, dNTP, and either Cy3 or Cy5 cyanine dye was added to samples and reference DNA, respectively. Samples were then labeled at 37˚C for two hours, followed by 65˚C for ten minutes and then cooled to 4˚C.

Arrays were scanned using the Agilent G2565CA Microarray Scanner System and analysed using BlueGnome BlueFuse Multi software (versions 2.6 or 3.0).

2.2 Pre-clinical Trial

2.2.1 Mouse Model

All animal procedures were performed in accordance with the UK Home Office guidelines. *Tsc1*$^{+/-}$ mice on the Balb/c background were used as described by Wilson et al (2005). They were generated in the Institute of Medical Genetics, Cardiff University.

2.2.2 Treatments

Tsc1$^{+/-}$ mice were treated for 2 months from 12 months old with vehicle (2.5% PEG, 2.5% Tween 2.5% DMSO, by i.p; n=8.), rapamycin (5 mg/kg by i.p; n=10.), nelfinivir (80mg/kg by gavage; n=10), sunitinib (45mg/kg by gavage; n=10), or a

rapamycin/sunitinib combination (n=10) 5 times a week. Drug treatments are summarized in Table 2.1. Kidneys from one mouse in each treatment group were taken for molecular analysis and were thus not included in totals and statistical analysis. After treatment, animals were humanely killed and tissues were collected for histological and molecular analysis.

Treatment	No of *Tsc1*$^{+/-}$ mice	Dosage	Duration	Frequency	Admin.	Vehicle
Vehicle (2.5% PEG, 2.5% Tween, 2.5% DMSO)	8	up to 250 µl	2 months	5 times a week	IP	n/a
Rapamycin	10	5 mg/kg	2 months	5 times a week	IP	2.5% PEG, 2.5% Tween, 2.5% DMSO
Nelfinavir (Pfizer)	10	80mg/kg	2 months	5 times a week	gavage	10% ethanol
Sunitinib (Pfizer)	10	45mg/kg	2 months	5 times a week	gavage	0.5% methyl cellulose
RS	10	Full dosage of each	2 months	5 times a week	IP/gavage	2.5% PEG, 2.5% Tween, 2.5% DMSO/ 0.5% methyl cellulose

Table 2.1 Drug treatments in *Tsc1*$^{+/-}$ mice. IP: intraperitoneal injection.

2.2.3 Histological analysis

Kidney sections were prepared for histological analysis as described by Kalogerou et al (2011). Kidneys were fixed in 10% buffered formalin saline for 24 hours. They were then paraffin-embedded and sectioned. Slides were prepared with 5µm sections taken at 200µm intervals and stained with haematoxylin and eosin (H&E).

Slides were then scanned to create virtual HE slides. Photographs of individual renal lesions were then taken with Aperio ImageScope system with an included reference length for scale. Lesions were then measured (by a blinded examiner) using ImageJ software to find both total area and cellular area.

2.2.4 Statistical Analysis

All statistical analysis was performed using GraphPad Prism software (version 6.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com). One-way ANOVAs and subsequent Tukey Multiple Comparison Tests were used to compare mean tumor number and size between treatment groups. $p< 0.05$ was considered statistically significant.

CHAPTER 3

Genetic and clinical studies of the *TSC2/PKD1* contiguous gene deletion syndrome

3.1 Introduction

The *TSC2/PKD1* contiguous gene syndrome is a rare disease in which both the *TSC2* and *PKD1* genes are involved in a deletion mutation. These genes lie adjacent to each other on chromosome 16p13.3 (European Polycystic Kidney Disease Consortium, 1994). The contiguous gene syndrome is characterised by the presence of polycystic kidney disease (PKD) in the presence of TSC and its accompanying manifestations. It is distinguished from TSC alone by a more severe cystic phenotype as opposed to the few cysts often seen later in life in typical TSC. Diagnosis of PKD can often occur early in life or even prenatally. Genetic approaches are now available to confirm the underlying genetic change in such cases (see Section 1.6.2). Treatment includes management of hypertension that often accompanies PKD, management of the complications of chronic kidney disease (CKD) and in some cases dialysis or kidney transplant.

Much is left unknown about kidney disease in the *TSC2/PKD1* contiguous gene syndrome, especially with regard to the mechanisms of disease and natural history (section 1.6.3 and 1.6.4.3). On the basis of a cross sectional study it has been suggested that patients with a contiguous deletion show a relatively rapid decline in kidney function, often reaching end stage renal disease (ESRD) by early adult life

(Sampson et al, 1997). However, follow up and prospective studies are needed to more clearly define the course of disease. Here we further investigate the natural progression of renal disease in patients with the *TSC1/PKD2* contiguous gene syndrome. We compare kidney function and age at ESRD onset in patients with a *TSC2/PKD1* deletion and those with a mutation of *TSC2* alone.

3.2 Methods

For the present study we ascertained patients with the *TSC2/PKD1* contiguous gene syndrome from the records of the genetic diagnostic laboratory at the Institute of Medical Genetics, Cardiff. Patients with *TSC2* mutations alone were ascertained from the records of genetic diagnostic laboratories at the Institute of Medical Genetics or at Cincinnati Children's Hospital, as well as via advertising on the web site of the Tuberous Sclerosis Association (UK). Once written consent was obtained (see section 2.1.2.3 and Figure 2.1), medical records were requested and reviewed. Further blood testing for both genetic and kidney function testing was requested if necessary.

Information collected from medical records included presentation, age at diagnosis of TSC and method of diagnosis, age and method of diagnosis of renal disease, history of hypertension and its treatment, details of results of renal imaging and records of kidney function tests enabling estimation of glomerular filtration rate (eGFR) from serum creatinine values. In children under 18, height measurements were included in order to calculate eGFR by the Bedside Schwarz Equation method (Schwartz & Work, 2009 & Schwartz et al, 2009). Adult eGFR values were calculated using MDRD method (Levey et al, 1999). If ESRD was reached, age and treatment

was noted as were dates and causes of death. *TSC2/PKD1* deletion mutations were characterised by MLPA, FISH and aCGH (see section 2.1.5 and Figure 3.1). All statistical analysis was performed using STATA 12 software with $p< 0.05$ considered statistically significant.

3.3 Results

3.3.1 Patients

We were able to recruit a total of 44 patients with a confirmed *TSC2/PKD1* deletion. Approximately 52% of these patients were female and 48% male (See Table 3.1). Our group included 5 affected parent-offspring cases. Patients 2 and 3 are mother and daughter, 14 and 15 are father and son, 22, 23, and 24 are a mother and two sons, 37, 38, and 39 are a mother, daughter, and son, and 40 and 41 are a father and daughter. The mean age of patients with a *TSC2/PKD1* contiguous deletion was 32 with a standard deviation of 13.

Likewise, 69 patients with intragenic mutations affecting *TSC2* alone were recruited. In *TSC2* mutation cases recruited from Cincinnati Children's Hospital sex was unknown as a result of anonymization. Of the patients recruited via the Institute of Medical Genetics or via the Tuberous Sclerosis Association (who were not anonymized) 62% were female and 38% were male.

3.3.2 Molecular Genetic Findings

All cases with TSC2/PKD1 contiguous deletions had molecular confirmation of their status using a variety of different techniques. Historical cases previously had

deletions confirmed by a combination of pulsed field gel electrophoresis and fluorescence in situ hybridisation (FISH). FISH utilises cells from peripheral blood samples and labels them with a fluorescent probe (in this case, for the *TSC2/PKD1* region of chromosome 16). One can then recognize if both copies of chromosome 16 have the gene(s) of interest intact. More information regarding FISH can be found in Section 2.1.5.3, and examples of cells labelled for the *TSC2/PKD1* region can be seen in Figure 3.1.

More recently diagnosed cases had deletions confirmed by a combination of multiplex ligation-dependent probe amplification (MLPA) and array comparative genomic hybridisation (aCGH) along with FISH. MLPA, a variation on polymerase chain reaction (PCR), utilises multiple fluorescent oligonucleotide probes to amplify small, separate regions along the length of our genes of interest. The probes are paired in such a way that they only amplified if the region of DNA they target is present. They then are ligated to one another and fluoresce, giving a signal read-out to the accompanying software. More information regarding MLPA can be found in Section 2.1.5.2 and examples of results obtained can be seen in Figure 3.2.

aCGH looks for gene abnormalities on a genome-wide scale. It compares patient DNA to a reference DNA sample and looks for differences between the two. It utilizes thousands of probes covering the entire genome, with sample DNA and reference DNA probes labelled with different coloured fluorophores. The accompanying software then reads the ratio of the two coloured probes for a particular region of interest. One can then see if the patient's DNA gives less signal than the reference, signifying a deletion in that region. More information regarding

the aCGH process can be found in Section 2.1.5.4 and examples of collected data can be found in Figure 3.3.

There was very good concordance between the different techniques used to identify and characterize deletions. In some cases mosaicism was suspected following MLPA or aCGH analysis as signals for probes in the deleted region were reduced by less than 50%. Confirmation and quantification of mosaicism was undertaken by counting cells with and without deletions following FISH with the probe JH2A that maps to the *TSC2/PKD1* locus (Figure 2.2). However, very small deletions involving only the most 3' regions of the genes could not be characterised by FISH as the probe is larger than the deletions in these cases.

All cases in the data presented here have confirmed *TSC2/PKD1* contiguous deletions. Work is ongoing as more patients are enrolled in the study at large. A future publication will detail characterization of the patients' separate deletions.

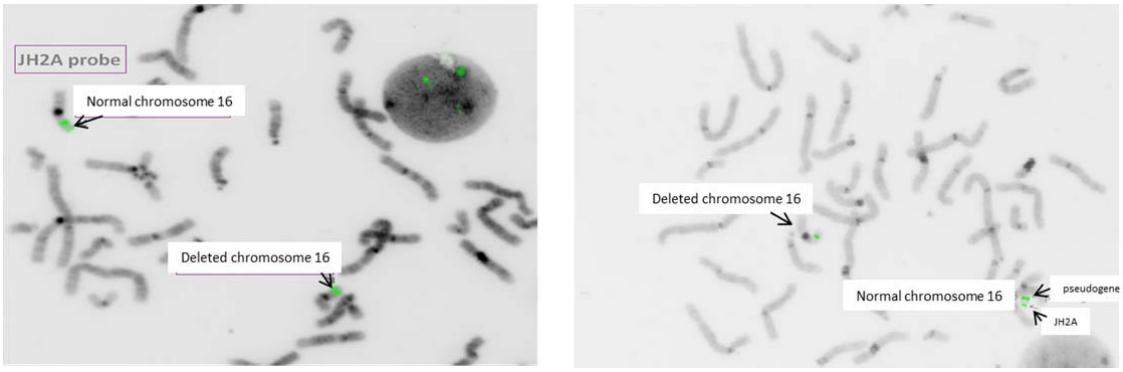


Figure 3.1 FISH analysis of the TSC2/PKD1 region. Two examples of mutation analysis results for fluorescence *in situ* hybridization (FISH) are shown. Each copy of chromosome 16 should have two green fluorescent signals present. One is the location of TSC2/PKD1 genes, and the other is a pseudogene region elsewhere on the chromosome. Each example shows a cell with two copies of chromosome 16. One is normal, showing both green signals. The other has a TSC2/PKD1 deletion, showing only one green signal. This was repeated for many cells from each patient, looking for deletions in this region. FISH was particularly used to identify mosaic patients. In this case, a proportion of cells would show deletions with rest showing two normal copies of TSC2/PKD1 present.

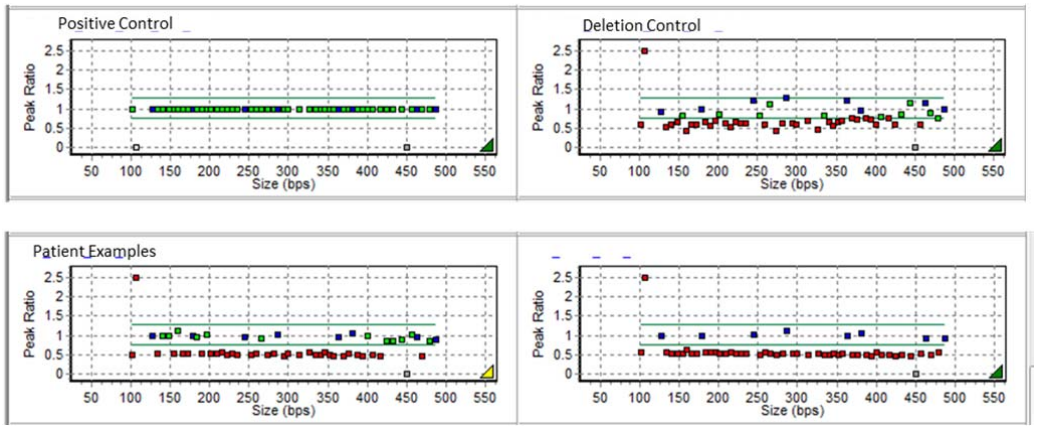
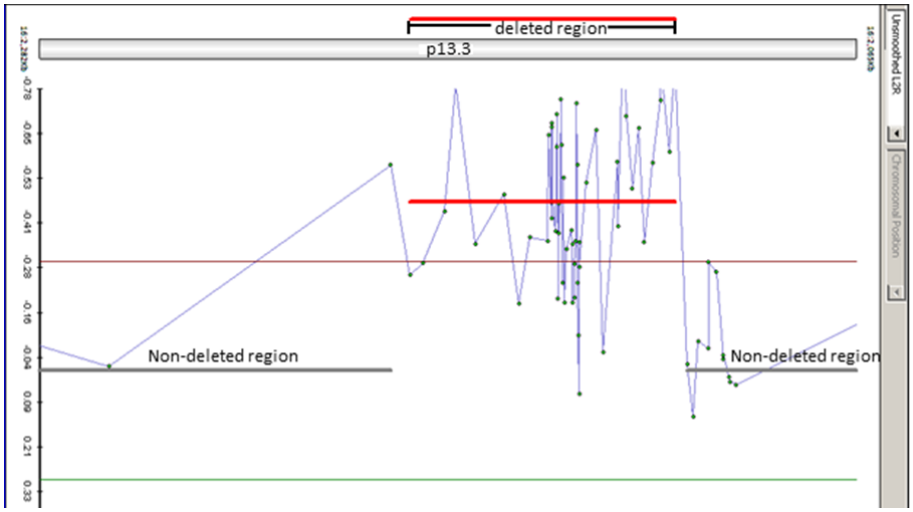


Figure 3.2 MLPA mutation analysis. Two examples of mutation analysis results for Multiplex Ligation Probe Amplification (MLPA). In the top row, positive and negative control data is shown. In the positive control, MLPA probes/their fluorescent read-outs all lie around a ratio of 1, signifying that region's presence in a normal amount (i.e., the TSC2/PKD1 region is not duplicated or deleted). Each probe is signified by a separate square. In the deletion control, most of the probes lie below 1, signifying their presence is lower in amount than expected, i.e., deleted. Both patients' samples below show similar results to the deletion control. Almost all the probes read below a ratio of 1, signifying that region is deleted. Thus both patients have TSC2/PKD1 gene deletions.

A



B

Figure 3.3 aCGH mutation analysis. One example of mutation analysis results for Array (aCGH). **(A)** shows the software read out for a very small section of this patient's DNA in the TSC2/PKD1 region. Each fluorescent probe found in this region is signified by a green dot. As described in section 3.3.2, the ratio of fluorescence in patient and reference DNA is calculated. A normal ratio signifies no deletion in the patient, as is seen above where it is noted "non-deleted region" between the two thin horizontal lines. There is a section in the middle, though, which has an abnormal ratio. Even though in this case the middle region is above the two thin lines, this signifies a deletion (re: software use guidelines). **(B)** shows more of a summary screen or schematic of these results. The band entitled "Genes" shows where the TSC2 and PKD1 genes should lie. The band above entitled "Current" shows results for the same patient. This region is deleted in our patient, as stated in the red bar entitled "deleted region." Exon locations are depicted below for informational purposes.

3.3.3 Clinical Presentation

The age at which polycystic kidneys were first identified ranges from 33 weeks gestation to 43 years old with an average age +/- SD being 10.1 years +/- 12.0 (See Table 3.1). Of the 44 cases, 15 (34%) were diagnosed to have PKD under the age of one year, 18 (41%) between 1 and 18 years, and 9 (20%) over the age of 18 (See Figure 3.4). In other words, a majority of the cases were diagnosed with polycystic kidneys in childhood (76%). For two cases, their age at diagnosis is unknown. Notably, cases with mosaicism included some relatively mildly affected parents whose condition was diagnosed only following the diagnosis of their more severely affected non-mosaic offspring.

The most common identification of PKD, noted in 28 (64%) of patients, was through abdominal/renal Ultrasound Scan (USS) ordered as a part of the routine work up for a TSC diagnosis. Six patients (14%) presented with abdominal distension and PKD was consequently discovered by imaging.

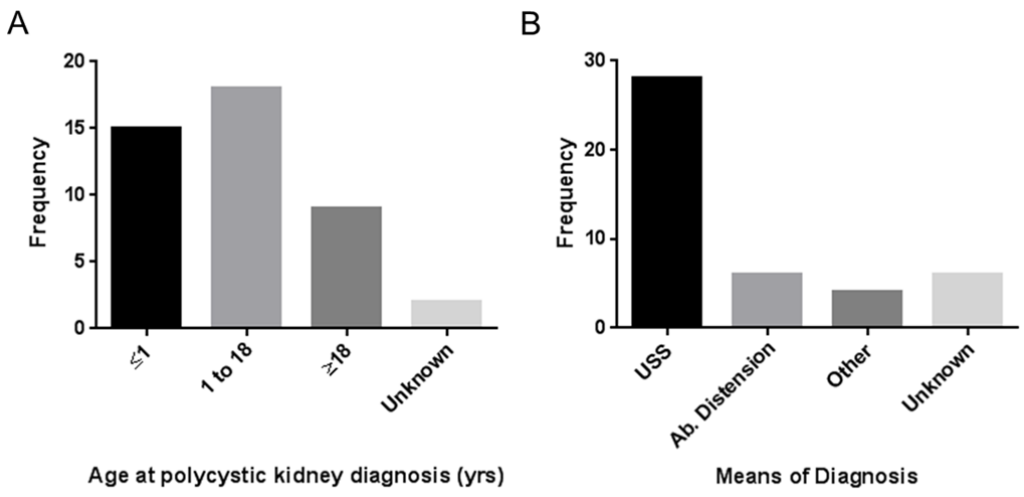


Figure 3.4 Diagnosis of polycystic kidneys. Frequency of diagnosis within certain age groups is shown in **A**. Most patients were diagnosed with polycystic kidneys by age 18. Frequency of various means of diagnosis is shown in **B.** Most patients were diagnosed via Ultrasound Scan (USS) of the kidneys while some were diagnosed upon presentation with abdominal distension.

Pateint	Sex	Age at Diagnosis	Means of Diagnosis/ Presentation	Commencement of anti-hypertensives	ESRD (years)
1	Female	2 mo	USS	None	-
2	Female	35 yrs	unknown	≤46 yrs	-
3	Female	4 yrs	family history	24 yrs	-
4	Male	3 mo	USS	9 yrs	21
5	Female	17 yrs	USS	29 yrs	-
6	Female	7 yrs	USS/CT	None	20
7	Male	33 wks gestation	USS	2 yrs	-
8	Male	28 yrs	USS	25 yrs	-
9	Female	6 mo	USS	None	-
10	Male	4 mo	abdominal distension	3 mo	-
11	Male	11 mo	abdominal distension	26 yrs	19
12	Male	2.5 yrs	abdominal distension	18 yrs	18
13	Male	6 mo	USS	None	-
14	Male	10 yrs	USS	18 yrs	20
15	Male	35 yrs	family history	None	-
16	Female	Unknown	unknown	None	18
17	Female	4 yrs	abdominal distension	15 yrs	-
18	Female	Unknown	unknown	None	-
19	Male	9 mo	unknown	11 mo	22
20	Male	9 mo	USS	None	-
21	Female	≤2 yrs	USS	2 yrs	-
22	Male	12 yrs	USS	15 yrs	-
23	Female	early 20s	unknown	41 yrs	-
24	Male	4 mo	USS	4 mo	-
25	Female	12 yrs	unknown	None	-
26	Male	9 mo	US	6 mo	-
27	Male	7 mo	abdominal distension	7 mo	18
28	Female	3 yrs	USS	None	-
29	Female	4 mo	abdominal distension	1 yr	17
30	Female	43 yrs	USS	None	-
31	Female	21 yrs	USS	None	26
32	Female	≤16 yrs	USS	≤25 yrs	-
33	Female	3 yrs	USS	3 yrs	-
34	Female	5 yrs	haematuria	14 yrs	29
35	Male	1 mo	USS	4 mo	21
36	Female	9 yrs	USS	None	-
37	Female	15 yrs	USS	26 yrs	-
38	Female	≈30 yrs	gynocological investigations	29 yrs	40
39	Male	9 yrs	USS	≤22 yrs	-
40	Female	2 yrs	USS	None	-
41	Male	30 yrs	USS/family history	None	-
42	Male	9 yrs	USS	None	-
43	Male	32 yrs	USS	38 yrs	39
44	Male	9 mo	USS	None	-

Table 3.1 Patient Summary. Each patient's presentation is summarized here including sex, age and means of diagnosis, treatment with anti-hypertensives, and age at end-stage renal disease (ESRD), if applicable. USS: Ultra Sound Scan.

3.3.3.1 Hypertension

27 patients (61%) required anti-hypertensive treatment. Age at first treatment ranged from as young as three months to approximately 46 years. About half of those patients requiring treatment commenced anti-hypertensives during childhood, with the other half starting them at 18 years or older.

3.3.4 Renal function in *TSC2/PKD1* deletion syndrome and comparison with *TSC2* only cases

Renal function data is reported via estimated glomerular filtration rate (eGFR) in Figure 3.5. Patients with *TSC2/PKD1* contiguous deletions show lower eGFRs at presentation ($p<0.0064$) and lower final eGFRs ($p<0.0002$) than patients with *TSC2* mutations alone. The drop between first and final eGFRs was also considered significantly greater in patients with contiguous deletions ($p<0.0113$). One patient with a contiguous deletion (Patient 38, see Table 3.1) was transplanted at age 40 and subsequently showed increases in eGFR. This is reflected in Figure 3.5 A. Original eGFR data for each individual patient can be found in Appendix 2.

Additionally, patients with contiguous deletions showed more advanced kidney disease as expressed by Chronic Kidney Disease (CKD) stages (determined by eGFR values). Patients with TSC2/PKD1 contiguous deletions showed higher initial CKD stages ($p<0.002$) and final CKD stages ($p<0.007$).

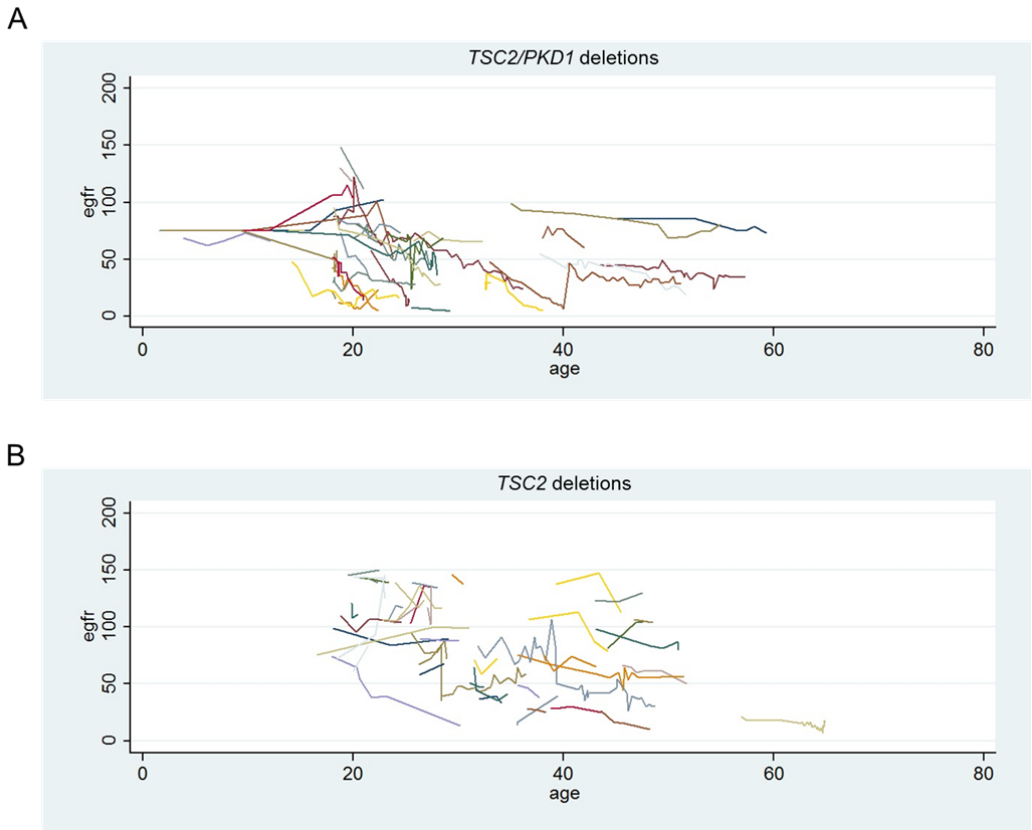


Figure 3.5 Renal function in patients with tuberous sclerosis and polycystic kidneys. Estimated glomerular filtration rates (eGFR) are shown for patients with *TSC2/PKD1* contiguous deletions (**A**) and those with *TSC2* mutations alone (**B**). Patient 38 was transplanted at age 40 and showed improved renal function (seen in **A**). Original eGFR data for each individual patient can be found in Appendix 2.

CHAPTER 4

Pre-clinical investigation of novel drug treatments for TSC

4.1 Introduction

The current goals of TSC treatment remain chiefly to control symptoms rather than targeting of underlying pathophysiology. One recently developed exception to this rule is treatment with rapamycin and its analogues for some renal, neurological, and possibly other manifestations (see section 1.3). In addition to "rapalogues", many other drugs are being investigated in the pre-clinical setting for management of renal manifestations of TSC. Nelfinavir is a protease inhibitor (PI) chiefly used in HIV treatment and has been shown to have anti-cancer effects (Ikezoe et al, 2000 and others). Sunitinib is a receptor tyrosine kinase (RTK) inhibitor already approved for the treatment of renal cell carcinoma (RCC) (Chow & Eckhardt, 2007). Both of these agents are potentially attractive for TSC treatment due to their activity in relevant signaling pathways (AKT/protein kinase B/mTOR) and anti-proliferation effects (see 1.4.5.1 and 1.4.5.2). This chapter describes my work investigating the effects of these drugs on tumour growth and development *in vivo* in a *Tsc1*$^{+/-}$ mouse model.

4.2 Methods

This study utilized a *Tsc1*$^{+/-}$ mouse model described by Wilson et al, 2005. The transgenic construct in this model results in an out of frame deletion of exons 6-8 of

Tsc1 generating a null allele associated with homozygous embryonic lethality and with renal and extra-renal tumours and learning and socialization deficits in heterozygous animal (Wilson et al, 2005). Forty eight mice were treated with either vehicle, rapamycin, sunitinib, nelfinavir, or a rapamycin/sunitinib combination for two months (see Table 2.1). After drug therapy was concluded, mice were sacrificed and their kidneys removed for analysis. Kidneys were sectioned and HE slides were prepared (see sections 2.2.3.1 and 2.2.3.2) and scanned. Lesions were photographed with a size reference and subsequently both lesional total areas (i.e. including fluid filled cystic areas and cellular areas) and lesional cellular areas alone were measured. All statistical analysis was performed using Prism GraphPad software with $p< 0.05$ considered statistically significant. Slide preparation was performed by Jian Yang and Paulina Samsel, and subsequent analysis was performed by Anna Hellmann Thamann.

4.3 Results

4.3.1 Kidney lesions in *Tsc1*$^{+/-}$ mice

We used a *Tsc1*$^{+/-}$ mouse model to explore the kidney manifestations of *Tsc1* heterozygosity. This model develops a relatively severe kidney phenotype showing a variety of lesion types, and all mice in this study (48 total) reflected this. The three lesion types documented were cysts, papillary lesions, and solid lesions (Figure 4.1).

Cysts found in this model are simple, fluid filled lesions classified pathologically by a single, organised layer of epithelial cells differing from the surrounding kidney tissue. Cysts varied greatly in size.

Papillary lesions are charcterised by a less organised and more complicated lining. These lesions do contain fluid, but the proportion of abnormal cells/fluid is significantly greater than cysts. Papillary lesions include mostly epithelial cells but other cell types may be present (e.g. smooth muscle-like). The lesions vary markedly in size.

Solid lesions are completely filled by cells with very little to no fluid. Cell type varies (epithelial, smooth-muscle like) as well as lesion size. In our model, solid lesions generally occur more in the cortex than within the deeper renal parenchyma.

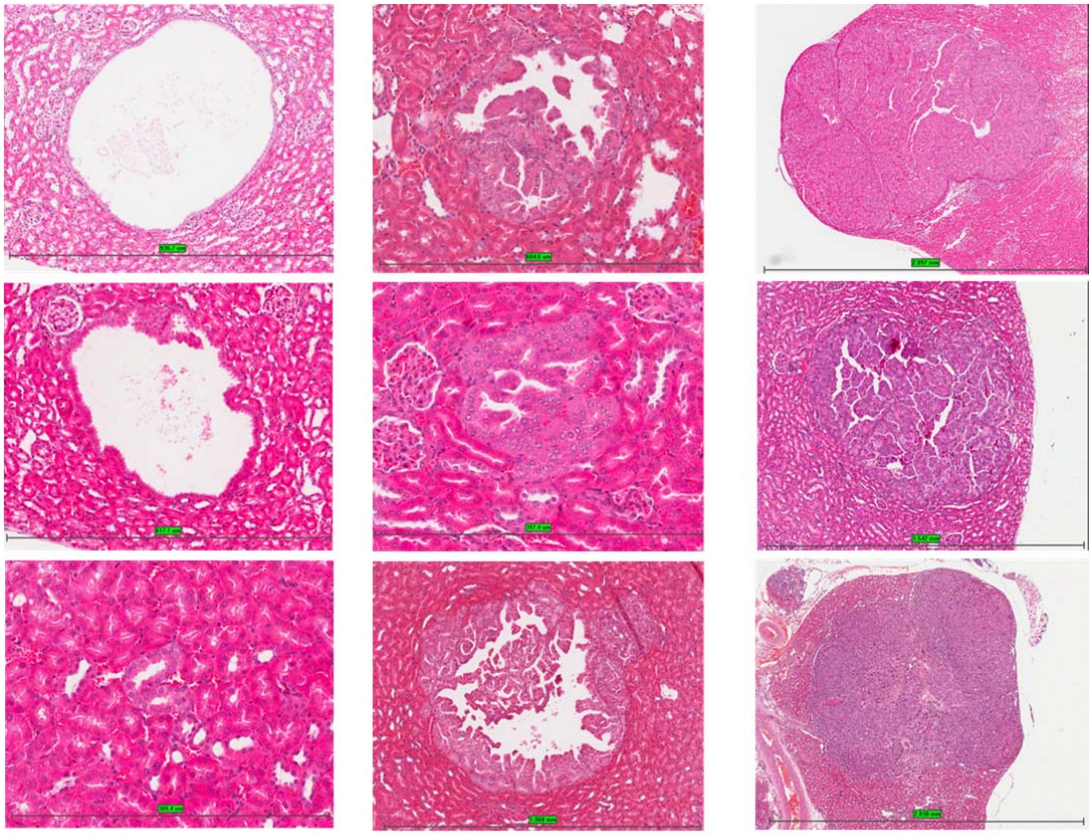


Figure 4.1 Histopathology of kidney lesions in *Tsc1* $^{+/-}$ mouse model. HE sections showing cystic, papillary, and solid lesions. Note the simple border of the cystic lesions in the left panel and the lack of fluid filled (white) space in the papillary and solid lesions. Also note green boxes showing measurement in um with black scale bar below. All slides were prepared by Jian Yang and Paulina Samsel and analysed by Anna Hellmann Thamann.

4.3.2 Drug treatment alters number of renal lesions found in the *Tsc1*$^{+/-}$ mouse model

The aforementioned *Tsc1*$^{+/-}$ mouse model was employed to explore the effects of a variety of drug treatments on kidney lesions. Mice were treated with either vehicle, rapamycin, nelfinivir, sunitinib, or a rapamycin/sunitinib combination for two months and were consequently sacrificed. Table 2.1 shows drug treatment regimens for each group. Kidney sections were prepared and scanned for analysis. The total number of lesions was then recorded for each kidney and mouse, as well as the numbers of cysts, papillary lesions and solid lesions, respectively. Raw data for each individual mouse can be found in Appendix 1.

As shown in Figure 4.2A, mice treated with rapamycin or the rapamycin/sunitinib combination therapy showed a significant decrease in total number of kidney lesions. Neither treatment with sunitinib nor nelfinivir alone had a significant effect on the total number of lesions.

Furthermore, mice treated with either rapamycin alone or in combination with sunitinib show a significantly decreased number of papillary and solid lesions (Figure 4.2C, D). Interestingly, mice in these two treatment groups showed no change in the number of cysts (Figure 4.2B). Mice treated with either nelfinivir or sunitinib alone showed no significant change in the number of cysts or papillary lesions (Figure 4.2B, C). Both groups, however, did show a significant decrease in the number of solid lesions (Figure 4.2D), though these changes are not as marked as those in mice treated with rapamycin, i.e., treatment with rapamycin causes a significantly larger decrease in number of solid lesions.

In summary, treatment with rapamycin (either alone or in combination with sunitinib) was associated with a reduction in the total number of kidney lesions that reflected a reduced number of papillary and solid lesions while causing no change in the numbers of cysts. Nelfinivir and sunitinib alone caused a decrease in the numbers of solid lesions but no change in total lesion number or that of cysts or papillary lesions.

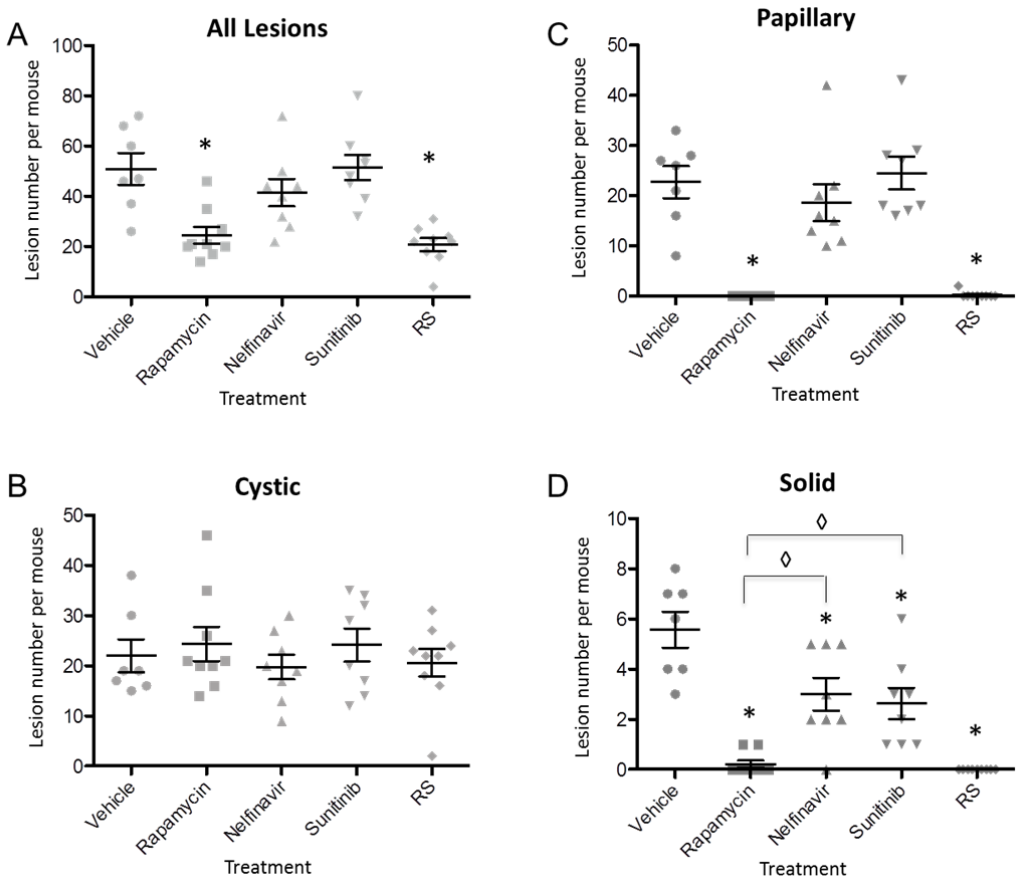


Figure 4.2 Number of kidney lesions after drug treatment. Number of lesions per mouse after treatment with vehicle, rapamycin, nelfinivir, sunitinib, or rapamycin/sunitinib (RS), respectively. ***A*** shows number of all lesions total, while ***B, C, and D*** show number of specific types of lesions. Black bars signify mean +/- SD. **, p< .05 compared to Vehicle; ◊, p<.05 compared to rapamycin.* Original data for each individual mouse can be found in Appendix 2.

4.3.3 Drug treatment alters lesion size in *Tsc1*$^{+/-}$ mouse model.

In addition to looking at the effects of drug treatments on the number of lesions in these mice, we explored effects on the size of cysts, papillary and solid kidney lesions. The area of each lesion (at the largest section) was measured in μm^2 using ImageJ software. The total sum of lesion areas was calculated for each mouse, and was also broken down into sum of all cystic area, papillary lesion area, and solid lesion area, respectively. That is, we calculated the kidney area occupied by all lesions in general, cysts, papillary lesions and solid lesions.

As shown in Figure 4.3A, mice treated with rapamycin or a rapamycin/sunitinib combination show a significant decrease in the total lesion area. Mice in these groups also show a significant decrease in the areas of papillary and solid lesions (Figure 4.3C, D). Similar to the effects of these therapies on number of cysts, mice in these groups also show no decrease in cystic area (Figure 4.3B). To the contrary, it appears as though cystic size actually increases after Rapamycin or Rapamcin/Sunitinib treatment, though this difference is not statistically significant compared to vehicle. Mice treated with either nelfinivir or sunitinib alone show no change lesion area, be it in total lesion area or cystic, papillary, or solid area (Figure 4.3A-D).

In summary, treatment with rapamycin (either alone or in combination with sunitinib) appears to cause decreases in general lesion size and, in particular, size of papillary and solid lesions. These treatments do not cause any significant change in cyst size. Nelfinivir and sunitinib cause no change in lesion size whatsoever.

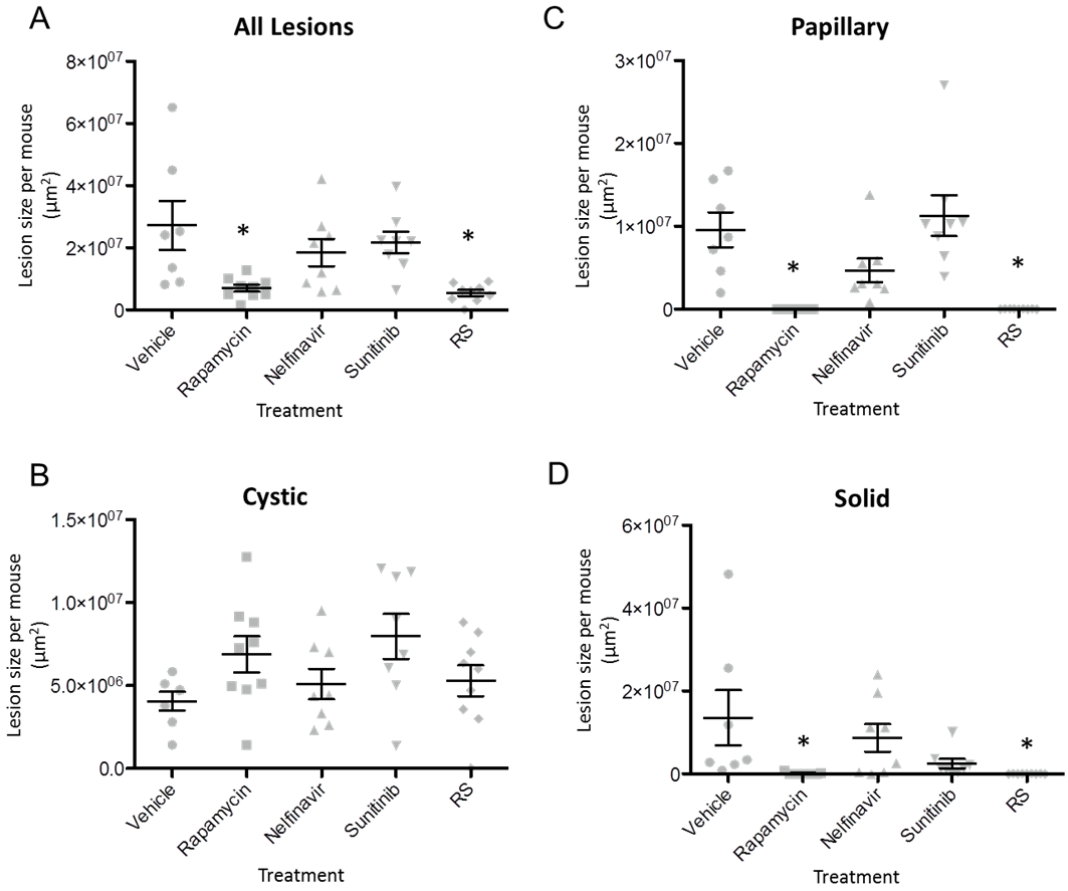


Figure 4.3 Lesions size after treatment. Total sum of lesions areas after treatment with vehicle, rapamycin, nelfinavir, sunitinib, or rapamycin/sunitinib (RS), respectively. ***A*** shows total size of all lesions, while ***B, C, and D*** show size of specific types of lesions. Black bars signify mean +/- SD. *$p<0.05$ *compared to vehicle.* Original data for each individual mouse can be found in Appendix 1.

CHAPTER 5

Discussion

5.1 Introduction and Project Aims

Tuberous Sclerosis Complex (TSC) is a rare genetic disorder in which either the *TSC1* or *TSC2* gene is mutated. It is characterised by benign growths (hamartomas) in a variety of organs, including the kidneys. Renal disease is common and problematic in the TSC patient population and includes angiomyolipomas and cysts and, very rarely, other types of renal tumours. *TSC2* lies adjacent to *PKD1* (the autosomal dominant polycystic kidney disease 1 gene) on chromosome 16p13.3 (European Polycystic Kidney Disease Consortium, 1994). In a few percent of TSC patients, multi-kilobase deletion mutations involve both *TSC2* and *PKD1*, leading to the TSC2/PKD1 contiguous gene syndrome. This disorder is characterised by the presence of polycystic kidneys in the presence of TSC, and is distinguished by a much earlier onset and more severe cystic phenotype than the scattered renal cysts that are often seen later in life in TSC. Traditional treatment for TSC and for the TSC2/PKD1 contiguous gene syndrome is based chiefly on management of symptoms and complications rather than on targeting the underlying pathophysiology. Renal treatment is inadequate in both the general TSC patient population and those with the contiguous gene deletion syndrome, but perhaps more so in the latter group.

The present study had dual aims involving pre-clinical and clinical investigations to improve understanding of renal disease in TSC. The first aim was to better define the natural history the *TSC2/PKD1* contiguous gene syndrome. Clinical histories of patients with either the contiguous gene syndrome or with mutations affecting only *TSC2* were collected and clinical presentations and change in kidney function with time were compared.

The second aim was to explore nelfinivir, sunitinib, and rapamycin as candidate drug treatments for renal manifestations in a *Tsc1+/-* mouse model. These agents were selected for study because of their different effects in signaling pathways thought to be relevant to TSC pathophysiology. Nelfinivir is a protease inhibitor that also has inhibitory effects on AKT; sunitinib is a relatively promiscuous tyrosine kinase inhibitor whose activities include antiangiogenic effects while rapamycin is a much more specific inhibitor of mTOR. The effects of these agents on number, size, and cellular content of renal lesions were investigated in pre-clinical trials. Thus this project aimed to improve understanding of renal disease in TSC.

5.2 Chronic kidney disease in the *TSC2/PKD1* contiguous gene syndrome and Comparison to *TSC2*-associated kidney disease

For the present study we ascertained patients with the *TSC2/PKD1* contiguous gene syndrome and compared them to patients with *TSC2* mutations alone. Medical records were requested and reviewed, and genetic status was confirmed via FISH, aCGH, and MLPA. We recorded clinical data from medical records including presentation and diagnosis, history of hypertension and its treatment, details of

results of renal imaging and records of kidney function tests enabling estimation of glomerular filtration rate (eGFR) from serum creatinine values.

Overall, our data show that renal disease is more severe in patients with *TSC2/PKD1* deletions than those with *TSC2* mutations alone. Patients with contiguous deletions showed lower presenting and final eGFRs and a greater drop between them than the control group. CKD staging (as determined by eGFR) also showed more advanced kidney disease in patients with contiguous deletions. Interestingly, there was no significant difference in change in CKD status between the two groups, though there were trends that could be significant with larger sample sizes.

We also present here some descriptive data about the *TSC2/PKD1* contiguous gene syndrome patient population. This group presented for a variety of reasons at various ages, but did tend to present earlier than their TSC2 control counterparts (data not shown) and very often in childhood. This is somewhat expected since kidney disease is indeed more serious and more rapidly progressing. Many patients were also treated for hypertension, though not all.

5.2.1 Study Limitations

As with any clinical study, there are limitations to the study I report here. The major challenge with this work is recruiting enough patients to have an adequate sample size. This is especially challenging with a rare disease such as tuberous sclerosis or the even rarer *TSC2/PKD1* contiguous gene syndrome. There are also often difficulties with obtaining all the necessary medical records to obtain a full clinical picture. Even though the data we report here are statistically significant, our results

could be stronger with more patients and clinical data. Work is ongoing and we are actively collecting more data. This should provide an even more accurate picture of renal disease in this population and more clearly illustrate the differences between the two groups. Furthermore, a more detailed analysis of ESRD in each group is needed.

5.3 Pre-clinical analysis of drug treatments for TSC

We employed a *Tsc1*$^{+/-}$ mouse model to explore the kidney manifestations of *Tsc1* heterozygosity and the impact of treatments on them. This model develops a relatively severe phenotype with a variety of renal lesion types and our results reflect this. All mice in all experimental groups show cystic, papillary, and solid lesions (see Figure 4.1). Cysts are simple and fluid-filled, surrounded by a single layer of epithelial cells. Papillary lesions are classified by a less organised border with multiple layers of cells, some of which branch into the fluid filled center. Overall their cell to fluid filled area ratio is significantly larger than that of cysts. Solid lesions have little to no fluid and show a variety of cell types. They are less organised, but most look encapsulated. Solid lesions were almost always seen in the kidney cortex. All lesion types showed wide size variation.

We utilized this model to test a variety of drug treatments for the treatment of TSC. Specifically, we wanted to explore the effects of these drugs on kidney lesion growth and progression. Mice were treated with either vehicle, rapamycin, nelfinivir, sunitinib, or a rapamycin/sunitinib combination, and kidney sections were prepared for analysis. We recorded the total number of lesions for mice in these groups as well as frequency of cysts, papillary and solid lesions, respectively.

Furthermore, we recorded the maximum cross sectional area of each individual lesion and then calculated total cross sectional area of all lesions. This was also broken down to look at area of cysts, papillary and solid lesions.

5.3.1 Effects of rapamycin on cystic and solid renal lesions

Our results show that rapamycin-treated mice (either alone or in combination sunitinib) have a smaller number of total renal lesions, and that these lesions are smaller than in control (vehicle treated) mice (see Figures 4.2 and 4.3). Interestingly, while rapamycin and rapamycin/sunitinib treatments proved successful in reducing the number and size of both papillary and solid lesions, cystic lesions showed no difference from control mice. There were virtually the same number of cysts across groups, and these lesions showed no decrease in size after rapamycin treatment.

This could be for a variety of reasons. In general, the more cellular papillary and solid lesions could shrink (or not develop to such an extent) because cell proliferation is halted/decreased and because cells shrink or die. In terms of proliferation, one might not expect a large effect to be seen in established cystic lesions. Cysts may be undergoing proliferation as they become larger and accommodate more fluid, but the pace and amount of cell division may not be as great as in faster-growing solid or papillary lesions. Both of these lesion types have much greater cell content and therefore are expected to be more significantly affected by a treatment targeting cell proliferation. Furthermore, treatment affecting cell size would not be expected to show a significant effect on cystic lesions. Cysts are mostly fluid and their cellular content is small compared to their

overall size. Consequently, if a treatment (like rapamycin) were successful in shrinking cells this may not be reflected in total number or size of cysts.

In a rather fascinating turn of events, treatment with rapamycin or rapamycin/sunitinib actually appeared to increase the size of cystic lesions (Figure 4.3). Compared to the vehicle, this difference was not considered statistically significant with $p<0.05$. If our cutoff were $p<0.1$, though, it would qualify for significance. This was a very unexpected observation, and has no obvious explanation. One could assume that this difference in size is most likely from added fluid since rapamycin has been shown time and time again to decrease (or at least halt) cell proliferation/growth. Why would rapamycin cause a shift of more fluid into cysts? Perhaps there are more signaling changes going on inside these cells than currently understood. For example, one could argue there is a difference in polarity of cystic epithelial cells causing them to shift fluid from the kidney interstitium into the cyst rather than the other way around. Perhaps rapamycin exacerbates this problem in some way in these particular epithelial cells. It would be very interesting to explore in greater detail why this phenomenon occurred, though perhaps not completely clinically relevant if rapamycin keeps showing it is no help in treating renal cysts.

In the context of human disease, sirolimus (rapamycin) and everolimus (a rapamycin analogue) were successful in shrinking angiomyolipomas in patients with TSC but did not have any significant effect on cysts or in patients with ADPKD (Bissler et al, 2008 & 2013, Davies et al, 2011, & Perico et al, 2010). It is not yet clear whether rapalogues could affect renal cyst size in TSC. In the clinical trials in

TSC AMLs also re-grew once treatment was discontinued, but the extent to which this reflected a change in cell size as opposed to recurrence of cell proliferation is not known. In our mouse model, if papillary or solid lesions have cells that shrink significantly upon mTOR inhibitor treatment, a significant decrease in overall lesion size could be easily detected. On the other hand, even if every cell in a cyst were to shrink, a much smaller decrease in lesion size would occur. It would be interesting to further characterize actual cell size in renal lesions in the mouse model pre- and post-treatment.

5.3.2 Nelfinavir and Sunitinib

While treatment with rapamycin showed significant reductions in lesion number and size, treatment with nelfinavir or sunitinib did not prove as successful. Mice treated with either of these drugs alone carried the same total number and size of lesions as control mice (see Figures 4.2 and 4.3). When the data was broken down into cystic, papillary, and solid lesions, both drugs did show some promise in decreasing the proportion of solid tumours. Mice treated with nelfinavir or sunitinib alone had significantly less solid lesions than control mice. However, the effect was not as great as seen with rapamycin treatment. Even though treatment with nelfinavir or sunitinib resulted in a smaller number of solid tumours, the area covered by these solid tumours remained the same, suggesting that while some lesions responded others did not. Neither of these drugs showed a decrease in the number or size of cysts or papillary lesions (Figures 4.2 and 4.3). Furthermore, there was no significant advantage to treatment with the sunitinib/rapamycin combination over rapamycin alone.

Treatment with both nelfinavir and sunitinib has been successful in inhibiting growth and proliferation of cancer cells in a variety of other studies (see also section 1.4.5.1). Nelfinavir was shown to impair proliferation and spark apoptosis in multiple myeloma cell lines as well as in a mouse model (Bono et al, 2012). Furthermore, nelfinavir was shown to have synergistic effects in combination with rapamycin in Diffuse Large B cell Lymphoma (DLBCL), a Non-Hodgkin's lymphoma. In DLBCL, like TSC, cells have an over-active mTOR signaling pathway (Petrich et al, 2012).

Sunitinib has also previously been shown to inhibit AKT signaling, thereby inducing apoptosis and growth arrest in cancer cells (see also section 1.4.5.2). Sunitinib specifically induced apoptosis in colon cancer cells (Sun et al, 2012), and induced growth arrest of medulloblastoma tumour cells (Yang et al, 2010). Interestingly, the drug was also shown to cause growth factor receptor inactivation leading to Tsc1/Tsc2 inhibition of mTOR (Tran et al, 2013). Thus, the mechanisms of action of both sunitinib and nelfinavir may include mTOR mediated activities that would be relevant to TSC.

Nelfinavir and sunitinib have not been investigated previously in a TSC setting. . If they were effective in decreasing proliferation or growth in this context then we would have expected to see more significant decreases in papillary and solid lesions. However, neither drug showed such effects and the results of these pre-clinical trials do not suggest that these agents should be pursued in clinical trials unless they can be shown to be more effective, perhaps through combination with other agents.

5.3.3 Study Limitations

As with any pre-clinical study, there are limitations to the studies I report here. Animal studies have proven essential in biomedical research in order to derive evidence on which clinical trials can be based. However, drugs may act differently in different species and so evidence from mouse studies cannot be taken in isolation. Preclinical studies using human derived cell lines, including tumour lines, also have an important role to play. Nonetheless, the signaling pathways targeted by drugs like rapamycin are highly conserved and this is likely reflected in the efficacy of this drug in both mouse and human TSC.

The mouse model we employed was heterozygous for *Tsc1*. In the human clinical setting, *TSC2* heterozygosity tends to lead to a more serious renal phenotype. There could, though, be differences in molecular aspects of signaling and specific drug actions in *Tsc1* vs. *Tsc2* lesions and/or cells. However, since both TSC1 and TSC2 protein activity are essential for mTOR regulation, mTOR inhibitors are predicted to be effective in both TSC1 and TSC2-associated disease. Recent clinical trials have borne this out as both patient groups appear to respond equally well (Julian Sampson, personal communication). By contrast, TSC2 (or perhaps TSC1) may have additional functions which could lead to differential efficacy of other treatments.

Additionally, the mouse model used here is unlikely to be directly comparable to human cases with a TSC2/PKD1 contiguous deletion in whom inactivation of the PKD1 gene is associated with renal cystogenesis. So little is known about the pathophysiology of the contiguous gene syndrome that at present treatment of their TSC and PKD is not the subject of any clinical trials.

5.4 Treatment in the TSC/PKD contiguous gene syndrome

Currently, treatment for both TSC and the TSC/PKD contiguous gene syndrome is in response to symptoms rather than preventative. There is also very little data available to support approaches to slow disease progression in either syndrome. Kidney function screening is key as kidney damage is often only symptomatic when there is very advanced loss of function. If kidney function becomes too poor, dialysis and/or transplant are the last line of treatment.

Current treatment for renal disease in both the TSC only and TSC/PKD groups is inadequate. It is important for both sets of patients that more targeted options are developed to prevent or slow the progression of disease. Patients with contiguous deletions are in a particularly dangerous situation. Our results indicate that renal failure is more likely in this group, and also occurs more quickly than in patients with TSC alone. Presently, the only option for clinicians is to monitor closely and deal with problems such as hypertension as they arise. Work in the pre-clinical Tsc1 models in this study showed that rapamycin was effective in treating solid lesions, but not cysts. However, our group has shown that early intervention with rapamycin can prevent cyst development in a Tsc2 mouse models (Yang et al 2014). Studies of early intervention in people with TSC and with the TSC2/PKD1 contiguous gene syndrome are therefore required.

In recent years, rapamycin and everolimus have proven to be useful therapies for treatment of several aspects of TSC. This highly targeted therapy for the genetic basis of the disease is successful in shrinking AMLs and SEGAs but has not been

investigated in the contiguous gene syndrome. It is imperative for this particular patient population that similar targeted therapies be investigated.

5.4.1 Rapamycin, sunitinib, and nelfinavir as treatment in TSC/PKD contiguous gene syndrome

Based on our results and those previously described, it is not likely that sunitinib or nelfinavir would be indicated for treatment of renal disease in the TSC/PKD contiguous gene syndrome. In our mouse study, neither drug had any effect on cysts. While solid tumours may occur in the TSC/PKD contiguous gene syndrome, they are not the most common or damaging problem, and therefore should not be the main focus of treatment development.

It is not likely that rapamycin would be very effective in treating the contiguous gene syndrome once disease is advanced. Our results indicate that rapamycin is not efficacious in the treatment of established renal cysts. This is supported by clinical trials with rapamycin in both TSC and ADPKD (Bissler et al, 2008 & 2013, Davies et al, 2011, & Perico et al 2010). However, rapamycin has been shown to be effective in preventing renal cysts in a Tsc2 mouse model (Yang et al .2014) and hence prevention and early treatment strategies should be explored. Furthermore, treatment with rapamycin could still be indicated in patients with the contiguous gene syndrome even if impact on cysts is small as most patients do demonstrate significant wider symptoms of TSC.

Our pre-clinical studies did not include mice with a contiguous deletion. Cysts occur in both TSC and the TSC2/PKD1 deletion syndrome, but their formation could be by

different mechanisms. More information via actual contiguous gene syndrome pre-clinical trials is needed. This would also require better pre-clinical models.

5.4.2 Future drug treatment options and preventative therapy

Ideally the future should see more drug exploration for the treatment of the contiguous gene syndrome. While nelfinavir and sunitinib were unsuccessful in our study in relation to regarding cysts, they were rational choices for investigation as they are already clinically available. It would be sensible explore other clinical approved drugs such as other cancer therapies or drugs targeting specific signaling pathways. Another option for investigation is preventative (or prophylactic) therapy with the drugs tested here and/or others. This could be especially useful in the contiguous gene syndrome as renal disease is often more serious and faster-progressing and preventive treatment is an important aim.

There are already pre-clinical studies indicating that there may be a critical time period during organogenesis that is pivotal for the severity of some types of renal cystic disease (Lantinga-van Leeuwen et al, 2007 & Piontek et al, 2007). Certain aspects of disease have been shown to be more severe if mutation occurred before or during this critical time period, and less severe if afterwards. For example, cilia function has shown to be impaired in renal tubule epithelial cells in TSC. The degree of function-loss, though, depends on when the mutation occurred in development (Patel et al, 2008). Treating patients through this time period could perhaps lead to a less severe course of disease on the other side of this "critical window". One case study in humans showed that early mTOR inhibition could prevent development of renal lesions (Kotulska, Borkowska, & Jozwiak, 2013). A monozygotic twin female

treated with everolimus (a rapamycin analogue) from age 4 showed no renal lesions two years later while her untreated twin did.

Rapamycin could be more successful in treating cystic disease prophylactically for a few reasons. First, because this treatment timing could get patients through the critical time in development of cysts and therefore slow disease progression and decrease severity. Second, once cysts are well-established they could be harder to shrink via inhibition of cell proliferation or growth (reasons outlined above in section 5.3.1). "Catching" cysts with rapamycin as they are developing or beforehand could have a more positive effect. This and other drugs need to be investigated for their effectiveness on different time-scales and as preventative treatment in cystic disease specifically.

5.4.3 Preclinical studies for the TSC/PKD contiguous gene syndrome

In order to identify further treatment options for the TSC/PKD contiguous gene syndrome, additional pre-clinical studies are necessary. The challenge so far is that there are no good pre-clinical models for this specific disease state. Insights into signaling have been gained through investigations into TSC or PKD alone, but none have looked specifically at these pathways when a contiguous deletion is involved. This is a major roadblock to targeted treatment development for this patient population.

One study did attempt to investigate a TSC/PKD mouse model. Bonnet et al (2009) created a *Tsc1*$^{+/-}$*Pkd1*$^{+/-}$ mouse model by mating *Tsc1*$^{+/-}$ mice with *Pkd1*$^{+/-}$ mice. However, the double heterozygotes did not recapitulate the severe human

TSC2/PKD1 deletion syndrome. One potential solution for this problem may be development of a mouse with one single deletion knocking out both *Tsc2* and *Pkd1.* An actual contiguous deletion, rather than two separate mutations, may develop a phenotype closer to that of humans and therefore be more useful in testing potential therapies and looking further into signaling.

In vitro studies could also shed much more light on the genetic mechanisms behind disease progression and cyst formation. Some studies have already started looking into signaling connections between *TSC2* and *PKD1* (Shillingford et al, 2006 & Distefano et al, 2009, see section 1.5.3.2). Investigations show that PC1 and TSC2 interact, and specifically the carboxy terminal tail of PC1 helps localize TSC2 to repress mTOR (Dere, Wilson, Sandford, & Walker, 2010). Primary cilium changes found in $TSC2^{-/-}$ cells, on the other hand, seem to be PC1-independent (Hartman et al, 2009). Information on the relationship between these two proteins could definitely be helpful, but virtually no studies exist with a contiguous deletion in cell culture.

5.5 Renal function and hypertension monitoring in TSC and TSC/PKD

It is of extreme importance that both renal function and hypertension be monitored closely in patients with the TSC2/PKD1 contiguous gene syndrome. Since at present there is no preventative therapy, treatment relies heavily on watching kidney function and intervening via surgery, dialysis, transplant, etc. when needed. This is of particular importance since patients are often asymptomatic until renal function is already very poor, which in turn limits treatment options further.

Patients with *TSC2/PKD1* contiguous deletions or *TSC2* deletions are often treated for hypertension early but this is overall unpredictable from patient to patient. Monitoring blood pressure is extremely important since hypertension itself can lead to renal problems. This could lead to further decline in both groups, but could be especially damaging in the setting of contiguous deletions as their renal function is already declining more rapidly. Method of hypertension control also differs between patients and is left up to the treating physician's discretion. Interestingly, a recent study suggesting that early treatment of hypertension with angiotensin-II receptor antagonists could possibly slow AML development (Siroky et al, 2014). This could have indications for hypertension control in these patients in the future.

5.6 Future directions

Work is ongoing to further define the natural disease progression of the TSC2/PKD1 contiguous gene syndrome. More clinical data is being collected for control patients with TSC2 deletions only, and this will help show a stronger statistical difference between the two groups. After this study concludes, more research is needed to develop adequate treatment for patients with contiguous deletions. First, a mouse TCS2/PKD1 deletion model should be created for pre-clinical studies. Second, it is imperative that early intervention and prevention studies both pre-clinically and clinically be prioritized. This particular group of patients needs more research attention in order to produce better outcomes.

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Appendix 1:

Mouse	Treatment	Total Number Lesions	Cysts	Papillary	Solid	Total Lesion Size	Cyst Lesion Size	Papillary Lesion Size	Solid Lesion Size
M221	Vehicle	26	15	8	3	8941582.769	4711136.75	1954138.581	2276307.438
M231	Vehicle	72	38	28	6	65156173.67	4757110.052	12172723.87	48226339.75
M245	Vehicle	46	16	27	4	25288421.86	5845601.611	16692544.95	2750275.299
M257	Vehicle	60	19	33	8	44985504.41	3801898.384	15660490.14	25523115.89
M290	Vehicle	37	17	16	4	8138081.82	2800178.759	4592638.499	806809.062
M313	Vehicle	47	19	21	7	13571428.33	1435312.285	8686425.929	3395412.6
M327	Vehicle	68	30	26	7	24128791.17	5102344.381	7198217.477	11828229.31
M224	Rapamycin	17	16	0	1	10006452.08	9184177.807	0	822274.277
M253	Rapamycin	20	20	0	0	4978963.528	4978963.528	0	0
M260	Rapamycin	20	20	0	0	4787829.812	4787829.812	0	0
M271	Rapamycin	46	46	0	0	12747901.63	12747901.63	0	0
M288-1	Rapamycin	14	14	0	0	5108092.996	5108092.996	0	0
M289	Rapamycin	27	26	0	1	7455201.44	7283376.247	0	171825.193
M323	Rapamycin	21	21	0	0	8823974.015	8823974.015	0	0
M325	Rapamycin	35	35	0	0	7603936.922	7603936.922	0	0
M354	Rapamycin	21	21	0	0	1437591.161	1437591.161	0	0

Mouse	Treatment	Total Number Lesions	Cysts	Papillary	Solid	Total Lesion Size	Cyst Lesion Size	Papillary Lesion Size	Solid Lesion Size
M226	Nelfinavir	44	23	16	5	23697458.23	7000761.38	5494642.186	11202054.66
M237	Nelfinavir	32	19	11	2	26992712.03	4406122.762	3052352.281	19534236.99
M255	Nelfinavir	50	30	20	0	12019148.16	9531128.555	2470831.156	0
M259	Nelfinavir	22	9	10	2	6319297.804	3331435.602	2622552.381	365309.821
M280	Nelfinavir	72	27	42	3	42136553.96	4344416.407	13814163.02	23977974.53
M287-1	Nelfinavir	44	17	22	5	21457405.45	7300416.504	3052920.799	11104068.15
M291	Nelfinavir	28	13	13	2	8607758.836	2303387.395	5890831.854	413539.587
M341	Nelfinavir	40	20	15	5	5932614.559	2604397.764	811375.893	2516840.902
M244	Sunitinib	39	20	17	1	14745807.06	5029357.36	8731126.916	985322.785
M249	Sunitinib	60	32	28	3	17804670.01	6065562.46	10466967.9	1272139.648
M282	Sunitinib	45	17	27	1	22118047.25	11569315.87	10264942.52	283788.859
M284	Sunitinib	55	35	16	4	28242941.78	11858666.92	6329512.735	10054762.13
M284-1	Sunitinib	80	34	43	3	22193994.56	6858842.714	13175660.3	2159491.542
M292	Sunitinib	48	29	18	1	22401895.47	12060688.31	10244909.13	96298.031
M314	Sunitinib	53	14	29	6	39657467.42	8959363.479	27040663.47	3657440.474
M321	Sunitinib	32	12	18	2	6462524.418	1350726.393	3889720.607	1222077.418

Mouse	Treatment	Total Number Lesions	Cysts	Papillary	Solid	Total Lesion Size	Cyst Lesion Size	Papillary Lesion Size	Solid Lesion Size
M240	RS	31	31	0	0	8803524.342	8803524.342	0	0
M310	RS	16	16	0	0	6339601.672	6339601.672	0	0
M318	RS	24	24	0	0	9187105.879	8217226.46	0	0
M333	RS	18	18	0	0	2994520.356	2994520.356	0	0
M342	RS	27	27	0	0	6022075.426	6022075.426	0	0
M345	RS	23	23	0	0	3566942.079	3566942.079	0	0
M347	RS	22	22	0	0	4736207.729	4736207.729	0	0
M353	RS	4	2	2	0	32840.08	12331.701	0	0
M356	RS	22	22	0	0	7011647.152	7011647.152	0	0

Appendix 2: Original eGFR Data

Patient	Age (Years)	eGFR
1	6	>75
	13	>75
	15	>75
	18	93
2	22	71
	21	81
3	43	45
	44	44
	44	44
	45	45
	47	43
	48	41
	48	43
	49	42
	49	49
	50	39
	51	40
	51	38
	51	36
	51	34
	51	37
	51	37
	52	32
	52	33
	52	37
	52	37
	53	37
	53	35
	54	33
	54	24
	54	24
	54	36
	55	34
	55	36
	55	34
	56	34
	56	34

Patient	Age (Years)	eGFR
	56	34
	57	34
4		
	18	12
	18	11
	18	11
	19	12
	20	8
	20	6
	20	7
	20	8
	20	8
	20	8
	20	7
	20	6
	20	6
	22	23
5	20	68
	20	73
	21	81
	21	68
	21	69
	22	65
	22	64
	23	55
	23	50
	24	49
	24	63
	24	53
	24	49
	25	55
	26	45
	26	50
	26	51
	26	50
	26	58
	27	51
	27	45
	27	45
6		

Patient	Age (Years)	eGFR
7	3	68
	6	62
	7	66
	9	73
	12	66
8	32	66
	25	66
	31	65
	28	67
	27	74
9	23	52
	22	83
	22	101
	21	89
	9	>75
10	19	52
	20	53
	19	73
	20	53
	21	42
	21	33
11	25	8
	26	7
	27	6
	27	5
	28	5
	28	4
12	18	21
	18	15
13		
14	14	48
	14	48
	14	43
	14	43

Patient	Age (Years)	eGFR
	16	17
	17	23
	18	21
	19	11
	19	11
	19	11
	19	11
	19	9
	19	9
	20	10
	20	15
	21	24
	22	19
	22	15
	24	19
	24	16
	24	16
	16	17
	18	21
15	37	55
	38	53
	38	50
	40	42
	40	42
	40	45
	40	46
	40	45
	41	45
	41	50
	41	49
	42	42
	42	48
	43	43
	44	46
	44	45
	45	42
	46	39
	47	39
	47	37
	48	32
	48	34
	49	23

Patient	Age (Years)	eGFR
	49	23
	50	26
	50	26
	50	25
	51	19
16		
17	20	122
	34	30
	34	39
	33	40
	31	45
	30	45
	30	42
	30	48
	29	54
	28	57
	28	57
	23	68
	24	68
	18	87
	29	52
	32	38
	34	34
	27	57
	27	64
	25	73
	25	67
	24	68
	24	68
	23	65
	23	74
	22	62
	21	77
	21	90
	20	97
	20	91
	19	94
	18	80
	18	87
	31	49
	32	41

Patient	Age (Years)	eGFR
	33	38
	34	32
	35	26
	35	24
	35	25
	35	25
	35	25
	36	24
18		
19	18	42
	18	42
	18	36
	18	36
	19	35
	19	32
	19	23
	19	31
	19	33
	20	26
	20	27
	20	21
	20	20
	21	19
	21	17
	21	17
	21	12
	21	7
	22	7
	22	6
	22	5
	22	5
20	11	>75
	18	106
	18	106
	19	115
	19	104
	20	104
21		

Patient	Age (Years)	eGFR
22	13	>75
	15	>75
23	40	71
	39	76
	39	77
	38	71
	38	79
	38	69
	40	60
24	6	>75
	6	>75
	8	>75
	6	>75
	2	>75
	1	>75
	6	>75
25	34	73
26	18	50
	18	55
	18	39
	18	49
	18	58
27	5	91
	4	75
28	18	>75
	18	122
29	0	64
	1	69
	1	79
	2	101
	3	86
	4	88
	13	21
	15	9

Patient	Age (Years)	eGFR
30	45	85
	52	86
	56	75
	57	75
	58	78
	58	78
	59	73
31	72	57
	23	30
	23	33
	24	30
	24	25
	24	17
	24	19
	25	9
	25	10
	25	11
	25	10
	25	15
	25	12
32	23	72
	27	60
	26	68
	26	54
	26	63
	25	24
	25	70
	25	69
	25	69
	25	72
	25	65
	28	69
33	16	53
34	18	30
	18	34
	18	34
	18	30
	18	25
	18	22

Patient	Age (Years)	eGFR
	18	22
	19	25
	19	32
	20	39
	21	36
	21	31
	22	31
	24	29
	25	27
	25	28
	24	30
	24	28
	24	29
	19	25
35	18	52
	18	51
	18	51
	18	49
	18	48
	18	35
	18	48
	18	46
	18	46
	18	38
	19	38
	19	33
	20	24
	20	19
	20	18
	20	19
	20	19
	20	14
36	28	86
37	18	95
	78	76
	21	70
	22	65
	24	57
	25	41
	26	43

Patient	Age (Years)	eGFR
	26	35
	26	38
	27	27
	27	27
	28	28
38	33	48
	34	32
	35	30
	36	23
	37	16
	38	16
	38	15
	38	15
	38	12
	39	11
	39	11
	39	10
	39	10
	40	6
	49	30
	49	29
	49	32
	48	33
	48	30
	48	30
	47	32
	47	25
	47	27
	46	27
	45	34
	45	31
	44	33
	44	34
	44	31
	44	31
	43	34
	43	34
	43	37
	42	39
	42	37
	41	31
	41	35

Patient	Age (Years)	eGFR
	41	41
	40	46
	40	38
	51	28
	50	29
	50	35
	50	30
39	20	81
	18	85
	16	86
	15	
	23	79
	22	81
	21	70
	20	81
	19	81
	24	73
40	12	>75
	19	71
	27	43
	25	59
	24	55
	24	58
	23	53
	21	60
	23	53
	26	66
	27	41
	27	56
	27	52
	27	41
	27	49
	27	56
	27	52
	27	46
	28	36
	28	38
41	39	152
	54	80
	54	80

Patient	Age (Years)	eGFR
	52	74
	49	69
	34	99
	48	80
	51	70
	53	74
	46	84
	45	
	44	
	41	90
	40	
	33	
	35	93
	39	
42	18	130
	19	118
43	33	29
	32	37
	37	5
	37	5
	32	30
	34	30
	37	8
	38	5
	37	5
	32	23
	37	5
	36	10
	34	22
	34	30
44	14	75